Symposium on Oxygen Binding Heme Proteins
Structure, Dynamics, Function and Genetics

Asilomar Conference Grounds

Pacific Grove, California

October 9-13, 1988

Program and Abstracts

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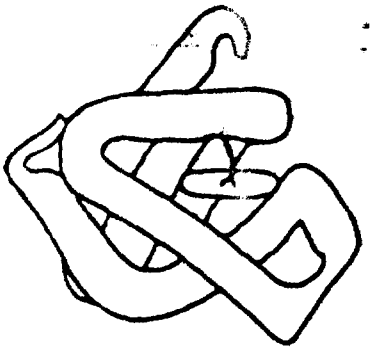
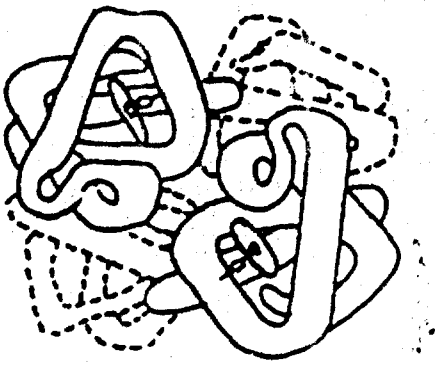
August 15, 1989





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Gerd N. La Mar, Co-chair

David A. Case, Co-chair

James D. Satterlee, Secretary

John S. Olson

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The symposium was designed to bring together active scientists involved in both theoretical and experimental advances in the structure, function, dynamics and genetics of myoglobins and hemoglobins. Abstracts of the presentations are included. Keywords: Genetics; proteins; Molecular dynamics; Molecular Structure; Raman spectroscopy; blood substitutes; (KT)

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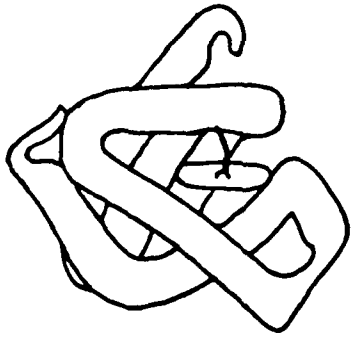

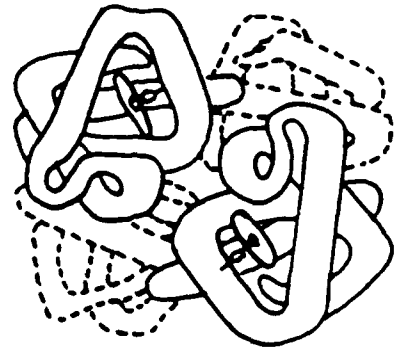

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* *

Symposium on Oxygen Binding Heme Proteins: Structure, Dynamics, Function and Genetics

October 9-13, 1988; Asilomar Conference Grounds; Pacific Grove, California

General Information

Formal sessions. All formal sessions will be held in the Chapel. Morning sessions begin at 8:30 am, evening sessions at 7:30 pm. The program begins on the next page. Times listed for the lecturers *include a ten-minute discussion period.* In addition to the lectures, we have scheduled seven discussion periods. It is our intent that these begin with consideration of the invited posters listed in the program, and consider also contributed posters on similar subjects. Those presenting invited posters are asked to come to the podium during the discussion periods.

We have tried our best to arrange sessions around lecture and poster presentations that have a common theme. In a conference as focussed as this one, however, it is inevitable that many contributions will cross subject boundaries. We ask forbearance from those who find the divisions confusing or feel that their contributions have been poorly assigned. All of the poster presentations will be available for viewing throughout the week, and we trust that additional connections between topics will be brought up during the discussion periods.

The foyer of Merrill Hall will serve as a social gathering area during the conference. Refreshments will be available there following the evening sessions.

Poster sessions. Poster sessions and social events will take place in Merrill Hall. Each poster has been assigned a number that includes its session (as a Roman numeral,) and a sequence number (as an Arabic numeral.) These numbers are printed on the abstract page for each contribution, by the name of the author in the index at the back of this booklet, and on cards at the corners of the posterboards. The first few posters in each session are assigned to the invited posters, as listed in the program.

All posters should be mounted as soon as possible, and must be removed by *9 am, Thursday morning.* Although posters will be available for viewing during the entire week, we ask that the presenters be available to answer questions during the afternoons assigned to their session: *viz.:* Monday, sessions II, III and IV; Tuesday, sessions V and VI; Wednesday, sessions VII and VIII.

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Symposium on Oxygen Binding Heme Proteins: Structure, Dynamics, Function and Genetics

October 9-13, 1988; Asilomar Conference Grounds; Pacific Grove, California

Program

All lectures will be in the Chapel.
Poster sessions and social events will take place in Merrill Hall.

I. Sunday evening: Plenary session

Gerd La Mar and David Case, chairpersons

7:20	Welcoming remarks
7:30	Max Perutz, MRC Laboratory of Molecular Biology. *Allostery's Many Faces*
8:30	Quentin Gibson, Cornell University. *A Perspective on Hemoglobin Kinetics*
9:30	Reception, Merrill Hall

II. Monday morning: Genetics

Lowell Hager (University of Illinois), chairperson

8:30	Kyoshi Nagai, MRC Laboratory of Molecular Biology. *The Role of Distal Residues in Hemoglobin Studied by Protein Engineering*
9:20	Stephen Sligar, University of Illinois. *Mechanisms of Molecular Recognition in Heme Proteins*
10:00	Steve Boxer, Stanford. *Electrostatic and Dynamic Interactions in Human Myoglobin Mutants*
10:30	Coffee break

(Monday morning program continues on the next page.)

III. Monday morning: Structure in crystal and solution

Greg Petsko (MIT), chairperson

11:00 John Kuriyan, Rockefeller Univeristy. *Probing Motion and Disorder in Crystal Structures by Molecular Dynamics*

11:30 Peter Wright, Scripps Clinic. *Structure and Dynamics of Myoglobin in Solution*

12:00 Group photo

12:10 Lunch

Monday afternoon: Poster session

3:00-4:30 Poster viewing. Wine and cheese will be served. *(Merrill Hall)*

III. Monday evening: Structure (cont.); poster discussions II and III

Greg Petsko, chairperson

7:30 Teizo Kitagawa, Institute for Molecular Science, Okazaki. *Iron-Ligand Stretching Vibrations and Quaternary Structure of Hemoglobin.*

8:20 Invited poster discussion: structure in crystal and solution. *Greg Petsko, discussion leader.*

James Alben, Ohio State University
Nai-Teng Yu, Georgia Tech
Eric Oldfield, University of Illinois
Denis Rousseau, Bell Labs
Gerd La Mar, University of California, Davis

9:10 Invited poster discussion: genetics. *Lowell Hager, discussion leader.*

Hideo Shimada, Keio Univeristy
Austen Riggs, University of Texas
Daniel Shih, Oregon Health Sciences University
Dale Webster, Illinois Institute of Technology

10:00 Adjournment

IV. Tuesday morning: Dynamics and ligand binding

Bob Austin (Princeton University,) chairperson

8:30	Hans Frauenfelder, University of Illinois. *Ligand Binding to Heme Proteins: Structure, Spectroscopy, Dynamics and Function*
9:20	John Olson, Rice University. *Dynamics of Ligand Binding to Myoglobin: Quantum Yields, Geminate Recombination, Isonitriles and E7 Mutants*
9:50	Joel Friedman, Bell Labs. *Structure, Dynamics and Reactivity in Hemoglobin and Myoglobin.*
10:20	Coffee break
10:50	Keven Peters, University of Colorado. *Time-resolved photoacoustic calorimetry: enthalpy and volume changes following photodissociation of CO from carboxymyoglobin*
11:20	Invited poster discussion: dynamics and ligand binding. *Bob Austin, discussion leader.* Doug Magde, U.C. San Diego James Hofrichter, National Institutes of Health Robin Hochstrasser, University of Pennsylvania Bob Austin, Princeton University Paul Champion, Northeastern University
12:10	Lunch

Tuesday afternoon: Poster session

3:00-4:30	Poster viewing. Wine and cheese will be served. *(Merrill Hall)*

V. Tuesday evening: Structure and Function

Gregorio Weber (University of Illinois,) chairperson

7:30	Maurizio Brunori, University of Rome. *Kinetics and Cooperativity of Binding of CO to Hemoglobins*
8:20	Larry Parkhurst, University of Nebraska. *Rapid and Precise Determination of Adair Constants for Oxygen Binding to Hemoglobin*
8:50	Brian Hoffman, Northwestern. *Protein Dynamics: Photophysics and Electron Transfer in Zinc-Substituted Heme Proteins*
9:20	Invited poster discussion: structure and function. *G. Weber, discussion leader.* Robert Noble, SUNY Buffalo Klaus Gersonde, Fraunhofer Institute Vijay Sharma, U.C. San Diego Emilia Chiancone, University of Rome

VI. Wednesday morning: Hemoglobin structure and intermediates

Maurizio Brunori (University of Rome), chairperson

8:30 Gary Ackers, Johns Hopkins. *The Three Allosteric States of Tetrameric Hemoglobin*

9:20 Takashi Yonetani, University of Pennsylvania. *Ligand Interactions and Cooperativity in Hemoglobin using Symmetric and Asymmetric Fe-Co Hybrid Hemoglobins*

9:50 Stanley Gill, University of Colorado. *Double Ligand Binding Studies of Human Hemoglobin.*

10:10 Coffee Break

10:40 Kyohiro Imai, Osaka University. *Inherent Problems in Evaluation of Hemoglobin Oxygenation Parameters*

11:20 Invited poster discussion: hemoglobin structure and intermediates. *M. Brunori, discussion leader.*

S. Walter Englander, University of Pennsylvania
Arthur Arnone, University of Iowa
Bob Liddington, Harvard University
Ben Luisi, University of Chicago
Gregorio Weber, University of Illinois

12:10 Lunch

Wednesday afternoon: Posters and special discussion

3:00-4:00 Poster viewing. Wine and cheese will be served. *(Merrill Hall)*

4:00-5:30 Special discussion on hemoglobin, with an emphasis on intermediate states. *Gary Ackers, discussion leader.* Session will include movie contributions from R. Liddington, B. Luisi and D. Case. *(The Chapel)*

VII. Wednesday evening: Hemoglobin (continued)

Kaspar Winterhalter (ETH, Zurich), chairperson

7:30 Martin Karplus, Harvard University. *Simulations of Hemoglobin and Myoglobin*

8:20 Bill Eaton, National Institutes of Health. *Kinetic Cooperativity in Hemoglobin*

8:50 Tom Spiro, Princeton University. *Dynamics of the Hemoglobin Quaternary Structure Switch from Time-Resolved UV Resonance Raman Spectroscopy*

9:20 Invited poster discussion: hemoglobin. *K. Winterhalter, discussion leader.*

Chien Ho, Carnegie-Mellon University
Winslow Caughey, Colorado State University
Catherine Royer, University of Illinois
Ron Elber, University of Illinois, Chicago

10:00 Adjournment

VIII. Thursday morning: Clinical prospects

Alan Schechter (NIH), chairperson

8:30 Robert Winslow, Letterman Army Research Institute. *Blood Substitutes: Current Status and Problems*

9:20 James Manning, Rockefeller University. *Carboxymethylated Cross-Linked Hemoglobin A as a Potential Blood Substitute*

9:50 Ron Nagel, Albert Einstein College of Medicine. *Genetics of Hemoglobin S*

10:20 Coffee break

10:50 H. Franklin Bunn, Harvard Medical School. *Regulation of the Erythropoietin Gene: Evidence that the Oxygen Sensor is a Heme Protein.*

11:20 Inivted poster discussion: clinical prospects. *A. Schechter, dicussion leader.*

Joseph Bonaventura, Duke University
Celia Bonaventura, Duke University
Joe Walder, University of Iowa
Alan Schechter, NIH

12:10 Lunch and conference adjournment

Allostery's Many Faces

M. F. Perutz,

Medical Research Council Laboratory of Molecular Biology,

Cambridge, U.K.

It is over 20 years since Monod, Wyman and Changeux, and Koshland, Nemethy and Filmer formulated their rival theories of allostery. For many years hemoglobin has been the only allosteric protein whose stereochemical mechanism was understood. Crystallographic studies have now unravelled the mechanisms of one metabolic enzyme, phosphofructokinase, one biosynthetic enzyme, aspartate transcarbamylase, and of one genetic repressor, the tryptophan repressor of E.coli.

What, if anything, is common to the mechanisms of action of these proteins? Do they confirm the predictions of either Monod or Koshland? Is hemoglobin a prototype of allosteric enzymes, an "honorary enzyme" as Monod used to call it? Has Nature solved the problem of feedback inhibition in more ways than one? We can now begin to answer those questions.

Q.H. Gibson.

ABSTRACT.

The first 50 years of hemprotein kinetics after Hartridge and Roughton (1923) were largely devoted to the collection of data, at first by flow methods, later supplemented by relaxation procedures, primarily flash photolysis. The three gaseous ligands bind at quite different rates, and also show very different variability across species, and, in the case of hemoglobin, between allosteric states. These results, and differences in apparent quantum yield, can be rationalized by taking into account new flash photolysis data using very short laser pulses. The new data allow the movement of ligand within the protein molecule to be taken into account, and, in combination with the study of genetically engineered mutants, promise a highly detailed account of ligand binding which may be related to the wealth of structural information already available.

THE ROLE OF DISTAL RESIDUES IN HAEMOGLOBIN STUDIED BY PROTEIN ENGINEERING (review)

Kiyoshi Nagai, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, ENGLAND

We have engineered mutant human haemoglobins in which His-E7 and Val-E11 of the α and β chains have been substituted and studied their equilibrium and kinetic ligand binding properties, crystallographic structure and vibrations of bound ligand by resonance Raman spectroscopy. The replacement of Val-E11α with Ala, Ile, Leu has no significant effect on the oxygen equilibrium curves but in the β subunit the same mutations alter the oxygen affinity. X-ray crystallographic studies show that the Ile-E11β side chain hinders oxygen binding since an Ile side chain attached to an α helix cannot rotate freely about the C_α–C_β bond and its rigidly fixed side chain extends to the oxygen binding site. The Leu-E11β side chain is flexible and does not hinder the oxygen binding. The replacement of Val-E11α with Ile does not cause any structural change and merely expels the water molecule hydrogen bonded to the distal histidine. The α haem pocket is larger and can accommodate the Ile side chain without affecting the oxygen affinity. We have measured the ν(Fe-CO), ν(C-O) and δ(Fe-C-O) of carbonmonoxy Hb, δ(Fe-N-O) of nitrosyl Hb, ν(Fe-N_3) of azidemet Hb, ν(Fe-CN) of cyanomet Hb by resonance Raman spectroscopy to see the effect of these new side chains on the Fe-ligand bond. None of these vibrational frequencies were affected by these Val-E11 mutations. This implies that protein is flexible to accommodate these mutations without significantly distorting the Fe-ligand geometry.

Substitution of His-E7 with Gly in myoglobin and the α subunits of intact, R-state haemoglobin causes a 10-14 fold decrease in oxygen affinity, a 4-6 fold increase in carbon monoxide affinity, and a 10-60 fold increase in methyl isocyanide affinity. In contract the same mutation produced little or no effect on the rates and affinity of oxygen, CO or methyl isocyanide binding. These results show that His-E7 plays an important role in myoglobin and the α subunit of haemoglobin but its effect is less significant in the β subunit. The vibrational properties of the Fe-C-O bond and absorption spectra of deoxy Hb, however, are affected whether or not the E-7 residue is hydrophobic. His-E7 is highly conserved throughout the evolution of globins but is replaced by Gln in elephant myoglobin and the α subunit of opossum haemoglobin. The crystallographic structure of the His-E7→Gln shows that a hydrogen bond can be formed between O_2 and the Gln side chain therefore this mutation is conservative.

Our protein engineering study of haemoglobin has proved powerful in understanding its structure and function relationship.

MECHANISMS OF MOLECULAR RECOGNITION IN HEME PROTEINS

Stephen G. Sligar, Barry A. Springer, Karen D. Egeberg,
J. Dezz Ropp, and Mark L. Chiu

Departments of Chemistry and Biochemistry
University of Illinois, Urbana, IL 61801

Equilibrium and dynamic structures of heme proteins dictate the mechanisms and specificity of polypeptide chain folding, macromolecular association, electron transfer physics, recognition of substrates, and the chemistry of catalysis. In order to probe the structure-function correlations in heme proteins we have utilized site-directed mutagenesis of cloned or totally synthetic genes of sperm whale myoglobin, Aplysia myoglobin, rat liver cytochrome b_5, putidaredoxin, and cytochrome P-450_{cam}. Precise understanding of the physics and chemistry of the fundamental processes described above requires structural, catalytic, dynamic, spectroscopic and biochemical information. In the case of sperm whale myoglobin, we have obtained gram quantities of the following mutants. In examining distal pocket effects, Histidine E7 (H64) has been replaced by Gly, Cys, Lys, Tyr, Val, Met, Arg, Phe, Asp, and Thr. Valine E11 (V68) has been replaced with Ile, Phe, Ala, and Glu. E7/E11 double mutants Gly/Ile, Gly/Phe, and Gly/Ala have been constructed and over-expressed. The contribution of Arg 45 to the salt linkage at one pocket access channel has been examined by replacing this residue with Gly, Asp, Ser, and Asn. The role of axial ligands in the bio-inorganic chemistry of myoglobin has been realized by removing the normal histidine at F8 (H93) in favor of Cys and Tyr.

Determining the precise effects of our sperm whale myoglobin mutations on protein function requires extensive collaborations, partial results of which will be presented at this meeting by Drs. Phillips (X-ray structure), Olson and Gibson (ligand binding and geminate recombination), LaMar and Wright (NMR), Frauenfelder (ligand binding and infrared spectroscopy), Champion (Raman), Debrunner (EPR), and Peters (photoacoustic spectroscopy). In my presentation, I will describe the basis for molecular recognition in the myoglobin system. Supported by grants from the National Institutes of Health.

ELECTROSTATIC AND DYNAMIC INTERACTIONS IN HUMAN MYOGLOBIN MUTANTS

R. Varadarajan, D. Lambright, S. Balasubramanian and S. G. Boxer
Department of Chemistry, Stanford, CA 94305

ABSTRACT

A full length cDNA clone for human myoglobin was isolated and expressed as a fusion protein in high yield in E. coli. The fusion protein can be reconstituted with hemin and then cleaved with trypsin to generate fully functional human Mb. We have used site directed mutagenesis to explore electrostatic and dynamic interactions in native and mutant proteins. Site specific mutants have been prepared in which residue Val 68 is replaced by Glu, Asp, Asn, and Ala, residue Lys 45 by Arg, and residue Asp 60 by Glu. Characterization of the purified mutant proteins using isoelectric focusing, absorption, CD, and NMR spectroscopy indicates that the overall structure of the heme pocket as found in the native protein is highly conserved in all of the mutants. Consistent with this is the observation that the mutants are all stable to tryptic digestion. The consequences of the Val mutations on the redox properties of heme group were examined by thin layer spectroelectrochemistry. Cyanide binding constants were measured in order to obtain estimates of the electrostatic interaction between potentially charged substituents at residue 68 and the heme iron. In addition, the apoproteins were prepared and reconstituted with a derivative of chlorophyll, Zn pyrochlorophyllide *a* in order to determine the effects of such amino acid substitutions on chromophore spectral properties. Dynamic properties for mutant proteins have been investigated on the femtosecond to kilosecond time scale by following the kinetics of ligand binding and proton exchange. Substantial effects are observed on both the rate of CO recombination and distribution of NO recombination rates (collaboration with J. W. Petrich and J. L. Martin, ENSTA) following flash photolysis for several of the mutants. CO off-rates and proton exchange kinetics are also perturbed. Although the structural changes induced by these mutations are remarkably small, the effects on dynamic properties are complex as well as surprising.

Probing Motion and Disorder in Crystal Structures by Molecular Dynamics

John Kuriyan
The Rockefeller University
1230 York Avenue
New York, NY 10021

Diffraction studies of protein crystals can, in principle, reveal detailed information about protein dynamics and disorder. Unfortunately, the large amplitude motions found in proteins leads to a paucity of diffraction data and prevent the use of methods that have been successfully used to study mobility in small molecule crystals. In addition, large scale motions can distort the results of standard crystallographic analysis, particularly when there is conformational heterogeneity or highly anisotropic motion. We have been concerned with two problems: (i) how to evaluate highly refined crystal structures in order to discover deficiencies or errors in the model and (ii) how to devise X-ray refinement models which capture the essential features of the dynamics of proteins and yet are frugal in their use of free parameters.

The application of molecular dynamics to crystallographic refinement problems ("simulated annealing") has been shown to reduce the number of manual interventions needed to refine a protein structure to completion (A.T.Brünger, J.Kuriyan and M.Karplus (1987) *Science*, 235, 458-460). This method involves modifying the molecular mechanics potential energy function to include the crystallographic residual. This "R-factor/Energy" molecular dynamics method is now well established as the refinement protocol of choice for crude initial structures. We have extended the use of the method recently to estimate the accuracy of highly refined structures, to search for alternative structures in proteins and to evaluate the need for including anisotropic temperature factors.

Our approach is to take a well refined structural model and duplicate it so that there are two structures that are now invisible to each other in terms of the internal nonbonded energy, but are completely equivalent in terms of the X-ray structure factor calculation. We then assign random velocities to the "twin" structures and gently heat up the system to 500K or so and run molecular dynamics (including the X-ray structure factor term) for several picoseconds. The system is then quenched and conventional least-squares refinement is carried out to convergence. The final refinements are done with single structures (i.e., one or the other of the "twin" structures is deleted) or with both structures simultaneously present. The single structure refinements give estimates for the reproducibility of the structure. The "twin" structure refinements yield information about possible disorder and anisotropic motions. Our initial calculations have been on Ribonuclease-A against 1.5Å data (S.K.Burley and G.A.Petsko) and Crambin (0.9Å data, W.A. Hendrickson). The "twin" refinements result in large (0.4Å to 1.0Å) shifts between the two structure in many regions of both Ribonuclease and Crambin. The kinds of displacements observed are: (i) The two structures are in different torsional minima. All of these are sidechain atoms, mainly on the surface. Some of these had been located in the original crystallographic model but a few were newly discovered by the "twin" annealing procedure; (ii) The two structures have fairly close torsional angles, but still exhibit distinct hydrogen bonding; (iii) The most common reproducible shift involved no large changes in torsional angles or H-bonding patterns, but rather was the result of correlated displacements of atoms over several residues. Analysis of data for Crambin suggests that these displacements are best modelled as anisotropic thermal ellipsoids, rather than alternative conformers.

Structure and Dynamics of Myoglobin in Solution

Peter E. Wright, Claudio Dalvit, Surinder S. Narula, Tom Pochapsky and Linda Tennant, Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037.

High resolution NMR methods are being used in this laboratory to probe the structure and dynamics of sperm whale myoglobin in solution. Experiments are focused on the diamagnetic CO and O_2 complexes and the five-coordinate zinc myoglobin (a diamagnetic analogue of the deoxy state). A variety of two-dimensional ^1H NMR methods have been used to make extensive assignments for all of these complexes. Multiple quantum experiments have proved to be particularly important in this regard. Assignments have been made both on the basis of networks of NOE connectivities to the heme and by identifying NOEs between adjacent residues in the amino acid sequence. In the case of MbCO, assignments are now available for approximately 60% of the residues. The availability of such extensive resonance assignments throughout the protein allows a detailed comparison to be made of the structure of MbCO in crystal and solution. Both the observed NOEs and the measured ring current shifts confirm that the solution and crystal structures are very similar, although some small but potentially important differences are observed, eg., in the proximal histidine coordination geometry. The conformation of zinc myoglobin appears to be very similar to that of the deoxy protein. The sequential assignment methods provide assignments for a very large number of backbone amide proton resonances. Amide exchange rates have been measured and these provide novel insights into the conformational flexibility of myoglobin on a global basis. Backbone amide exchange from the E-helix is very slow and is highly correlated. Amide exchange from the F-helix is relatively fast, as might be expected since ligand binding is accompanied by movement of this helix. Some information on side chain dynamics is also available and its relevance to the ligand binding processes of myoglobin will be discussed.

Iron-Ligand Stretching Vibrations and Quaternary Structure of Hemoglobin

Teizo Kitagawa

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Cooperativity of Hemoglobin (Hb) has been explained by assuming a reversible transition between the high affinity (R) and low affinity (T) quaternary structures. This theory has reproduced a number of experimental data satisfactorily, but still there were significant features which appeared inconsistent with it. A basic problem to be clarified is what is the main structural factor that directly controls the affinity and whether this factor changes in accord with the T - R transition. In order to answer this question we have investigated resonance Raman spectra of Hb.

We demonstrated previously that the Fe-histidine(F8) stretching mode (ν_{Fe-His}) of deoxyHb yields different frequencies between the T and R structures. It was pointed out by other groups that a few porphyrin modes also exhibited slight shifts proportional to changes of the ν_{Fe-His} frequency while other modes remained unshifted and also that photodissociated HbCO with higher affinity gave distinctly higher ν_{Fe-His} frequencies. Our recent study on asymmetrically Co substituted Fe-Co hybrid Hb[1] revealed that the O=O and Fe-CO stretching frequencies changed little upon changes of the number of ligands from 1 to 4 while the ν_{Fe-His} mode exhibited definite frequency changes. Furthermore, a site specifically mutated Hb[2], in which α42-Tyr of Hb A is replaced by Phe, gave the ν_{Fe-His} band at the frequency of the R structure deoxyHb. Consequently, it is reasonable to assume that the affinity is controlled by the Fe-His bond but not by an interaction to the sixth ligand.

We found that the ν_{Fe-His} frequencies of various mutant deoxyHbs with varied oxygen affinity change systematically with K_1, and this was interpreted satisfactorily by assuming the strain in the Fe-His bond which was originated from the free energy changes in the globin moiety. This matches the Perutz's strain model and means that the magnitude of the strain is a continuous variable but is not limited to two kinds of values. Moreover, the ν_{Fe-His} band of the photodissociated transient FeCO subunits of the asymmetric Fe-Co hybrid Hbs[1] showed a shift from the frequency of fully deoxyHb when two CO molecules were bound to two α(Fe) subunits or one CO molecule was bound to a single β(Fe) subunit. This implies that binding of a single ligand to the α or β subunit gives rise to different states and binding of two ligands finishes the structural changes despite the fact that the oxygen affinity is altered significantly after three ligands are bound. This inconsistency between the structural changes and functional properties should be clarified in future studies.

1. S.Kaminaka, T. Ogura, K. Kitagishi, T. Yonetani, and T. Kitagawa, to be published.
2. K. Imai, K. Fushitani, G. Miyazaki, H. Morimoto, K. Ishimori, I. Morishima, T. Kitagawa, Y. Wada, D. Shih, Y. Tame, and K. Nagai, to be published.

Asilomar 1988

Symposium on Oxygen Binding Heme Proteins

Ligand Binding to Heme Proteins: Structure, Spectroscopy, Dynamics, and Function

Hans Frauenfelder
Departments of Physics, Chemistry, and Biophysics
University of Illinois at Urbana-Champaign
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The binding of small ligands to monomeric heme proteins is one of the simplest biological processes. Nevertheless, it turns out to be a very complicated phenomenon that can teach us a great deal about control, specificity, and protein dynamics. The fact that binding is a dynamic and not a static phenomenon follows already from the observation of Perutz that the average structures of myoglobin and hemoglobin do not show channels through which CO or O_2 could reach the heme iron. Protein motions must open transient channels.

Flash photolysis experiments over wide ranges in time and temperature provide insight into both the static and dynamic features of ligand binding. At temperatures below about 200 K, only geminate rebinding occurs; in general two processes I and I*, with very different properties, can be observed. Above 200 K, geminate and binding from the solvent compete. The crucial characteristics can be described with two simple relations.

To understand binding one must connect structure, spectroscopy, dynamics, and function. Structure and spectroscopy are linked by relating the stretch frequency of bound CO to the angle between the CO dipole and the heme normal, measured by a photoselection technique. Spectroscopy and function are connected by measuring the binding of CO to substates with different CO stretch frequencies.

Dynamics is investigated by observing the relaxation of spectroscopic markers after a pressure jump. Evidence for three different relaxation processes emerges. The time and temperature dependence of these processes suggests which parts of the protein participate.

The experimental data relating structure, spectroscopy, and dynamics lead to a number of conclusions concerning the biologically important parameters and suggest how structural features control specificity and function. The data also suggest further experimental and theoretical work, particularly involving mutated proteins.

Dynamics of Ligand Binding to Myoglobin: Quantum Yields, Geminate Recombination, Isonitriles, and E7 Mutants

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Ligand binding to myoglobin can be described by the three step, linear scheme first proposed by Frauenfelder and co-workers in 1975:

$$\text{MbX or A} \underset{k_{BA}}{\overset{k_{h\nu}I + k_{AB}}{\rightleftharpoons}} \text{B} \underset{k_{CB}}{\overset{k_{BC}}{\rightleftharpoons}} \text{C} \underset{k_{XC}}{\overset{k_{CX}}{\rightleftharpoons}} \text{x + Mb}$$

where in photolysis experiments $k_{h\nu}I$ is the rate of photochemical formation of the first geminate state. The recent picosecond studies of Magde's and Martin's groups (Jongeward, et.al. (1988) J. Am. Chem. Soc., 110, 380; Petrich et. al. (1988) Biochemistry 27, 4049) have shown unambiguously that at room temperature the intrinsic photochemical yield of state B is ≈ 1.0 for all ligands, heme proteins, and model compounds. Any decrease in the overall quantum yield measured with long light pulses (>0.5ms) is due to geminate recombination from states B or C. Their work plus the previous studies of Hochstrasser, Noe, Friedman, and co-workers indicate that state B represents a true "geminate pair" in which the ligand and the iron atom are separated by Van der Waals distances since the recombination rates for this intermediate, k_{BA} and k_{BC}, are extremely large, 10^{11}-10^{10} s^{-1}, and similar to those measured for sterically unhindered model heme compounds. In contrast, ligand recombination and escape from state C occurs on the nanosecond time scale, and this kinetic intermediate appears to represent a ligand molecule within the heme pocket but farther removed from the iron atom. We have examined the physical nature of these transient species by measuring the effects of ligand chemistry and size on the kinetic parameters describing recombination. Structural interpretation of these results were made possible by X-ray crystallographic studies determination of the crystal structure of the ethyl isocyanide complex of sperm whale myoglobin and by kinetic measurements with mutants in which E7 (64) His was replaced by Gly, Val, and Phe.

Four major conclusions were obtained. First, the differences between the overall quantum yields of the gaseous complexes are determined primarily by different rates of recombination from state B (i.e. k_{BA}/k_{BC} = 0.03, 4.4, and 440 for native MbCO, MbO_2, and MbNO, respectively), whereas the rate parameters for state C are similar for all three ligands (i.e. $k_{CB}/k_{CX} \approx 0.5$; $k_{CX} \approx$ 15 μs^{-1}; $k_{XC} \approx$ 50 $\mu M^{-1}s^{-1}$ for MbO_2 and MbNO). Second, in native myoglobin state B is not easily detected for small isontriles ($k_{BA}/k_{BC} \approx 0.1$-$0.2$); the low overall quantum yields observed for these complexes are due to extensive recombination from state C ($k_{BC}/k_{CX} \approx$ 30-100). E7 His to Gly or Val substitution increases the rate of entry into and exit out of the heme pocket for all ligand molecules (i.e. k_{XC} increases 10-400 fold and k_{CB}/k_{CX} decreases 2-50 fold). Fourth, these kinetic data coupled with the location of the two alkyl carbon atoms in the ethyl isocyanide complex of native myoglobin suggest strongly that state C represents ligand molecule kinetically "stuck" in the back of the heme pocket by the distal histidine. This idea is supported by our kinetic results for the E7 Phe mutant, which exhibits "native-like" geminate recombination time courses, and by the previous molecular dynamics calculations of Case and colleagues.

Structure, Dynamics and Reactivity in Hemoglobin and Myoglobin

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Advances in pulsed laser technology and in weak signal detection have made time-resolved resonance Raman spectroscopy a valuable tool for probing structural dynamics in systems containing a resonance Raman active chromophore. The role of protein dynamics in the control of biologically relevant functional properties is of current interest. Only a very few proteins have been sufficiently well characterized to allow for a detailed probe of structural dynamics. Because hemoglobin and its Raman spectrum have been very well characterized, it is possible to apply picosecond and nanosecond time-resolved resonance spectroscopy to fundamental questions concerning dynamics, structure, and function in this protein.

A discussion will be presented of the emerging and evolving picture of the interplay among structure, structural dynamics and function based on recent time resolved Raman and absorption studies on a wide variety of hemeproteins at *both ambient and cryogenic conditions.*

KINETICS AND COOPERATIVITY IN THE BINDING OF CO TO HEMOGLOBINS

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In hemoglobin, the kinetic basis of cooperativity is manifested either in the combination or in the dissociation rate process, depending on the type of ligand. The low ligand affinity of the T-state is associated to an increase in the dissociation rate constant for oxygen and nitric oxide, and to a decrease in the combination rate constant for carbon monoxide (as shown by classical experiments). A satisfactoy quantitative description of cooperativity demands an understanding of the structural basis of these phenomena.

Carbon monoxide has been extensively investigated as an informative probe ligand, by rapid mixing and photochemical techniques, using optical absorbtion and Raman spectroscopy. Its combination rate constant varies considerably with the type of protein (myoglobins and hemoglobins from different species, natural and artificial mutants), and the kinetic control has been attributed to proximal and/or distal effects. Kinetic experiments on monomeric hemeproteins (Mb, mini-Mb, Chironomus Hb) and heme model compounds will be reviewed; recent data on cooperative tetrameric hemoglobins (human, trout) will be presented and discussed to undestand (a) which kinetic barrier is controlled by the quaternary state of the macromolecule, and (b) what is the correlation between the rate of binding at the heme and the stereochemistry of the active site.

RAPID AND PRECISE DETERMINATION OF ADAIR CONSTANTS FOR OXYGEN BINDING TO HEMOGLOBIN. L. J. Parkhurst, T. M. Larsen, and T. Mueser. Dept. of Chemistry and School of Biological Sciences, University of Nebraska, Lincoln, NE 68588-0304.

We have completed the first phase of the development of a new device for the precise determination of oxygen equilibrium binding curves. The device employs a small stopped-flow apparatus built into a Cary 210 spectrophotometer interfaced to a micro-computer. In the present design, 0.08 mL of solution is in the optical path. Enzymatic depletion of oxygen is initiated by the rapid mixing, resulting in a decrease in oxygen concentration from approximately 250μM to less than 1 nM in about 20 minutes. Myoglobin is used in place of an oxygen electrode to serve as a homogeneous and rapid sensor of oxygen concentration. Dual wavelength spectrophotometry allows one to follow changes in the concentration of oxymyoglobin, and hence in the concentration of free oxygen at the oxy-deoxy Hb isosbestic, and follow oxygen release by hemoglobin at the oxy-deoxy Mb isosbestic. Typically 200-250 pairs of absorbances are obtained for each run. Knowledge of the details of the enzymatic reaction allows us to interpolate adequately the oxygen concentrations from 250μM to approximately 120μM, the region where the optical sensor is insensitive. If extrapolation methods are not employed at the end points of the isotherm, then six parameters must be fit if one assumes that the reaction can be described by the conventional binding polynomial. These parameters are the four Adair constants and the absorbances at zero and infinite concentrations of oxygen. After processing nearly 50,000 data points, we concluded that serious inconsistencies were appearing in the analyses. First, values for K_4 were in poor agreement with the value calculated from kinetics, which should provide an upper bound on that parameter. In some instances, values for K_4 were 3-10 times that estimated from the kinetics. Second, the residuals were poorly distributed, and were much larger than expected from the known errors in the absorbance data and for the propagation of error from the uncertainty in the oxygen concentrations. Third, the value for K_4 obtained in the overall fit differed significantly from that derived from extrapolations using only the top 2% of the binding curve. The source of the error was traced to the "spectrophotometric assumption", which states that changes in absorbance for hemoglobin are directly proportional to changes in saturation. The first convincing evidence that the assumption was untenable came from studies at the static Hb oxy-deoxy isosbestic which was found not to be isosbestic over the course of the deoxygenation. Further studies of the fractional saturation as a function of wavelength showed that the shape of the differential saturation curve was that expected for a function with three additional parameters that must be determined. These additional spectroscopic parameters occur in the numerator of the usual expression for Y as coefficients of the first three terms. Such spectroscopic differences are reasonable in view of the well-known differences in the spectra of R and T Hb and of the spectra of the individual chains within the tetramer. We suggest that at least in part, recent controversy over the values of the Adair constants derives from use of the incorrect equation to describe the measured optical changes. It is clear that only extensive and highly precise data will allow determination of all nine parameters. Toward that end, we are nearing completion of a new cell that will allow Hb in the concentration region of 1mM in heme to be studied, and a second device that will employ diode array detection for the collection of several thousand points per run.

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THE THREE ALLOSTERIC STATES OF TETRAMERIC HEMOGLOBIN

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During the course of ligand binding, tetrameric human hemoglobin can exist in ten molecular species (ligation states) reflecting the structurally-unique combinations of ligated and unligated subunits. While an understanding of the mechanism of cooperativity will clearly require much information on the structures and functional energetics of the partially-ligated tetrameric molecules, systematic information has been difficult to obtain. Recent developments, however, have provided promising new approaches to this problem. A study from this laboratory (1) using CN-met hemes in the ligated subunits demonstrated that the hemoglobin tetramer acts as a three-level combinatorial switch, i.e., each tetrameric species assumes one of three cooperative free energies depending on both the number of ligands bound and the specific configuration of ligated subunits. This finding of three cooperative free energies based on results of kinetic techniques has recently been confirmed using independent cryogenic techniques (2). The same result has been found in two other systems of chemical species that mimic intermediate oxygenation states (3). In one system subunits containing Mn^{3+} protoporphyrin IX are models for the ligated subunits; in the other, Mn^{2+} protoporphyrin IX serves as the analog of unligated heme while the ligated subunits contain normal hemes reacted with carbon monoxide. We have studied five ligation species in each system and find the same distribution of cooperative free energies as in the CN-met system. These results demonstrate that the three-state combinatorial nature of cooperative switching is not a special feature of the CN-met reactions, nor of the substitution of manganese for iron, but reflects a fundamental property of hemoglobin.

A finding of three cooperative free energies for hemoglobin tetramers does not by itself mean that there must be three corresponding molecular forms; a tetrameric system with only two allosteric states can have assembly free energies of partially-ligated species that are intermediate between those of the end-state species (4, 5). We have analyzed the observed distributions of cooperative free energies among the various ligation states in terms of mechanisms based on two interconvertible molecular forms (R and T) under the most general conditions in which (i) dimers may be cooperative (ii) ligand affinities of α subunits may be different within tetramers and dimers, and the same for β subunit affinities, and (iii) dimers need not be halves of R-state tetramers. It is found that the experimental distributions are totally inconsistent with even the most general model of the two-state class; thus at least **three molecular forms of tetramer are required**, each with a different value of cooperative free energy (heme-heme interaction). This result implies the existence of at least three corresponding molecular structures; while a degeneracy of multiple structures into only a few dominant free energy levels is frequently to be expected, the reverse situation is extremely unlikely.

The relationship of these findings to recent crystallographic structural results on partially-ligated hemoglobins and to oxygen binding will be discussed.

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Ligand Interactions and Cooperativity in Hemoglobin using Symmetric and Asymmeric Fe-Co Hybrid Hemoglobins.

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The mechanism of subunit cooperativty in allosteric hemoglobin (Hb) has been probed by measuring changes in the tertiary and quaternary structures and ligand affinity of Hb accompanying the stepwise binding of ligands using Co(II) protoporphyrin IX as a combination probe of functional active site analog, EPR spin label, NMR shift agent, and ligand discriminator. Asymmetric Fe-Co hybrid Hb tetramers, four protoheme groups of which are partially replaced with one, two, and three Co(II) protoporphyrins IX, have been prepared by recombination and cross-linking of appropriate dimers from symmetric Fe-Co hybrid HbAs [$\alpha(Fe)_2\beta(Co)_2$ and $\alpha(Co)_2\beta(Fe)_2$], HbA, HbC, CoHbA, and CoHbC. Both α_1-α_2 (1) and β_1-β_2 (2) cross-linked hybrids have been prepared under anaerobic (with IHP) and aerobic conditions, respectively. The Co(II)-containing subunits are unreactive with carbon monoxide. Thus, upon addition of carbon monoxide, these mono-, di-, and tri-Co(II)-substituted Fe-Co hybrid Hbs are converted to tri-, di-, and mono-carbon-monoxide-ligated species, respectively, which serve as analogs of respective intermediate states of ligation of Hb.

These physically isolated model intermediates are examined by: (I) EPR for the coordination state of deoxy Co(II) ions, (II) hyperfine-shifted exchangeable proton NMR for the metal-proximal His bonding, (III) ring-current shifted proton NMR for the tertiary structure of the distal heme pocket, (IV) exchangeable hydrogen-bonded proton NMR for the quaternary structure, (V) resonance Raman ($\nu_{Fe\text{-}His}$, $\nu_{Fe\text{-}CO}$, δ_{FeCO}, and ν_{OO}) for the metal-ligand coordination and orientation (3), and (VI) oxygen equilibrium measurements for the ligand affinity of the Co(II)-containing subunits (4).

We observe: (i) the αCo(II)-subunits in the low-affinity quaternary state always shows a highly hindered metal-proximal His coordination, (ii) ligation of carbon monoxide to an α-subunit induces minimal changes in the tertiary and quaternary structures and the ligand affinity, (iii) ligation of carbon monoxide to a β-subunit causes significant changes in the tertiary and quaternary structures and the ligand affinity of the three remaining subunits, and (iv) ligand binding causes setpwise changes in the T- and R-state marker hydrogen-bonded resonances and non-synchronous behaviors of the T- and R-state markers.

These observations suggest: (a) there may exist several intermediate quaternary (sub)states (designated as T_0, T_1, T_2, R_1, and R_o), (b) the initial ligation may take place in the α-subunits, (c) the ligation in one subunit may change in the ligand affinitis of other subunits even in the absence of complete T/R quaternary structural change, as monitored by hydrogen-bonded proton NMR, and (d) a simple two-state allosteric model is inconsistent with the present observations. Supported by NIH HL14508 and NSF DMB87-16796.

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DOUBLE LIGAND BINDING STUDIES OF HUMAN HEMOGLOBIN

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I wish to discuss results from our laboratory on precision ligand binding measurements utilizing thin layer optical changes induced by changes in gaseous ligand partial pressure. The technique enables use of high concentrations, where dimer formation can be minimized, and avoids the use of oxygen electrodes. First, double gas ligand studies employing oxygen and carbon monoxide, have been made to follow: 1) pure O_2 and CO binding processes, 2) competitive binding at near saturation and, 3) binding events at fixed ratios of partial pressures[1]. Second, experiments of the effect of the solution ligand, DPG or IHP, upon oxygen binding has produced a set of results for various limited fixed total concentrations of the organic phosphate in the presence of high hemoglobin concentrations[2].

Global data analysis for the relevant experiments is found to permit resolution of pertinent binding parameters to a reasonable level of certainty, generally much higher than possible for single ligand binding measurements[3,4]. The analysis of identical linked ligands (CO and O_2) or fixed limited amount of solution ligand (DPG, IHP), has been formulated in terms of either 1) model free basic stoichiometry reactions (Adair-like schemes) or (2) a modified two-state allosteric model[5]. In terms of the Adair analysis, one finds a consistently low value of triply ligated species for all the systems with human hemoglobin so far studied. This broad functional feature finds a structural basis 1) in terms of early observations of blocked access to β chain in the deoxy structure by Perutz[6] and 2) of the discovery of a doubly oxygenated (α chain) T structure of hemoglobin by Brzowski, et al.

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INHERENT PROBLEMS IN EVALUATION OF HEMOGLOBIN OXYGENATION PARAMETERS

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The four-step oxygen binding equilibria of tetrameric hemoglobin is generally expressed by the Adair equation,

$$Y = \frac{A_1p + 2A_2p^2 + 3A_3p^3 + 4A_4p^4}{4\,(1 + A_1p + A_2p^2 + A_3p^3 + A_4p^4)},$$

where Y is fractional saturation of Hb with oxygen, p is partial pressure of oxygen, and A_i (i = 1 to 4) is overall equilibrium constant for the ith step of oxygen binding,

$$Hb(O_2)_{i-1} + O_2 \rightleftarrows Hb(O_2)_i.$$

Intrinsic (corrected for statistical factors) stepwise equilibrium constants K_1 to K_4 are given by

$$K_1 = (1/4)A_1,\ K_2 = (2/3)(A_2/A_1),\ K_3 = (3/2)(A_3/A_2),\ K_4 = 4(A_4/A_3).$$

In our extensive hemoglobin studies, we determined highly accurate oxygen equilibrium curves of Hb with an automatic oxygenation apparatus (1-3) and analyzed them by a nonlinear least-aquares curve-fitting method (3,4) to obtain best-fit values of A's, K's, and other oxygenation parameters such as median oxygen pressure and maximal slope of the Hill plot (3). We stressed that it is very essential to the evaluation of these oxygenation parameters with reasonable accuracy that oxygen saturation must be determined in a wide range (typically, covering Y = 1% to 99.9%) with high accuracy, especially at both the extremes of saturation, so that heavier weights are applied on both the ends of the equilibrium curve.

Recently, Gill and his coworkers (5) also determined oxygen equilibrium curves using their thin-layer optical technique (6) and evaluated oxygenation parameters by a least-squares method. The most conspicuous feature of their results was that the A_3 value (β_3 value by their terminology) was always practically zero, indicating an unmeasurably low concentration of the triply oxygenated species throughout oxygenation process. The zero value of A_3 immediately indicated that $K_3 = 0$ and K_4 is undefinable. This result forms strong constant to ours that gave small but definitely positive A_3 values.

The best diagnostic way to examine this inconsistency between the two groups' observations is to test the asymptotic nature of the Hill plot by experiment since the upper asymptote must have a slope of unity if $A_3 \neq 0$ but a slope of two if $A_3 = 0$. OUr careful experiments directed to this point prove that this slope is actually unity and, hence, A_3 cannot be zero.

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KINETIC COOPERATIVITY IN HEMOGLOBIN. Lionel P. Murray, Eric R. Henry, James Hofrichter, Naoya Shibayama, Takashi Yonetani, and William A. Eaton. Laboratory of Chemical Physics, NIDDK, NIH, Bethesda, MD; Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA.

We have employed time-resolved absorption spectroscopy in photodissociation experiments with nanosecond lasers to investigate the origin of cooperativity in the kinetics of carbon monoxide binding to hemoglobin. For human hemoglobin the bimolecular rate of carbon monoxide binding to the R state at neutral pH and room temperature is about 60 times faster than the rate of binding to the T state. The minimal kinetic model for ligand binding and dissociation which incorporates geminate rebinding is a two-step process: entry of the ligand into the protein followed by binding to the heme (or escape back into the solvent) in the overall association reaction, and breaking of the heme-ligand bond followed by exit of the ligand from the protein into the solvent (or rebinding to the heme) in the overall dissociation reaction. This model immediately raises the possibility that the rates of entering and leaving the protein may be altered by the quaternary structure, and therefore contribute to kinetic cooperativity in the overall rates of binding or dissociation.

To answer this question we have studied diliganded metal-hybrid hemoglobins which can be switched between the R and T states by changes in solvent conditions. We have recently reported our results for the hybrid in which the iron(II) was replaced by cobalt(II) in the β subunits (Murray, Hofrichter, Henry, Ikeda-Saito, Kitagishi, Yonetani, and Eaton, Proc. Natl. Acad. Sci. USA 85, 2151-2155, 1988). Here we report the results for the carbon monoxide complex of hemoglobin in which the iron(II) of the α subunits has been replaced by the unreactive nickel(II). Lowering the pH from 8.0 to 6.5 switches the population of diliganded α(Ni)β(Fe-CO) tetramers from mostly R-state molecules to mostly T-state molecules, as judged by the amplitudes of the fast and slow bimolecular phases. The geminate yield, which is the probability that the ligand rebinds to the heme from within the protein, is found to be 35% for the R-state, while for the T-state it is less than 1%. These results are very similar to what was found for the α(Fe-CO)β(Co) hybrid hemoglobin.

According to the simplest kinetic model, the results indicate that carbon monoxide enters the protein in the R and T quaternary conformations at the same rate; the 60-fold decrease in the overall binding rate of carbon monoxide to the T state compared to the R state is almost completely accounted for by the decreased probability of binding after the ligand has entered the protein for both α and β subunits. The geminate yield in the T state is too small to measure the associated relaxation time, but because of the high sensitivity of our transient spectrometer the relaxation time of 100 ns can be obtained from the measurement of the associated spectral change of the deoxy photoproduct, assuming that this process competes with ligand rebinding. These results indicate that the low probability of binding in the T-state results from a decreased binding rate to the heme, and not from an increased rate of return of the ligand to the solvent. The scaling of the nanosecond geminate processes in the R and T states with the overall association rates in the millisecond time regime has the interesting implication that the functionally significant tertiary conformational changes associated with ligand photodissociation must occur on a sub-nanosecond time scale.

DYNAMICS OF THE HEMOGLOBIN QUARTERNARY STRUCTURE SWITCH FROM TIME-RESOLVED UV RESONANCE RAMAN SPECTROSCOPY. Chang Su, Young Park, and Thomas G. Spiro, Department of Chemistry, Princeton University, Princeton, New Jersey 08544-1009, USA.

Photolysis of the CO adduct of hemoglobin produces an unrelaxed deoxy protein which undergoes a series of changes, detectable via absorption and resonance Raman spectroscopy of the heme group, culminating in a ~ 20 μs transition between fast- and slow-recombining protein. This last relaxation is believed to involve the rearrangement of the subunits from the R to the T quarternary states. We have undertaken to monitor motions of the protein at points distant from the heme group by recording resonance Raman spectra with ultraviolet excitation. When RR spectra excited at 229 nm are compared for oxy and deoxy Hb, distinctive differences are seen for bands assignable to the ν_{8a} and ν_{8b} ring modes (1615, 1600 cm^{-1}) of tyrosine, and to the W_3 ring mode (~1555 cm^{-1}) of tryptophan. These differences are attributed to the altered H bonds of Tyr-α45 and Trp-β37 at the $\alpha_1\beta_2$ interface in the R vs. T states. When 229 nm excitation is used to monitor the RR spectrum of HbCO before and after a 10 ns 532 nm photolysis pulse, the difference spectrum is initially featureless. The characteristic R-T difference signals of tyrosine and tryptophan then grow in as the delay time between pump and probe pulses approaches 20 μs. At earlier times, ~5μs, an altered difference signal is seen for tyrptophan, but not tyrosine. These results imply that 1) the oxy minus deoxyHb UVRR difference signals for tyrosine and tryptophan do represent R-T structure markers, since they are not produced by ligand dissociation at early times; 2) these signals arise from the $\alpha_1\beta_2$ interface, the principal site of subunit rearrangement, and they monitor this rearrangement at ~ 20μs; 3) an intermediate is formed with an altered tryptophan signal, suggesting that the rearrangement is a stepwise process involving an initial alteration in the flexible joint region around Trp-β37.

BLOOD SUBSTITUTES - CURRENT STATUS AND PROBLEMS

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Alternatives to human red blood cells to be used for transfusion are being developed witn increasing intensity. Such "blood substitutes" would find many applications, including resuscitation fluids to be used in hemorrhagic shock and other emergencies, preservation of organs for transplantation, elective transfusion where crossmatching is difficult and in perfusion of the heart during angioplasty procedures. To be successful, a blood substitute must be at least as efficacious as blood: it must carry and release oxygen, it must not be inherently toxic, and it must be free of pyrogens and viruses. In addition, it must be retained in the circulation long enough to be useful, and it must be sterile. Although data are lacking, it is believed that oxygen binding of these solutions should be similar to that of blood under physiological conditions, the viscosity should be as low as possible, and the solution should not exert a higher colloid osmotic pressure than whole blood.

The widely held perception of an enormous commercial market has stimulated a profusion of potential products. Although much clinical experience has accumulated with the perfluorocarbon emulsions, hemoglobin-based blood substitutes are now thought to be more promising, because they carry more O_2 and are potentially less toxic. Microencapsulated hemoglobin also has been studied, but problems with RE blockade have slowed progress.

Unmodified hemoglobin, hemoglobin that has been internally crosslinked, polymerized crosslinked-hemoglobin, and hemoglobin that is conjugated to carriers such as dextran and polyethyleneglycol are also under intense study. To date, only unmodified hemoglobin and crosslinked-polymerized hemoglobin have been administered to human volunteers. However, a number of crosslinked hemoglobins from both human and animal sources appear to be at least as promising.

The apparently slow rate of progress in the field is due to problems of scaleup technology, control of methemoglobin formation, and lack of uniformity in evaluation of potential products. Although efficacy of hemoglobin-based blood substitutes is no longer a serious issue, toxicity is. Residual impurities contribute to a variety of toxic renal, hepatic, and cardiovascular effects, hyperoncotic solutions cause potentially serious shifts of water in the body, and free hemoglobin in the circulation could facilitate the formation of toxic O_2 radicals. Furthermore, some preparations activate complement and may bind bacterial endotoxins in vivo.

During the past 3 years, the US Army has supported the industrial scaleup of one such product, human hemoglobin crosslinked internally with Bis-(3,5-dibromosalicyl)-fumarate (DBBFHb). Although the product is not yet ready for human use, it fulfills many of the criteria of a blood substitute and supports life in totally exchange-transfused pigs.

CARBOXYMETHYLATED CROSS-LINKED HEMOGLOBIN A AS A POTENTIAL BLOOD SUBSTITUTE. James M. Manning. The Rockefeller University, New York, NY 10021.

Experimental conditions have been employed in which human adult hemoglobin A is treated with sodium glyoxylate and sodium cyanoborohydride under defined conditions to achieve selective modification of the NH_2-terminal residues of the α- and β-chains with carboxymethyl groups ($HbNHCH_2COOH$) with minimal reaction of the ε-NH_2 groups of lysine residues. The selectivity of the carboxymethylation reaction for the NH_2-terminal residues can be further enhanced if the reductive alkylation is carried out with deoxy hemoglobin instead of oxy hemoglobin. Under these conditions the amount of desired derivative is about 75% in a one-step procedure. Proof of structure was obtained by peptide mapping, amino acid analysis, and proton NMR of standards. The carboxymethylated hemoglobin tetramer has a much lower oxygen affinity (P_{50} = 30-35 mm Hg) than unmodified hemoglobin A (P_{50} = 9 mm Hg) with an unchanged Hill coefficient. X-ray diffraction analysis of the Hb derivative shows that the carboxymethyl group occupies nearly the same positions and interacts with the same residues as does the hemoglobin - CO_2 (carbamino) adduct ($HbNHCOOH$). Thus, the functional properties of the carboxymethyl and the carbamino derivatives of hemoglobin are quite similar but they differ in their stability. The carbamino derivative is quite labile whereas the carboxymethyl derivative is very stable. The presence of a covalently bound negative anion interacting with Ser-131(α) and Arg-141(α) and, on the β-chains interacting within the DPG cleft is the probable structural basis for the lowered oxygen affinity.

Any hemoglobin derivative that is to be considered as a blood surrogate must be prevented from dissociating into its constituent subunits. Glycolaldehyde, a latent cross-linking agent, reacts slowly with proteins and the cross-linking is subject to experimental manipulation to achieve enrichment of a particular population of cross-linked tetramers, i.e., 64,000 or 128,000 or greater molecular weight. After cross-linking of carboxymethylated hemoglobin the P_{50} of the mixture is reduced to about 14 mm Hg and there is a lowering of the Hill coefficient to a value of 2.0. Cross-linked, carboxymethylated hemoglobin species are also reactive with both CO_2 and chloride so that the ultimate P_{50} that is achieved is in the range of 25-30 mm Hg. Therefore, cross-linked carboxymethylated, hemoglobin A releases its oxygen more readily than does unmodified hemoglobin. The ultimate oxygen affinity of the hemoglobin derivative is a function of the state of ligation of the hemoglobin during the cross-linking reaction. Thus, if <u>oxy</u> hemoglobin is cross-linked then the oxygen affinity is increased, i.e., that conformation is frozen. If <u>deoxy</u> hemoglobin is cross-linked then the oxygen affinity is lower than that of unmodified Hb. Therefore, the final oxygen affinity of any cross-linked hemoglobin derivative will probably be a function of the oxygenation state of the hemoglobin when it is cross-linked. Studies on the site of cross-linking as well as on the clearance time of these cross-linked carboxymethylated derivatives are in progress. (Supported in part by the U.S. Army, DAMD-17-88-C-8169).

THE GENETICS OF THE β^S GENE.

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The understanding of the genetics of the β^S gene have advanced considerably in the last few years. Due to the methodological breakthroughs in molecular biology the questions of how many times the mutation arose in the world and where, are now answerable. In addition, practical bonuses from this seemingly abstract question have been derived.

Following the discovery by YW Kan of the a polymorphic site (a sequence that has inter-individual variations) detectable by HapI 3' to the β^S gene, and the further detection of many more polymorphic sites surrounding the β gene, each chromosome 11 could be defined by a haplotype (an individual set of polymorphic sites) in the area of the cluster of the β-like genes. Since the polymorphic sites are so close to each other (linked) they are inherited in block due to the low level of recombination. Furthermore, since each population has about 5 common haplotypes and several more uncommon ones, one can ask: in what haplotype background does the β^S gene sit? Initial analysis demonstrated that the β^S gene was associated with several haplotypes in Black individuals in this continent, suggesting strongly that the origin of the mutation was multicentric.

Work of our laboratory, in collaboration wit h Dominique Labie's group in Paris, has shown that the β gene arose at least three times in Africa, and in defined geographical areas of that continent in which one haplotype is exclusively or overwhelmingly associated with the mutation. Furthermore, haplotype analysis in India, has demonstrated that the β^S gene is associated almost exclusively with one haplotype in spite that the endogamic tribal population bearing the gene, live today isolated, dispersed, and surrounded by non-HbS bearing populations.

Further applications of these findings are:

1. Haplotype determination can help in prenatal diagnosis, and due to point 2, it can also help in predicting severity.

2. Haplotypes can help understand the phenotypic diversity of the disease. The β^S gene is linked to the γ gene that can modulate HbS polymerization. One of the African haplotypes and the Arab-Indian haplotype are associated with higher HbF production and less dense cells, than the other haplotypes, and consequently a milder phenotype.

3. Haplotypes can be used as anthropological markers, that help in the determination of gene flow, in the tracking of migration and the origin of gene in mediterranean populations and the Indian tribals. We have been able to define the origin of the β^S in Sicily, North Africa and by extension Greece and Turkey. The unicentric origin of Indian tribals can be deduced from their β^S/haplotype association. The data has also served to assess the composition by port of origin of Blacks in the New Continent.

4. Each haplotype represents a useful tool in defining the mechanism of HbF control.

REGULATION OF THE ERYTHROPOIETIN GENE: EVIDENCE THAT THE OXYGEN SENSOR IS A HEME PROTEIN. M.A. Goldberg, S.P. Dunning, and H.F. Bunn. Howard Hughes Medical Institute, Brigham & Women's Hospital, Harvard Medical School, Boston, MA, 02115.

Erythropoietin (Epo), the hormone that stimulates red blood cell production, is synthesized in the kidney and liver in response to hypoxia. The human hepatoma cell line, Hep3B, regulates its production of Epo in a physiologic manner. It can be induced by either hypoxia or cobalt chloride to markedly increase expression of Epo mRNA as well as production of biologically active and immunologically distinct Epo protein. However, the sensor mechanism through which these stimuli exert their effects is unknown. We have found that in addition to hypoxia and cobalt, nickel and manganese also stimulate Epo production in a dose-dependent fashion. The lack of an additive effect on Epo production by various combinations of these different stimuli provides evidence that they all work through a common pathway. Furthermore, cycloheximide experiments demonstrate that new protein synthesis is required prior to the induction of increased levels of hypoxia- or cobalt-induced Epo mRNA. Based on these findings we propose that the oxygen sensor is a heme protein which is exquisitely dependent on the oxygen tension to which Epo-producing cells are exposed. When the oxygen tension is sufficiently low, this heme protein is in a deoxy conformation and triggers increased expression of Epo. Conversely, when the oxygen tension is sufficiently high, the heme protein is in its inactive oxy conformation and does not stimulate Epo production. We tested this hypothesis by monitoring hypoxia-, cobalt-, and nickel-induced Epo production after blocking heme synthesis using desferrioxamine or 4,6-dioxoheptanoic acid. When heme synthesis was blocked, Epo production by all of these stimuli was markedly inhibited. These results indicate that cobalt and nickel also exert their effects through their respective metalloporphyrins. The inhibition of Epo production at low partial pressures of oxygen by carbon monoxide provides additional evidence that a heme protein is integrally involved in the oxygen sensing mechanism. It is known that cobalt protoporphyrin binds O_2 with low affinity and that nickel protoporphyrin does not bind O_2 at all. Furthermore, neither of these metalloporphyrins bind carbon monoxide. Hence, it is not surprising that we observed no inhibition of either cobalt- or nickel-induced Epo production by carbon monoxide. Incubation of the Hep3B cells under the various conditions described above in the presence of carrier-free [^{3}H]-leucine showed that the observed changes in Epo production were specific for Epo and could not be accounted for by differences in overall protein synthesis. In summary, these results strongly support the hypothesis that the oxygen sensing mechanism involves a specific oxygen receptor rather than a non-specific block in oxidative phosphorylation. We present evidence that the oxygen sensor is a heme protein that recognizes hypoxia by means of a ligand-dependent conformational change and transduces an intracellular signal to increase Epo production and secretion. These experiments provide an explanation for the mechanism by which cobalt stimulates erythropoiesis.

Expression of Bovine Myoglobin cDNA as a Functionally Active Holoprotein in *Saccharomyces cerevisiae*

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We isolated a cDNA clone for myoglobin mRNA from fetal bovine skeletal muscle using a DNA fragment of human myoglobin exon 2 as a probe. The complete coding sequence of myoglobin as well as the 3'- and part of the 5'-nontranslatable sequences with 546- and 66-base pairs, respectively, were determined. The amino acid sequence predicted from the nucleotide sequence was in agreement with that determined in the purified protein from adult bovine cardiac muscle (Han, K-K., Dautrevaux, M., Chaila, X., and Biserte, G. (1970) *Eur. J. Biochem.* 16, 465-471), except for eight amino acid residues: Val-99 → Ilu, Ilu-101→ Val, Asn-122 → Asp, Ala-124 → Gly, Gly-129 → Ala, Ala-142 → Met, Glu-144 → Ala and Lys-145 → Gln. When the myoglobin cDNA was expressed in *Saccharomyces cerevisiae* under the control of the *GAL7* promoter, myoglobin was synthesized as a functionally active holoprotein which bound molecular oxygen reversibly. The amount of myoglobin reached nearly 1 % of the total extractable protein in the yeast. N-terminal sequence analysis of the produced myoglobin revealed a glycine residue at the terminus, indicating that the N-terminus Met was removed in yeast by processing as in native muscle. The molecular size of the Mb protein was indistinguishable from that of adult bovine heart muscle upon 13.6 % SDS-polyacrylamide gel electrophoresis. Systematic replacement of amino acid residues in Mb protein by oligonucleotide - directed mutagenesis with the present expression system is underway to explore the structure - function relationship on this hemeprotein.

STRUCTURAL, FUNCTIONAL AND GENETIC STUDIES OF EARTHWORM HEMOGLOBIN.

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Invertebrate hemoglobins are widely distributed in most invertebrate phyla. Most have chains of 15-17 kDa and are either intracellular with masses to 65 kDa or extracellular with molecular weights to several million. Some hemoglobins of molluscs and arthropods have 2 to 20 myoglobin-like units in single polypeptide chains. Many of these hemoglobins have O_2 equilibria which are highly cooperative and strongly pH dependent. Others have equilibria which are non-cooperative and pH dependent. This great diversity of both form and function provides an attractive set of systems in which to investigate gene structure and evolution and the molecular basis of function. We have chosen to study the giant extracellular hemoglobin of the earthworm as an example of a cooperative multi-subunit system. This hemoglobin comprises four major heme-containing chains a, b, c and d in equal proportions. Non-heme chains with masses of 33-37 kDa are also present. The amino acid sequences of chains a, b, c and d give a molecular mass of 69664 Da for the abcd unit. Chains a, b and c form a disulfide-linked trimer. The native protein is known to comprise 12 subunits and to have a molecular weight near 3.8×10^6. We have determined the non-heme protein content to be about 2200 g per mole of heme-containing chain. These observations require that 4 abcd units are present in each one-twelfth subunit, or 192 heme-containing chains in all. The molecular weight thus accounted for would be 3,348,872. The contribution of the non-heme protein is approximately 422,000 Da or 35,200 Da for each one-twelfth subunit. This is consistent with the known presence of 33-37 kDa chains which appear to be required for assembly of the full-size molecule. On this basis the intact molecule would have 12 non-heme chains, 204 chains in all, and a calculated molecular weight of 3.77×10^6 which is close to that observed.

Oxygen binding measurements of the abc trimer shows that the trimer has at least one oxygenation linked Ca^{2+} - binding site. Structural studies indicate that chains a and c of the trimer are joined by a disulfide bond between cysteinyl residues at position 6 in each chain. This could place the NH_2-terminal sequences of chains a (ADDEDC-) and c (DEHEHC-) adjacent to one another. Such a structure with 7 carboxyl groups and 2 histidines is a prime candidate for the Ca^{2+} binding site. Oxygenation measurements of the whole molecule show that 0.77 protons are released and 0.37 Ca^{2+} ions taken up for each O_2 bound at pH 7.4, or 2 protons released per Ca^{2+} bound. Since the Bohr effect is zero in the absence of Ca^{2+}, proton release and Ca^{2+} uptake must be tightly coupled. We suggest that the two histidines in chain c at positions 3 and 5 may be the residues which release protons upon the binding of Ca^{2+}. These histidyl residues would form electrostatic links with residues at the same position in chain a in deoxyhemoglobin. Oxygenation would be accompanied by a perturbation in which the histidines would move away thus lowering their pK values. The movement, however, would depend on the presence of Ca^{2+} ions. Oxygenation of the abc trimer has a low cooperativity but addition of chain d results in a product with oxygenation characteristics closely resembling those of the intact molecule. The results show that the major features of oxygen binding by the intact molecule are present in the partially reassembled molecule obtained by adding chain d to the trimer.

The genes of vertebrate globins are characterized by the presence of 3 exons separated by 2 introns. A third intron, predicted to have existed in the ancestral globin gene and to have been lost in animal evolution, occurs in the genes of plant globins. We have cloned and sequenced the gene encoding globin chain c from Lumbricus terrestris. This gene has exactly the same exon-intron organization that characterizes genes of vertebrate globins. The exact positions of the splice junctions have been conserved, therefore, for at least 600 million years, the estimated time of divergence of annelids and the ancestors to vertebrates.

The Alkaline Bohr Effect of Hemoglobin Studied by Protein Engineering.

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Histidine (HC3)beta, the only chloride independent Bohr group to have been identified, forms a salt bridge with Asp (FG1)beta in deoxy-hemoglobin without mediation of a chloride ion, yet results obtained from proton NMR studies by Russu and Ho continue to contradict the chloride independency of this Bohr residue. His (FG1)alpha and Asp (F6)alpha are well ordered in the T state but they are both disordered in the R state. By deuterium exchange experiments, Ohe and Kajita found that this immidazole's pK shifted as much as that of His (HC3)beta upon ligation indicating that the immidazole group of His (FG1)alpha may also be a Bohr residue. In order to gain better insight into the involvement of these histidyl residues in the alkaline Bohr effect of hemoglobin A, we have replaced these histidyl groups with glutamyls by site-directed mutagenesis and produced the mutant hemoglobins using the protein expression system of Nagai and Thogersen. Differential maps of these engineered mutants and Hb A from X-ray analyses show that the structural changes of these engineered mutants are located only in the vicinity of the mutation sites. Oxygen equilibrium analyses of engineered mutants show that the substitutions of Gln for His at (HC3)beta and (FG1)alpha sites result in decreased alkaline Bohr effect of nearly 50% and 25% that of Hb A, respectively, in the presence of 0.1M chloride. Under chloride free HEPES buffer condition, these Gln (HC3)beta and Gln (FG1)alpha mutants exhibit <10% and 50% of normal Bohr effect ,respectively, indicating that His (HC3)beta and His (FG1)alpha in Hb A predominate in the chloride independent proton release during the course of hemoglobin oxygenation. Results obtained in this study are consistent with previous findings on the His (HC3)beta as a chloride independent alkaline Bohr group, and demonstrating that His (FG1)alpha indeed is also involved in the alkaline Bohr effect of hemoglobin as Ohe and Kajita predicted. Discussion on the difference between these two chloride independent Bohr groups is presented.

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A BACTERIAL GLOBIN GENE. Kanak L. Dikshit and Dale A. Webster, Department of Biology, Illinois Institute of Technology, Chicago, IL 60616.

A soluble dimeric hemeprotein, structurally and functionally similar to plant and animal hemoglobins, has been reported in the gram-negative bacterium, *Vitreoscilla*. We identified the genomic segment for *Vitreoscilla* hemoglobin (VtHb) production and cloned it in *E. coli* as a 1.4 kb *Hin*dIII-*Sal*I fragment of the *Vitreoscilla* genome. Subcloning of this fragment showed the presence of the *Vitreoscilla* globin gene (*vgb*) along with its promoter. A single copy of this gene on the *Vitreoscilla* genome was identified after Southern blot analysis. We determined the nucleotide sequence of *vgb* and its 5' upstream regulatory region after cloning on M13 mp18 and M13 mp19. The coding region of *vgb* spans 438 nucleotides and matches the previously reported primary protein sequence of VtHb (146 aa). The nucleotide sequence of *vgb* has homologous regions with some other globins and about 30% sequence homology with some leghemoglobins. However, the non-coding 5' upstream regulatory region is remarkably similar to the *E. coli* promoter region with sequences TATAAT at -10 and TTAAGA at -35. An approximately 500 base long RNA transcript specific for *vgb* was detected after Northern hybridization. The relative amount of this mRNA increased in cells grown at low levels of oxygen, suggesting a regulatory role for oxygen on the expression of *vgb*.

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Genetic Engineering of Low Oxygen Affinity Mutants of Human Hemoglobin

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We have used the principles of genetic engineering to design an approach which should improve the treatment of solid tumors by radiation therapy. Cell killing in solid tumors by x-irradiation depends, in part, on the extent of oxygenation of the cancerous cells within the tumor mass. Most solid tumors are not uniformly oxygenated, and parts of the tumor are significantly hypoxic. Radiation therapy of these tumors could be more effective if more oxygen can be delivered to the tumor. We have designed a series of low oxygen affinity mutants of human ß-globin which should release more oxygen to hypoxic tumors than is released by naturally occurring human ß-globin. We have chosen to synthesize human ß-globin genes which encode proteins with low oxygen affinity, including Hb Beth Israel and Hb Agenogi. Altogether we have 5 mutations at amino acid positions 90, 102 and 108. Futhermore, it is well known that the trout IV hemoglobin molecule exhibits a much lower oxygen affinity than these mutants and a strong Root effect. To see if a portion of the trout IV ß chain could confer low oxygen affinity to human hemoglobin, we have exchanged amino acids 86-100 of the human ß chain for the corresponding amino acids in trout IV. The oxygen affinity of these newly designed hemoglobin molecules remains to be tested after expression in *E. coli* and in transgenic mice.

SITE-DIRECTED MUTAGENESIS OF SPERM WHALE MYOGLOBIN: ROLE OF HIS E7 AND VAL E11 IN LIGAND BINDING

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Sperm whale myoglobin mutants were constructed from a totally synthetic sperm whale myoglobin gene (1) using site-directed mutagenesis to replace the highly conserved distal histidine (E7) and distal valine (E11) residues. The His E7 residue is thought to provide a key role in allowing myoglobin to discriminate between CO and O_2 binding, whereas the exact role of Val E11 remains in question. We have replaced His E7 with Gly, Val, Phe, Met, Arg, Cys, Thr, Asp and Lys and Val E11 with Ala, Ile and Phe to provide a range of side chain volumes and polarities. In addition, we have constructed double mutants where His E7 was replaced with Gly and Val E11 was replaced with Ala, Ile and Phe. All mutant proteins expressed in Escherichia coli to approximately 10% of the total soluble cell protein as heme containing myoglobin. All mutant proteins could be reduced with dithionite and bound O_2 and CO reversibly. However, removal of His E7 increased the rate of autooxidation 40 to 300-fold. The His E7 mutants showed 10 to 150-fold decreased O_2 affinities with the most dramatic change observed for the His E7-Val and His E7-Phe mutants where O_2 affinities decreased 100 and 130-fold respectively. These very low O_2 affinities are due mainly to the large O_2 off rates of 26,000 s^{-1} and 12,250 s^{-1} for the Val and Phe mutants respectively. CO affinities for the His E7 mutants increased by approximately 3 to 15-fold. The distal histidine of myoglobin thus discriminates between CO and O_2 binding by sterically hindering bound CO and by stabilizing bound O_2 through hydrogen bonding.

The CO affinities for Val E11-Ile and Val E11-Phe decreased 2 to 4-fold suggesting additional steric hindrance provided by the larger Ile and Phe residues. The CO affinity for the Val E11-Ala mutant increased only slightly, however the CO affinity for the His E7-Gly, Val E11-Ala double mutant increased approximately 20-fold. These data suggest that Val E11 may play a significant role in myoglobin ligand binding.

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ALTERATION OF SPERM WHALE MYOGLOBIN AXIAL LIGATION BY SITE-DIRECTED MUTAGENESIS

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Using site-directed mutagenesis, we have generated four mutant proteins of sperm whale myoglobin with altered axial heme-iron ligations. These mutant proteins were generated by substitution of the proximal histidine with tyrosine (HisF8-Tyr) and cysteine (HisF8-Cys), substitution of the distal histidine with tyrosine (HisE7-Tyr), and substitution of the distal valine with glutamate (ValE11-Glu). We have examined these mutant myoglobin (Mb) proteins in their various oxidation states by UV-visible, electron paramagnetic resonance (EPR), and resonance Raman spectroscopies. Comparisons to hemoglobin M mutants, in which the α or β chains have altered axial ligands, show many similarities.

The optical spectrum of met HisE7-Tyr Mb is nearly identical to Hb M Saskatoon (β HisE7-Tyr). This protein is easily reduced and binds CO, however, attempts to bind O_2 resulted in rapid autooxidation. EPR of the oxidized species shows a high-spin rhombic split signal ($g=6.64$, $g=5.33$), suggesting the distal tyrosine is the axial ligand. This high rhombicity is also seen with catalase and HbM Saskatoon which are tyrosine liganded. Resonance Raman confirmed that met HisE7-Tyr Mb is 5-coordinate high-spin with tyrosine as the axial ligand, while in the deoxy state the axial ligation switches to the proximal histidine (HisF8).

Heme-reconstituted HisF8-Tyr Mb can be reduced and binds CO while the oxy species is short-lived due to rapid autooxidation. EPR of the ferric protein shows at least two high-spin species. Resonance Raman reveals a 5-coordinate high-spin species with significant frequency shifts from wild-type Mb. Ferrous heme-reconstituted HisF8-Cys Mb binds O_2 stably in contrast to the tyrosine mutants, and binds CO with an optical spectrum similar to that of MbCO. EPR of the oxidized protein shows multiple high-spin species suggesting multiple ligation states.

ValE11-Glu Mb has distinctly different optical properties than wild-type Mb or the tyrosine Mb mutants. EPR shows a high-spin rhombic signal ($g=6.82$, $g=5.09$) and a small axial component ($g=5.96$), which is identical to that of Hb M Milwaukee (β ValE11-Glu) in which X-ray analysis has identified GluE11 and HisF8 as the axial ligands. Resonance Raman confirms a 6-coordinate high-spin species in the met state, and a 5-coordinate high-spin species in the deoxy state.

The Role of Distal Residues in Ligand Binding to R-state Human Hemoglobin : E7 and E11 Site Directed Mutants

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The roles of the strongly conserved distal residues E7His and E11Val in ligand binding were studied using a series of R-state $\alpha(\text{mutant})_2\beta(\text{native})_2$ and $\alpha(\text{native})_2\beta(\text{mutant})_2$ hemoglobins. The mutants examined were α E7His→ Gly, Gln ; α E11Val→ Ala, Ile, Leu ; β E7His→Gly, Gln, Phe ; β E11Val→ Ala, Ile, Leu. Comparison of data from native Hb and mutant proteins allowed assignment of distinct rates for the α and β subunits within R-state Hb. The native subunits of all hybrid proteins gave kinetic parameters equal to those observed for the corresponding subunit of normal R-state Hb.

For O_2 binding, α E7His→Gly and α E11Val→Ala both showed increased O_2 association rates, while the remaining α mutants showed normal O_2 association rates. A large increase ($k = 620\ s^{-1}$) in the α subunit O_2 dissociation rate was observed for the α E7His→Gly mutant, graphically demonstrating the importance of the α E7His--O_2 hydrogen bond in stabilizing the bound O_2 molecule. Smaller increases in the O_2 dissociation rate were observed for the α E7His→Gln and α E11Val→Ala proteins, with the remaining mutants showing native-like O_2 dissociation rates. Substantial increases in the CO association rate were observed for α E7His→Gly,Gln and α E11Val→Ala, whereas the α E11Val→Ile and α E11Val→Leu proteins showed native-like CO association rates. All α mutants showed native-like CO dissociation rates. The α subunit methyl isocyanide (MNC) association rate showed no obvious dependence on the nature of the residue at positions E7 and E11, with all α mutants showing normal MNC association rates. The α E7His→Gly and α E7His→Gln proteins both showed large decreases in the MNC dissociation rate, while the remaining α mutants yielded normal rates.

In contrast to the results for α subunits, the rate parameters for ligand binding to β subunits within R-state Hb showed a remarkable insensitivity to the nature of the residue at positions E7 and E11. For each of the ligands O_2, CO, and MNC, all β mutants showed normal ligand association rates except for β E11Val→Ile, which exhibited a 10-50 fold decrease in the association rate constants for the three ligands. All β mutants showed native-like O_2 and CO dissociation rates. With MNC, only the β E11Val→Ala and β E11Val→Leu proteins showed altered MNC dissociation rates, a large decrease in each case.

Most of the above results are readily interpreted with reference to available structural data for deoxy and liganded Hb.

Myeloperoxidase and Thyroid Peroxidase, Two Enzymes, with Separate and Distinct Physiological Functions, are Members of the Same Gene Family

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Myeloperoxidase, which is found in granulocytes and monocytes, plays a major role in the H_2O_2-dependent microbicidal systems of neutrophils. In the presence of H_2O_2 and halide, chiefly Cl^- *in vivo*, myeloperoxidase catalyzes the two-electron oxidation of Cl^-, leading to the formation of HOCl, which is ultimately toxic to the ingested organism. Human myeloperoxidase is primarily synthesized as a glycosylated precursor protein of 89-91 kDa, which is subsequently cleaved into the heavy and light chains of 55-60 kDa and 10-15 kDa, respectively. The mature protein is composed of two each subunit in a tetrameric structure of 120-160 kDa and contains two identical prosthetic groups per tetramer. The prosthetic group has been considered to be an iron chlorin. Evidence for the coordination of histidine to the iron center and the presence of the distal histidine residue have been proposed from the spectroscopic studies of the enzyme. On the other hand, thyroid peroxidase plays a central role in the biosynthesis of thyroid hormones: the iodination of tyrosine residues on thyroglobulin and the intramolecular coupling reaction of iodinated tyrosines, leading to the formation 3-3'-5-triiodothyronine (T3) and thyroxine (T4). The iodination reaction occurs via two-electron oxidation of I^-. The human enzyme is a monomeric protein, and its prosthetic group is considered to be closely related to protoheme. While the structural and functional properties of these two human peroxidases are quite different, their halogenation reactions share a unique feature in that compound I undergoes a two electron reduction to the native ferric state, without passing through compound II.

Recently the nucleotide and the deduced amino acid sequences of human myeloperoxidase and thyroid peroxidase have been established. Unexpectedly, we have found significant similarities in their nucleotide and amino acid sequences. The global similarities of the nucleotide and amino acid sequences are 46% and 44%, respectively. The similarities are most evident within the coding sequence, especially that encoding the myeloperoxidase functional subunits. These results clearly indicate that myeloperoxidase and thyroid peroxidase are members of the same gene family and diverged from a common ancestral gene. The residue at 416 in myeloperoxidase and 407 in thyroid peroxidase were estimated as possible candidates for the proximal histidine residue that link to the iron centers of the enzymes. The primary structures around these histidine residues were compared with those of other known peroxidases. The similarity in this region between the two animal peroxidases (amino acid 396-418 in thyroid peroxidase and 405-427 in myeloperoxidase) is 74%; however, those between the animal peroxidases and other yeast and plant peroxidases are not significantly high, although several conserved features have been observed. The possible location of the distal histidine residues in myeloperoxidase and thyroid peroxidase amino acid sequences are also considered.

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Expression of β6-Mutagenized Human β-Globin cDNAs in E. coli

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Sickle cell disease results from a single amino acid change of glutamic acid to valine at the sixth position (β6 Glu →Val) in the β-globin chain of hemoglobin. In an effort to clarify the role of β6 Val in sickling we have constructed a number of β6-modified globin cDNAs using synthetic oligonucleotide primers and a phosphothioate mutagenesis protocol (Amersham). A variety of amino acid substitutions were made at the β6 position in an effort to determine solubility, kinetics of polymerization and stability properties of *in vitro* reconstituted hemoglobin tetramers. Normal and β6-modified globin chains are being expressed in E. coli using the vector system developed by Nagai et al. (Nature 309:810, 1984). Expressed chains are isolated as λCII-β-globin fusion proteins using a simple and rapid reversed-phase FPLC protocol following solubilization of enriched fusion protein in low concentrations [(1-2% (v/v)] of formic acid in an attempt to minimize exposure to more harsh denaturants, and thereby hopefully facilitate cleavage and tetramer assembly *in vitro*. Confirmation of β-chain mutants is made by direct DNA sequence analysis of the expression vector as well as by micro-sequence analysis of electroblotted fusion protein using a pulsed-liquid protein sequencer (ABI Model 477A). Purified fusion proteins are then cleaved using activated Factor Xa resulting in authentic β globins containing modifications at the β6 position. Preliminary reassembly studies *in vitro* using normal β- and α-globin chains solubilized in the cleavage buffer (pH 8.0) indicate less than 10% efficiency in forming hemoglobin tetramers. Functional characterization and polymerization properties of the reconstituted hemoglobins are currently in progress. This work was supported in part by grants from the National Institutes of Health (RO1 HL32908) and by a Comprehensive Sickle Cell Center Award (HL38632).

MANIPULATION OF HEMOGLOBIN FUNCTION BY SITE-DIRECTED MUTAGENESIS

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Importance of the amino acid residues participating in the inter-subunit contacts at the α1-β2 interface in cooperative oxygen binding by hemoglobin was proved by studying hemoglobin mutants in which one of those residues is substituted. In deoxyHb, Tyr-42(C7)α1 forms a hydrogen bond with Asp-99(G1)β2 while that bond is broken in oxyHb. Therfore, it is predicted that Tyr-42α contributes to stabilizing the T quaternary structure. However, no direct way to prove this has been available since no natural mutant having a substitution for Tyr-42α has been discovered.

We prepared two artificial hemoglobin mutants in which Tyr-42α is replaced by His or Phe by protein engineering including a site-directed mutagenesis technique (1). The mutations were introduced into the human α globin gene by using M13 phage vector. The mutant α globins were synthesized in *E. coli* cells by using an expression vector pLcIIFXβ-globinFXα-globin(nic$^-$) which contained the λ phage pL promotor, DNA fragments coding for parts of λ CII protein and β globin, a short oligonucleotide, FX, encoding a tetrapeptide Ile-Glu-Gly-Arg, and the entire α globin gene (J. Tame & K. Nagai, unpublished). It was verified by mass spectrometry on tryptic digests that each synthesized α globin had the amino acid substitution aimed but no unexpected structural alteration.

Compared to Hb Tyr-42α (Hb A), Hb Phe-42α showed an 8 times higher oxygen affinity, a reduced (40% of normal) Bohr effect, almost completely diminished cooperativity (n = 1.1), and a very small response to IHP. Hb His-42α showed oxygen binding properties which are intermediate between those for Hb Phe-42α and Hb A. UV derivative and UV oxy-minus-deoxy difference spectra indicated that oxyHbs Phe-42α and His-42α assume a normal R quaternary structure whereas the quaternary transition to the T state is restricted highly in deoxyHb Phe-42α and considerably in deoxyHb His-42α. Proton NMR data were consistent with these spectrophotometric results. The T marker signal at 9.4 ppm assigned to the hydrogen bond between Tyr-42α1 and Asp-99β2 was completely absent from Hb Phe-42α. Resonance Raman scattering indicated that the Fe-N_ε(His-F8) bond is less stretched in deoxyHbs Phe-42α and His-42α than in deoxyHb A.

Thus, the present study proves that Tyr-42α is a key residue contributing to stabilizing the T state and, hence, to cooperative oxygen binding.

1. Nagai, K., Perutz, M.F. & Poyart, C. (1985) *Proc. Natl. Acad. Sci. USA* 82, 7252-7255.

Isolation of *Glycera dibranchiata* Monomer Hemoglobin IV cDNA

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The erythrocytes of the marine annelid *Glycera dibranchiata* contain hemoglobins that are separable upon gel filtration into high and low molecular weight fractions. The low molecular weight fraction consists of three major hemoglobin monomers (Kandler & Satterlee (1983) *Comp. Biochem. Physiol. 75B*, 499; Constantinidis, Kandler & Satterlee (1984) *Biochem. J. 225*, 131) with molecular weights of approximately 16kD. These monomers are separable by ion exchange chromatography using CM-cellulose columns.

The erythrocytes of this organism are nucleated. Consequently we have been able to construct a cDNA library from these erythrocytes, proving that at this phylogenetic level the blood cells are transcriptionally active. This task was accomplished by first performing partial N-terminal amino acid sequencing on the three major monomer globins (components II, III, IV). The sequencing results showed a region of identity. A mixed oligonucleotide was synthesized to be complementary to the mRNAs, and clones that hybridized to this probe were isolated from the cDNA library.

The first clone that was sequenced using Sanger's dideoxy chain termination method gave a predicted amino sequence that was identical to the first 26 amino acids of globin IV, except for an N-terminal methionine.

We note that our amino acid sequencing results reveal that the previously published protein sequence (Imamura , Baldwin & Riggs (1972) J. Biol. Chem. 247, 2785) is identical to the amino terminal sequence (first 25 amino acids) of the purified globin II.

These results confirm that the E-7 amino acid residue, most often a distal histidine in oxygen binding heme proteins, is leucine in globin IV. The very slow cyanide ligation exhibited by the *Glycera dibranchiata* methemoglobins (Mintorovitch & Satterlee (1988) *Biochemistry*, in Press; See other poster in this Symposium) is probably due to this substitution. Site-directed mutagenesis of this leucine (work now in progress) will show to what extent this hypothesis is true.

EXPRESSION OF HUMAN β-GLOBIN c-DNA IN E. COLI, STREPTOMYCES & YEAST.

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A number of plasmids have been constructed in an effort to develop systems with which one can obtain experimentally useful quantities of native recombinant globins. Of those expressed in E. coli, several have a Factor X_a recognition sequence (FX) fused to β-globin which in turn was fused to varying lengths of the N-terminal ends of: 1) NS1, a flu virus protein, 2) R32, a P. falciparum protein, 3) E. coli SSB protein, & 4) E. coli galactokinase (GalK). In all cases, the fusion proteins were produced at levels of at least 10 mg/liter of culture. However, in all cases the fusion proteins were insoluble, i.e. confined to the cell lysis pellet. β-Globin c-DNA was also inserted behind the "Trc" promoter in pKK-233-2 (Pharmacia). Induction resulted in the appearance of both soluble and insoluble β-globin at levels only slightly lower than those observed for the fusion proteins above.

In an effort to secrete β-globin into the periplasm of E. coli, the β-globin gene was inserted behind an OmpA secretion signal sequence. Although the fusion protein appeared to be correctly processed, β-globin was not secreted. An attempt was also made to secrete β-globin in Streptomyces based upon the system developed by H. Lichenstein et al. (J. Bacteriol. (1988) 170, 3924), which efficiently exported full length E. coli Galactokinase. The plasmid was constructed having a GalK-FX-β-globin sequence behind a β-galactosidase secretion signal sequence. However, under conditions where Galactokinase was secreted, GalK-FX-β-globin remained with the cells.

Two yeast plasmids containing β-globin were constructed, one with a constitutive promoter (TDH) and β-globin, and the other with an inducible promoter (metallothionein) where ubiquitin was fused directly to β-globin. In both cases, soluble and insoluble β-globin was obtained. The amount of soluble β-globin increased when the cells were grown with raffinose, rather than glucose, as the carbon source.

ELECTROSTATIC INTERACTIONS IN RECOMBINANT HUMAN MYOGLOBIN MUTANTS

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ABSTRACT

A full length cDNA clone for human myoglobin was isolated and the protein expressed in high yield in E. coli. Residue Val68 was replaced by Asn, Asp, and Glu using site directed mutagenesis. These proteins were characterized by isoelectric focusing and by absorption, CD, and nmr spectroscopy. These studies demonstrated that Mb is able to tolerate substitution of the buried hydrophobic residue Val68 by Asn, Asp and Glu. The temperature dependence of the redox potential as well as cyanide dissociation constants were measured for wild type and mutant proteins. In the metaquo derivatives of the Glu and Asp mutants, the negative charge at residue 68 is stabilized by a favourable coulombic interaction with the heme iron. In the absence of this interaction the relatively non-polar protein interior cannot stabilize an isolated buried negative charge and the carboxylate is either protonated or stabilized via a salt bridge with the nearby distal histidine. Hence in the Asp and Glu mutant proteins, both reduction and cyanide binding are accompanied by proton uptake by the protein. Very large changes in redox potential are observed for the Asp and Glu mutants.

The apoproteins were prepared and reconstituted with a chlorophyll derivative. Absorption and fluorescence spectra were quite similar for wild type and all mutant proteins reconstituted with this derivative. From the pH dependence of the absorption spectrum of Zn pyrochlorophyllide *a* in the Glu mutant, the pK_a of the buried glutamate residue was estimated to be 8.9. This increase of 4.4 pH units, over the value for Glu in aqueous solution, provides a measure of the polarity of the protein interior. These results do not support the hypothesis that electrostatic interactions between chromophore and charged residues in the protein are the primary cause of the redshifts observed in the spectra of chlorophylls associated with photosynthetic protein.

LIGAND AND PROTON EXCHANGE DYNAMICS IN HUMAN MYOGLOBIN MUTANTS

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ABSTRACT

Site specific mutants of human myoglobin (Mb) have been prepared in which lysine 45 is replaced by arginine (K45R) and aspartate 60 by glutamate (D60E), in order to examine the influence of these residues and their interaction on the dynamics of ligand binding and proton exchange. The structural consequences of these substitutions were examined by 1- and 2-D ^{1}H NMR. Dynamics were probed by measuring exchange rates for the distal and proximal histidine imidazole NH protons as a function of pH in the metcyano forms, flash photolysis of the CO forms, and the kinetics for the replacement of CO with NO.

The electronic absorption as well as ^{1}H NMR spectra of the CO forms of these proteins are virtually identical, indicating that the structure of the heme pocket is unaltered by these mutations. We observe substantial changes in the kinetics of both CO binding and proton exchange for the mutant K45R, whereas the mutant D60E exhibits behaviour indistinguishable from wild type human Mb within experimental error. K45R has a faster CO on-rate for bimolecular recombination and slower CO off-rate, relative to the wild type. The kinetics for CO binding are independant of pH (6.5-10) as well as ionic strength (0-1M NaCl). The exchange rate of the distal his NH in the metcyano form is substantially lower for K45R than wild type while the proximal his NH exchange rate is unaltered. The exchange behavior correlates well with the experiments of Lecomte et al. (1985) who compared exchange rates for Mbs having lysine at position 45 with sperm whale Mb which has arginine at this position. No such correlation exists for the CO kinetics indicating that the distal his exchange rates reflect primarily the lys→arg substitution at position 45 while the CO kinetics are sensitive to other amino acid differences as well. These experiments demonstrate that a relatively conservative change of a surface residue can substantially perturb ligand and proton exchange dynamics in a manner which is not readily predicted from the static structures.

LIGAND INTERACTIONS IN HEME PROTEINS STUDIED BY PHOTODISSOCIATION AT LOW TEMPERATURES

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A variety of important studies have contributed to our understanding of molecular interactions in heme proteins that control biological oxidation-reduction, and oxygen binding and transport processes. Studies with oxygen carriers (hemoglobin and myoglobin), and oxygen reducing systems such as cytochrome c oxidase, have made use of ligand photodissociation from iron by absorption of visible light. Use of short laser pulses in other laboratories has allowed study of various relaxation processes following photodissociation at room temperature. Use of low temperatures reduces the kinetic energy of the system so that individual processes may be studied. We now distinguish between kinetic energy associated with photodissociation from the electronically activated state and kinetic energy that results from absorption of subsequent photons. These in turn are distinguished from dissipation of kinetic energy to the surroundings. Photodissociation of simple heme proteins (carboxymyoglobin and carboxyhemoglobin) result in the formation of two populations of photodissociated carbon monoxide (B_1 and B_2). These are distinguished by the CO vibrational stretching frequency. The B_2 state has a lower CO frequency and can be preferentially populated by additional increments of kinetic energy resulting from secondary photon absorption. This is readily observed at 10 K. We conclude that B_2 represents a lower energy ground state, and that conversion of B_2 to B_1 with increased temperature (e.g. to 30 K) is entropically driven. Infrared studies indicate that vibrational changes associated with photodissociation are limited almost exclusively to the porphyrin and its axial ligands. We observe that vibrational changes at the hemoglobin cysteine sulfhydryl groups are very small and do not correspond to changes that are observed at room temperature. Photodissociation of oxyhemoglobin yields a similar pattern, but with greater fractional relaxation to the ligated state associated with geminate recombination processes. X-ray absorption studies of oxymyoglobin yield first shell radial distances from iron that are in good agreement with crystallographic studies, but in addition yield radial information about all porphyrin ring atoms.

Acknowledgement. This work was supported in part by grants from the National Institutes of Health (HL-28144 and RR-01739), The Ohio State University Instruction and Research Computer Center, and the Bremer Foundation.

RESONANCE RAMAN STUDIES OF LIGAND BINDING TO *E. coli* SYNTHESIZED HUMAN HEMOGLOBIN MUTANTS

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The technique of site-directed mutagenesis has produced human hemoglobin A and mutant Hbs in *E. coli* having : (1) His(βE7) replaced by Gln, Val, Gly or Phe, (2) His(αE7) replaced by Gln or Gly, (3) Val(βE11) replaced by Ala, Met, Leu or Ile, (4) Val(αE11) replaced by Ala, Leu or Ile, and (5) Phe(CD1) replaced by Gly or Tyr. Soret-excited resonance Raman spectroscopy was employed to investigate the effects of these amino-acid replacements on the bonding interactions between the heme iron (ferrous or ferric) and some exogenous ligands such as dioxygen, carbon monoxide, nitric oxide, cyanide or azide. We have detected the stretching vibrational modes of the Fe^{II}-O_2, Fe^{II}-CO, Fe^{II}-NO, Fe^{III}-CN^-, and Fe^{III}-N_3^- bonds, which are sensitive to changes in ligand geometry and bonding strength. The substitution of His(E7) by Gln in either α- or β-subunits shows no effects on the Fe-O_2 and Fe-CO stretching vibrations, presumably because the N_ε atom of the Gln(E7) can interact with the ligand in the same way as the N_ε atom of the His (E7) (Nagai et al., 1987). While the nature of the E11 residue affects the kinetic behavior of the CO binding, we found no effects on the Fe-CO and C-O stretching frequencies by the Val(αE11) or Val(βE11) replacements. Interestingly, for the oxy Hb mutants, the Fe-O_2 vibrational mode is sensitive to the mutations at the E11 position. The replacement of His(αE7) or His(βE7) by Gly has a profound effect on both Fe-O_2 and Fe-CO vibrations. On the other hand, the Fe-NO vibration exhibits no sensitivity by both E7 and E11 mutations. In contrast, the Co-NO stretching frequency for the nitrosyl cobalt Hb/Mb is very sensitive to distal H-bonding (Yu et al., 1986).

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Oxygen-17 NMR of $^{17}O_2$ Bound to a Picket Fence Porphyrin, Myoglobin and Hemoglobin

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Using solid-state ^{17}O NMR spectroscopy we have investigated Fe-O-O bonding in several oxygen-transport proteins, and model systems. Results on $^{17}O_2$-picket fence porphyrin suggest that both static and dynamic structural information on the Fe-O_2 unit can be obtained via ^{17}O NMR spectroscopy. Thus, we find that the isotropic chemical shifts of the bridging and terminal oxygens are at 1200 and 2000 ppm, respectively, that the anisotropies of the shielding tensors are very large for both oxygens ($\Delta\delta$ = 1800 ppm for the bridging oxygen and $\Delta\delta$ = 3400 ppm for the terminal oxygen), and that the Fe-O-O bond angle is about 130°, with fast rotations around the iron-oxygen axis, at room temperature. Our results also indicate that the ^{17}O nuclear quadrupole coupling constants (QCC) must be relatively small for both oxygens, contrary to initial expectations of very large QCC values arising from the proposed analogy between bonding in the ozone molecule, and the Fe-O_2 unit in oxyhemoglobin.

We have also obtained ^{17}O NMR spectra of human oxy-hemoglobin and sperm whale oxy-myoglobin at liquid nitrogen temperatures. The rigid lattice ^{17}O NMR spectra of these proteins are very similar to those of $^{17}O_2$-picket fence porphyrin. Preliminary progress on model hemerythrin Fe-O clusters, and on oxyhemerythrin, will also be outlined.

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COORDINATION STATES IN FERRIC APLYSIA MYOGLOBIN

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ABSTRACT

Resonance Raman spectra of ferric Aplysia myoglobin in the met and the azide-bound forms have been studied as a function of pH to determine the coordination states of the heme iron atom. The high frequency region of the resonance Raman spectrum has been shown in the past to be a very sensitive indicator of the spin and coordination states of the iron atom in other heme proteins. The data reveal that at high pH the iron atom in the met preparation is six-coordinate and is in a high/low spin equilibrium. As the pH of the protein is lowered and passes through the acid/alkaline transition, at about pH 7.5, the heme becomes five-coordinate. As the pH is lowered further, no other changes in the spectrum are detected. Thus, the heme remains five-coordinate down to pH 4, the lowest value studied. For ferric azide-bound Aplysia myoglobin, at both low and high pH the iron is six-coordinate in a high/low spin equilibrium. These data indicate that changes in heme coordination below pH 6 can not account for the pH induced differences in ligand binding kinetics or the reported changes in the optical absorption spectrum [Giacometti, et al. (1981) J. Mol. Biol. 146, 363-374]. Instead, our data suggest that at low pH, protonation of an amino acid residue in the heme vicinity may cause changes in the environment of the heme influencing both its optical properties and the ligand entrance to the binding site.

The Electronic/Magnetic Properties and Molecular Structure of the Heme in Cyano-met Myoglobin as Influenced by Distal Residues: An ^1H NMR Study.

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We have utilized 2D ^1H NMR NOESY to assign the signals for all of the heme pocket residue side chains in the paramagnetic, low-spin ferric complex of cyanide-ligated sperm whale myoglobin, Mb. The resulting dipolar shifts, together with the X-ray coordinates from the MbCO structure and the values of the principal components of the magnetic susceptibility, have allowed us to quantitatively map the paramagnetic dipolar field of the heme iron. The magnetic coordinates were found to have the major axis tilted 15° from the heme normal.

The 1D nuclear Overhauser effect was used to assign all of the heme cavity residue signals in elephant cyano-met Mb which has the distal E7 histidine replaced by glutamine. The proximal side stereochemistry is highly conserved. On the distal side, Phe CD1 is unaltered and Gln E7 occupies a position close to that of the His E7 it has replaced. However, we find for elephant Mb an additional residue in the heme pocket ~4.7 Å from the iron not found in sperm whale Mb which we identify as Phe CD4. The additional residue may account for the steric effects detected both by CO binding and resonance Raman studies. Analysis of the change of the pattern of dipolar shifts with the E7 His → Gln replacement indicates that the magnetic axes rotated so as to bring the major axis close to the heme normal. We interpret this rotation of the magnetic axes as indicating that the bound cyanide ligand is less tilted (by ~6°) from the heme normal in elephant than sperm whale Mb.

RESONANCE RAMAN STUDIES OF SITE DIRECTED MUTANTS AND SINGLE CRYSTALS OF MYOGLOBIN

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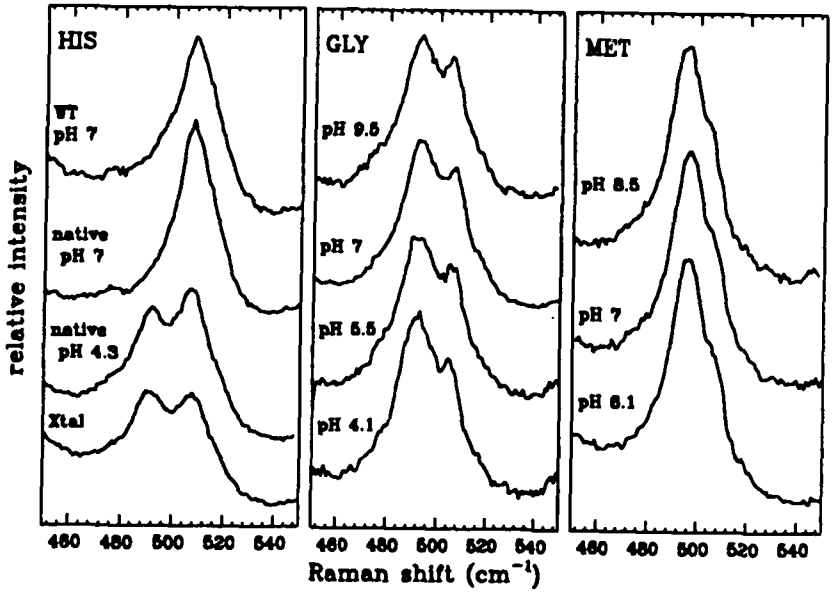
Studies of ligand binding to heme proteins are often complicated by "distal pocket effects." The ambiguities surrounding the interactions of the ligand with the distal pocket generally reflect a lack of experimental control over this facet of heme protein structure. However, with the recent advances in gene synthesis and bacterial expression, it has become possible to produce large quantitites of heme proteins having specifically designed amino acid sequences. Such proteins (site directed mutants) can be utilized in various spectroscopic and kinetic experiments to help uncover the structure/function relationships of specific amino acid residues.

In this respect we have focused on the role of the distal histidine (E7) in sperm whale myoglobin (Mb). We have examined the u.v.-visible absorption and resonance Raman spectra of a variety of E7 mutants as well as their complexes with ligand (CO, O_2). Raman spectra of crystallized native myoglobin have also been obtained for the first time. Additional work involving native Mb has demonstrated the spectroscopic and kinetic effects of pH and temperature. Here, we use the site directed mutants to test a simple model of conformational and proton exchange dynamics. The model helps to account for the temperature and pH dependence of multiple states of Mb•CO observed using infrared, Raman, and x-ray spectroscopies. The figure delineates some key observations involving the Fe-CO Raman frequencies.



PROTON RESONANCE ASSIGNMENTS IN THE SPECTRUM OF HUMAN HEMOGLOBIN BY 2D-NMR.
Evaluation of tertiary structural changes in the heme pocket.

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Proton Nuclear Magnetic Resonance (NMR) spectroscopy has proved to be a very useful tool for investigating the structural and functional properties of the human hemoglobin (HbA) in solution. Its effectiveness may be considerably increased by extending the set of proton resonances which are assigned to the specific atoms in the protein. For this aim we have undertaken a systematic study of one-dimensional (1D) and two-dimensional (2D) NMR spectra of human hemoglobin and its isolated chains in the monocarboxylated form.The assignment procedure was based on the partial identification of the amino acid spin system in the J-correlated (COSY) spectra and on the nuclear Overhauser effect connectivities (from NOESY spectra) between the neighboring groups. Firstly, assignments were made in the more simple spectra of α(CO) and β_4 (CO) chains. The resonances of the majority of the heme groups and of a considerable number of amino acids (mainly from the heme pocket) have thus been identified. These results were of valuable help in the analysis of 2D spectra of the HbA tetramer. We were able to assign the proton resonances of many heme groups and of 30 amino acid side chains from the two subunits of HbA(CO). This represents the first report of a successful application of 2D NMR techniques to a functional protein of such high molecular weight (MW=64,500).

Comparison of the ring-current shifts of the assigned resonances with those calculated from the known crystallographic coordinates suggests a close similarity between the tertiary heme pocket conformations in solution and crystal state. A significant difference was noted for the Leu-141 in β subunits which, in solution structure, appears to have stronger contacts with the heme groups than in crystal structure. Also, the present results permit to characterize some tertiary changes of the heme pocket when the isolated subunits are assembled in the hemoglobin tetramer. As expected, these structural variations indicate the existence of R state quaternary constraints of low intensity which may explain the functional difference between the free and assembled Hb subunits. The present assignments provide sensitive spectroscopic probes for the characterization of the ligand- and effector-induced structural changes in normal hemoglobin and its natural variants. This aspect is currently under study in our laboratory.

MOLECULAR DYNAMICS OF MYOGLOBIN

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The results of three molecular dynamics simulations of myoglobin are presented. The initial simulation was a 150 ps molecular dynamics trajectory of CO-myoglobin (MbCO) at 300 K. Properties calculated from this trajectory were used as reference values in the analysis of MbCO dynamics at 80 K and of CO ligand dissociation from MbCO.

Two 80 K trajectories were generated starting with different coordinates from the 300 K simulation. The simulation results are in qualitative accord with structural changes observed upon cooling MbCO from 300 to 80 K. At 80 K the molecule was trapped in distinct regions of configuration space. This suggests that conformational disorder exists in myoglobin crystals at low temperatures. Comparison of MbCO fluctuations at 300 K and 80 K confirms that the room temperature flexibility of the protein is determined by the mobility of the loop regions and by sidechain torsional motions; at 80 K this mobility is considerably reduced.

In a 100 ps simulation of the dissociation of CO from MbCO at 300 K, attention was focused on structural changes that follow the Fe-C bond breaking. During the simulation a transition toward a deoxy-like tertiary structure was observed. The structural relaxation involved a series of processes over a time scale from 50 fs to 80 ps. The results provide a microscopic description of the dynamics of the MbCO->Mb+CO transition to supplement the X-ray and photodissociation experiments.

SYNTHESIS, STRUCTURE AND SPECTROSCOPIC PROPERTIES OF TWO MODELS FOR THE ACTIVE SITE OF THE OXYGENATED STATE OF CYTOCHROME P450

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Two dioxygen adducts of thiolato-rion(II) porphyrins, $|Fe(O_2)(SC_6HF_4)TPpivP||K \subset 222|$ 1a and $|Fe(O_2)(SC_6HF_4)TPpivP||Na \subset 18c.6|$ 2 were synthesized by reaction of O_2 with five-coordinate, high-spin, cryptated alkali metal thiolate-iron(II) "picket fence" porphyrinate. They were characterized by visible and I.R. spectroscopy: $\lambda m(\log\varepsilon)$ = 360(4), 427 (4.69), 560 (3.69), 610 (3.40)nm for both compounds; $\nu(O^{16}-O^{16})$ = 1139 cm^{-1} in chloro and fluorobenzene for 1a and 2.

Single crystals of composition $|Fe(O_2)SC_6HF_4)TPpivP||K \subset 222||K \subset 222||SC_6HF_4)|C_6H_5Cl\cdot H_2O$ 1b were obtained by diffusion of pentane-xylene mixtures into cholorobenzene solutions of 1a at -5°C. Single crystals of composition $|Fe(O_2)(C_6HF_4)TPpivP||Na \subset 18c.6|$ were obtained by slow diffusion of pentane into benzene solutions of 2. the X-ray structures of 1b and 2 were studied at 20°C (1b) and -100°C (1b and 2). 1b: space group $P2_1/c$ (monoclinic) a = 16.806(5), b = 14.331(4), c = 52.000(15) Å, β = 92.95(2)°, V = 12507 Å^3, Z = 4, D_{cal} = 1.28 g·cm^{-3} (t = 20°C). The final R_1 factor was 0.085 for 5238 reflections having $I>3\sigma(I)$. 2: space group $P2_1/c$ (monoclinic), a = 13.107(3), b = 27.055(4), c = 25.029(4) Å, β = 96.84(2)°, V = 8812 Å^3 Z = 4 D_{cal} = 1.18 g·cm^3 (t = -100°C). The final R1 factor was 0.088 for 6587 reflections having $I>3\sigma(I)$. The iron atom is, in both compounds, bonded to the four Np nitrogens, the sulfur atom of the axial thiolate and one oxygen atom of the axially end-on bonded dioxygen molecule. The average Fe-Np distance found in 1b (1.994(4) Å) is not significantly different from that found in 2 (1.993(3) Å). The Fe-S bond length is 2.367(3) in 1b and 2.365(2) Å in 2. The Fe-O1 distances with the oxygen atom of O_2 bonded to iron are respectively 1.837(9) and 1.850(4) Å. The end-on bonded O_2 molecule is disordered in both complexes 1b and 2. This disorder is however slightly different from one structure to the other. Whereas the terminal oxygen atom of O_2 occupies two positions in 1b (τ(O2A) = 78%, τ(O2B) = 22% at 20°C), it occupies three sites in 2 (τ(O2A) = 51%, τ(O2B) = τ(O2C) = 24.5% at -100°C (τ = occupancy). The estimate of the magnitude of the non-bonded interactions between O_2 and its environment located in a sphere of 7 Å radius in 1b and 2 is consistent with a static disorder of O_2 in 1b and a dynamic distribution in 2.

Mössbauer measurements were performed with polycrystals of 1b and 2 in the temperature range 5-197K and 4.2-295K, respectively, and under externally applied magnetic field (6.76T) at 4.2K (1b and 2) and at 173K (2). The temperature variation of the Lamb-Mössbauer factor indicates that the iron atom in oxycytochrome P450 models is not very firmly bonded, more like in pentacoordinated than in other hexacoordinated hemes. From the specific temperature behavior of quadrupole splitting, asymmetry parameter and line width obtained for 2, the structural disorder of the terminal oxygen atom of O_2 is identified as dynamic distribution over three different sites. Corresponding values obtained for 1b are consistent with a static disorder of O_2. Magnetic spectra reveal that the oxycytochrome P450 models are purely diamagnetic.

Iron-oxygen Vibrations in Oxyferrous and Oxoferryl Heme Proteins and Model Compounds

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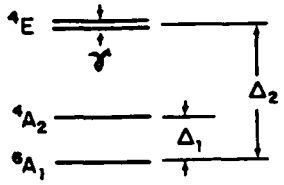
Resonance Raman and UV-visible absorption spectra of various oxygen-isotope substituted oxyferrous and oxoferryl species in solution are presented. Six-coordinate, imidazole-ligated model complexes of octaethylheme, protoheme dimethylester and heme a in solution at low temperature provide a basis to evaluate protein-induced changes in, not only the iron-oxygen vibrations, but also the structure sensitive macrocycle vibrations and the optical absorption spectra of both globin and peroxidase species. These spectral differences are interpreted in terms of variations in active site apo-protein structure. The variations of the Fe-O stretching frequencies of oxyferrous and oxoferryl forms of hemoglobin, myoglobin, horseradish peroxidase and myeloperoxidase with respect to those of the model complexes are discussed in terms of trans-ligand effects originating from the proximal imidazole as well as H-bonding and steric interactions of the distal protein residues with the exogenous oxygen ligands. The effects on the resonance Raman and optical spectra of the porphyrin ring substituents and protein-porphyrin interactions are also considered.

Quantitative Methods for Characterizing High-Spin Ferric Heme Electronic Energy Levels. Arthur S. Brill, Department of Physics and Biophysics Program, University of Virginia, Charlottesville 22901. Supported by NSF.

Electron paramagnetic resonance (EPR) reveals distributions in electronic energies which, in turn, reflect distributions in molecular structure. The spatial anisotropy of the high-spin ferric heme site splits the octahedral terms in such a way that several measureable properties of the low-field (g=6) resonances are affected. The number of parameters that can be experimentally determined, from solutions or single crystals, does not exceed the number of parameters of the 4-level model (shown to the left) which is 7 when the effective spin-orbit coupling constant and the widths of the 3 energy differences are included. With $\Delta_2 \approx 3\Delta_1$ as in aquo-ferric myoglobin, a spread in Δ_2 has an order of magnitude smaller influence upon observable magnetic resonance properties than has the same spread in Δ_1. While information about states at energies greater than Δ_2 is useful, these higher excited states have little direct effect upon the parameters obtained from measurements discussed here; the 4-level model is adequate for characterizing the results of such experiments. Measurements are made on frozen solutions ([heme] ~ 1mM, 33mM PO_4, pH 6.3) or single crystals at temperatures 4.2K and lower. The information at hand indicates that the energy distributions responsible for the observed phenomena arise mainly from structural inhomogeneity associated with frozen ensembles of protein conformation; a significant role for strain induced by formation of ice crystals is not supported by experimental results from the myoglobin and hemoglobin systems investigated here.



Parameters of the 4-level model for the heme protein in solution can be obtained from analysis of 1) low-field region of the EPR spectrum and 2) temperature dependence of EPR pulse saturation recovery kinetics. The low-field spectrum provides the ratio E/D of rhombic to tetragonal strengths of the ligand field, the admixture η^2 of the lowest 3 excited states into the ground state, and the spreads $\sigma_{E/D}$ and σ_η in these parameters (Brill et al., J. Inorg. Biochem. 28(1986)137). Formulas relate D, η^2, and E/D to Δ_1, Δ_2, and γ, but require knowledge of the effective spin-orbit coupling constant. The activation energy for spin-lattice relaxation is 2D, and non-exponential kinetics results from the distribution in D (which arises from distributions in Δ_1 and Δ_2). Comparison of the temperature dependence of observed and simulated recovery curves provides the widths of Δ_1 and Δ_2, and constrains the spin-orbit coupling to a narrow range. The measurements and analysis just described have been carried out on aquo-ferric sperm whale myoglobin in solution, quantifying the 4-level model, and showing additionally that the dimensionality of the vibrational space effective in the relaxation process is 3 (Levin and Brill, J. Phys. Chem. 92(1988)5103).

Parameters of the 4-level model for the heme protein in crystals can be obtained from 1) analysis of the EPR linewidth, at 2 microwave frequencies, as a function of orientation of the magnetic field within the heme plane and 2) as above for solutions. This method is based upon the dependence of the principal in-plane g-values upon Δ_1, Δ_2, and γ; spreads in these energies produce orientation-dependent linewidths. Not only can the 4-level model be quantified in this way, but the angular spread in the in-plane principal g-directions is obtained; this angular spread includes contributions from structural distributions (e.g. distributions in orientation/position of the proximal imidazole) and from lattice disorder. A single crystal investigation of this kind has been carried out for aquo-ferric sperm whale myoglobin (Fiamingo et al., Biophys. J., submitted).

FTIR Studies of CO Orientation in HisE7 Mutants of Carbonmonoxymyoglobin. *David Braunstein, Karen Egeberg#, Hans Frauenfelder, Judy Mourant, Pal Ormos, Stephen Sligar#, Barry Springer#, and Robert D. Young*, Departments of Physics,and #Biochemistry, Univ.of Illinois, Urbana, 61801, *Physics Dept , Illinois State Univ., Bloomingtion , IL 61761*

The production of a variety of novel HisE7 mutants of sperm whale myoglobin (Mb) by the Sligar and collaborators (1) has allowed us to probe the role of the HisE7 in determining the orientation of CO in the bound state. The CO adducts of seven mutants, four non-polar (His--> Gly, Val, Phe, Met),and three polar (His--> Tyr, Asp,and Arg) were prepared in a 75% glycerol-water solution at pH7. The ν(CO) bands of the bound and unbound CO were measured between 1900$cm^{-1}$to 2200cm^{-1} following flash-photolysis at 10K. The angle, α, between the CO dipole and the heme normal of CO bound, for each mutant, was obtained by measuring the linear dichroism of the ν(CO) bands(2).

The native form of MbCO has an IR spectrum with three bound ν(CO) bands corresponding to three CO orientations; which we interpret as three distinct protein conformations, that with the smallest angle at the highest peak frequency (2). One might expect several features in the IR spectrum of the mutants: (i) The absence of multiple ν(CO) bands in mutants with non-polar distals residues, and (ii) A linear correlation between the angle α and peak frequencies of the ν(CO). No such simple behavior was observed: (i) The Gly and Met mutants each displayed two bands, while the Arg mutant had only one, (ii) The angle α of the seven mutants ranged from 20° to 33° and the peak frequencies of the CO stretch bands varied between 1962 cm^{-1} to 1988 cm^{-1}. No clear correlations were apparent between angle and peak frequency for the seven proteins measured. (iii)Signifigantly, in the Gly mutant, the protein with the smallest distal residue, for which α=20°, the ligand is not aligned closer to the heme normal than in native MbCO and a number of the other mutants.

1. Springer, Barry, Egeberg, Sligar, Stephen G,. Rohlfs, Ronald J., Mathews, Antony J., & Olson, John S.(1988) **Science** *submitted*
2. Ormos, Pal, Braunstein, David, Frauenfelder, Hans, Lin, Shuo-Liang, Hong,Mi Kyung, Sauke, Todd B., Young, Robert D. (1988) **Proc. Natl. Acad. Sci.** *in press.*

Hydration and CO Binding in Myoglobin

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We have measured the FTIR spectrum of sperm whale carbonmonoxymyoglobin (MbCO) as a function of hydration in the temperature range 10 to 320 K. Of particular interest are the CO stretch bands (1910 - 1980 cm^{-1}) and the protein and the bound water regions. The CO stretch bands can be resolved into three lines A_0, A_1 and A_3 whose relative intensities and peak positions depend on humidity. At high temperatures the A substates interconvert in wet films. The system is frozen into a glassy state below a transition temperature (about 200 K). The results point to an important role of the hydration shell in the slaved glass transition of the ligand-protein solvent system [1].

In addition, we studied the effect of hydration on the CO rebinding kinetics following flash photolysis. The data taken on hydrated films are compared with the rebinding properties of MbCO in 75% glycerol-water.

[1] A. Ansari et al., Biophys. Chemistry **26** 337, (1987).

Crystallographic Studies of Myoglobin-Alkylisocyanide Complexes

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Interactions of oxygen and other ligands with myoglobin has been the subject of many biophysical studies, but a quantitative accounting for kinetic constants in terms of the structures of the protein and ligands is still lacking. A number of structures of myoglobin-ligand complexes have been solved, but it has proved difficult to predict kinetic constants for the binding of the different ligands. This state of affairs is not too surprising, since no one has yet been able to convincingly predict binding or kinetic constants from the structure of any protein ligand complex. However, because of the extensive studies on myoglobin, we believe that this molecule is an ideal one for developing quantitative structure-function relationships. We are examining the steric factors in the binding of ligands by solving the crystal structures of various kinetically well-characterized alkylisocyanide-myoglobin complexes (Rohlfs et al., J. Biol. Chem. 263:1803, 1988).

We have recently determined the structure of ethylisocyanide bound to myoglobin. Crystals were grown in the met form, reduced, and reacted with ethylisocyanide under nitrogen, and data were taken at 0° C. A total of nine crystals were used to measure 14,104 independent reflections to 1.68Å resolution. Absorption spectra were taken after data collection to verify that there was no conversion to the met form. Initial difference maps revealed the location of the ligand and some changes in the structure of the protein. Subsequent refinement has yielded a model structure with a crystallographic R-factor of 15%.

The ethylisocyanide ligand binds to the iron in a geometry similar to the binding of dioxygen and carbon monoxide, but with the additional ethyl group occupying more volume of the heme pocket. A water molecule found in the pocket in the oxy form is displaced by the ethylisocyanide. There are also some local changes in the protein structure. His 64 (E7) is disordered, occupying more than one position. The most prominent position is one in which the imidazole ring is "swung out" of the pocket, similar to the conformation found in the phenylmyoglobin structure (Ringe et al., Biochem. 23:2, 1984). This conformation for the histidine side chain requires a concomitant change in the position of Arg 45. Like the phenylmyoglobin structure, these changes suggest a pathway for ligand entry and exit. Other changes in the structure are minor, consisting of a small shift in the F helix (relative to the oxy-structure) and reordering of water molecules in the vicinity of the Arg 45 and His 64 side chains.

These kinds of results, taken with the structures of other ligands bound to wild-type and engineered myoglobins (see Sligar and Olson, this meeting) should expand the set of "boundary conditions" for theoretical studies of dynamic binding processes in myoglobin (e.g. see Kottalam and Case, JACS, in press), and hopefully will contribute to a quantitative description of the events involved in protein-ligand interactions.

This work supported by NIH grants AR32764 and GM35649, and grants C-612 and C-1142 from the Robert A. Welch Foundation.

EPR STUDIES OF THE RATE OF DIOXYGEN ROTATION IN COBALT-CONTAINING HYBRID HEMOGLOBINS

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In earlier epr studies of cobalt tetraphenylporphyrins (CoTPP) it was shown that the epr spectra were sensitive to the rate of rotation of dioxygen about the Co-O_2 bond. (1) For several derivatives of CoTPP having a single ortho-substituent capable of hydrogen-bonding to the terminal atom of coordinated dioxygen it was shown that the epr spectra were broad and glassy-like up until about 0 °C, whereas those with no ortho-substituent developed their familiar "isotropic" eight-line hyperfine structure at -80 °C. It was thus hypothesized that the "isotropic" eight-line pattern resulted from free and rapid rotation about the cobalt-oxygen bond. (1) The dioxygen adduct of cobalt-substituted myoglobin also gave a broad, glassy-like epr spectrum at ambient temperatures, suggesting that the rate of rotation of coordinated dioxygen within the myoglobin binding pocket was also fairly slow and thus restricted. (1)

We have recently extended our studies to four hybrid hemoglobins in an attempt to determine whether epr spectroscopy would be sensitive to differences in the rates of rotation of dioxygen for the α and β chains in the T and R states. For the purposes of this study, $[\alpha(Co)\beta(Fe)]_2$ and $[\alpha(Fe)\beta(Co)]_2$ were used as R state models and $[\alpha(Co)\beta(Zn)]_2$ and $[\alpha(Zn)\beta(Co)]_2$ as T state models. EPR spectra were recorded for samples of each of the above hybrids equilibrated with 1 atm O_2 gas, as a function of temperature. The progress toward averaging of the epr signal of the Co-O_2-containing subunits was measured as the difference in field positions, ΔH, for the midpoint of the rise and fall extrema of the derivative epr spectra. A plot of ΔH vs temperature for each hybrid showed that the $[\alpha(Zn)\beta(Co)]_2$ hybrid was unique in averaging more slowly than the other three (all of which behaved very similarly), thus indicating more restricted rotation of dioxygen in T-state β chain pockets than in the pockets of any other form. This finding is consistent with x-ray crystallographic data which shows that valine E-11 on the distal side of the T-state β chain pocket partially blocks the dioxygen binding site. (2)

(1) F. A. Walker; J. Bowen, J. Am. Chem. Soc. 1985, 107, 7632.

(2) B. Shaanan, J. Mol. Biol. 1983, 171, 31; M. F. Perutz, B. Shaanan, R. Fourme, J. Mol. Biol. 1984, 175, 159.

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^{1}H NMR CHARACTERIZATION OF BACTERIALLY EXPRESSED SPERM WHALE MYOGLOBIN

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The overexpression of sperm whale myoglobin (Mb) in E. coli TB-1 harboring the construct pMb 413 (derivative of pUC 19) has been reported (Springer, B. A. & Sligar, S.G., Proc. Natl. Acad. Sci. 84, 8961-65, 1987). Extensive sequence-specific ^{1}H resonance assignments in the diamagnetic ferrous MbCO complex permit a careful comparison of the solution structure of the bacterially-expressed Mb with that of Mb isolated from sperm whale.

Previously reported sequence-specific resonance assignments based on comparison of observed NOEs with the neutron structure of MbCO (Dalvit, C. L. & Wright, P. E., J. Mol. Biol. 194, 313-327, 1987) have been verified and extended by main-chain-directed sequential resonance assignments in the bacterially-expressed protein. NOE buildup data has been obtained for a large number of structurally significant intra- and interresidue contacts, and preliminary solution structures will be presented for the heme binding region of bacterially-expressed MbCO.

Ring-Current Calculations in Myoglobin

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A significant number of proton resonances have now been assigned for three diagmagnetic forms of sperm whale myoglobin: the dioxygen and carbon monoxide complexes, and a deoxy-like state where Zn is substituted for Fe. In almost all cases, the deviations of the observed chemical shifts from the "random coil" values seen in short peptides can be explained in terms of ring current contributions from aromatic side chains and from the heme group. We have used the ring current calibrations of Cross and Wright,[1] (which were based on data from cytochromes and model complexes,) to compare observed shifts with those calculated from several X-ray and neutron structures of myoglobin. These comparisons show the sensitivity of diamagnetic chemical shifts to small changes in structure, and demonstrate a remarkable similarity between the average solution and crystal structures.

The linear correlation coefficients between calculated and observed ring-current contributions are as large as 0.96, with typical root-mean-square errors of about 0.35 ppm. By this criterion, Zn-substituted myoglobin appears to be an excellent model for the deoxy protein, and the O_2 and CO complexes in solution closely resemble their crystalline forms. In general, structures at higher resolution show better agreement with the chemical shift data, and further improvement is possible if the crystal structures are subjected to a modest amount of energy minimization. There are small but significant differences, however, between even high resolution structures, such as the latest X-ray and neutron diffraction structures of MbCO.

We have developed computer programs to calculate the derivatives of the computed chemical shift with respect to atomic coordinates, so that one can use restrained molecular dynamics simulations to refine structures with respect to chemical shift data in ways analogous to those in common use for X-ray and NOE distance refinements. Propsects for using both chemical shift and distance information to determine a "solution structure" of myoglobin will be discussed.

1. K.J. Cross and P.E. Wright, *J. Magn. Res.* **64**, 220-231 (1985).

^{1}H NMR Studies of the Influence of Distal E7 Mutations on the Molecular and Electronic Structure of Sperm Whale Myoglobin.

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The ^{1}H NMR spectra of sperm whale myoglobin mutants possessing the substitution E7 His → Gly, Val and Phe have been recorded and preliminary analysis carried out in the diamagnetic carbonyl complex as well as in the paramagnetic high-spin met, low-spin cyano-met, spin-equilibrium azido-met and deoxy derivatives. The high-spin ferric derivatives yield spectra systematically different from that of the wild type which suggest that a water molecule does not occupy the sixth site in these mutants. The azide complexes, which exhibit a spin-equilibrium between the high-spin and low-spin states, reveal that relaxation of distal steric strain favors the high-spin state. The low-spin ferric complexes reveal that the major spectral perturbation can be interpreted in terms of a rotation of the magnetic susceptibility tensor axes so as to align the major axis with the heme normal. This reorientation of the susceptibility tensor indicates that reduced steric bulk for the E7 residue allows the bound cyanide ligand to adopt a more perpendicular orientation relative to the heme than found in the wild type.

Geminate Recombination in Heme Proteins and Model Systems by D. Magde, UCSD, LaJolla, CA 92093-0314.

All six-coordinated hemes investigated show photodissociation followed by geminate recombination. These include not only oxygen-binding myoglobins and hemoglobins from several species but also a number of cytochromes in which the iron is hexacoordinated to the protein and do not bind free ligands, as well as many synthetic model hemes. The ligands that are dissociated include NO, O_2, CO, imidazole, a variety of alkyl isocyanides, and several pyridines.

The key to understanding fast heme reactions is to distinguish four time scales corresponding to four distinct processes: (1) At times shorter than a few picoseconds one observes physical processes that are predominately electronic relaxations of five-coordinate (already dissociated) hemes. When we photolyse five-coordinate complexes, we observe the same changes over a few picoseconds that we see in the first few picoseconds following photolysis of six-coordinate hemes. This assignment has been made J.-L. Martin and coworkers, and we have little to add. (2) Over times of several tens of picoseconds one observes the binding reaction itself from a geminate pair that consists of a five-coordinate heme and its ligand, in their electronic ground states, slightly separated and/or rotated from their bound configuration but still in intimate proximity, the "contact pair." The process is the same whether one studies oxygen-binding proteins, cytochromes in which the ligand photolysed is an amino-acid side chain that can only rotate slightly, or a synthetic model compound and its ligand within a solvent cage. (3) Over times of many tens of nanoseconds one observes recombination of ligands that have spent that time trapped somehow in the protein as a "separated pair" that is certainly not the contact pair. This nanosecond process is always observed with the oxygen-binding proteins, but never observed with any model compounds or with cytochromes that do not contain ligands free to migrate elsewhere in the protein. (4) At the very longest times one observes diffusional, bimolecular combination reactions between ligand free in solution and unliganded heme. In the tetrameric hemoglobins on this time scale one can also observe effects related to conformation changes, as well documented by Hofrichter, Eaton, and coworkers.

Since the basic experimental observation is simply the amount of six-coordinate iron present as a function of time (supplemented by a few, very important kinetic Raman studies such as those of Friedman and coworkers), the model cannot be supported or refuted by measurements on just one system, no matter how detailed. The key is the intercomparison of the entire range of hemes and ligands. Given the model, with its two very distinct types of geminate pairs in very different time regimes, one may address long-standing questions of heme reactivity. A key parameter is the binding rate from the contact pair, which competes with diffusion of the ligand away from the iron. The fastest binding ligand is NO, which rebinds so efficiently that it is very difficult to measure anything except picosecond kinetics. The popular ligand CO is unique in rebinding so poorly that the picosecond process and the nanosecond process are only with special efforts. Oxygen is perfectly typical, but is sufficiently difficult to handle as to compromise early studies in picosecond laboratories. Many other ligands are like oxygen in that it is quite easy to distinguish the two time regimes.

Among our recent results are measurements of the activation barrier for escape from the protein-separated pair to the free species in solution. This barrier shows much greater temperature dependence than has been calculated by molecular dynamic simulations. Some feature that is being neglected in calculations must be important for real proteins in aqueous solution.

Abstract

Title: Subpicosecond IR Spectra of Photodissociating Carboxy Heme Proteins

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Picosecond time-resolved IR spectroscopy has been achieved using a new technique and in which the visible radiation is obtained from two dye lasers, each synchronously pumped by the frequency doubled output of a mode-locked and Q-switched Nd:YAG Laser and the tunable IR obtained from a diode laser. Single pulses (20 ps FWHM, 1-10 μJ/pulse may be selected from the dye lasers at a repetition rate of up to 1kHz. The IR laser is sampled by one of the dye laser pulses. This experimental arrangement has been used to study the photodisscociation of CO from carboxy-hemoglobin, -myoglobin and -protoheme, the observations being made on the bound CO stretch bands (1930-1970 cm^{-1}). All the systems have been observed to display pulsewidth-limited risetimes as expected for bleaching of the bound CO bands, and the transient bleaching spectra to mirror the steady-state absorption spectra of the CO-liganded species. In the case of the protoheme in high viscosity solvents there was recovery of the bound CO absorption on the picosecond timescale due to the geminate recombination, whereas no recovery was observed for the heme proteins. This suggests that in the case of the heme proteins there is a significant barrier to immediate rebinding introduced by relaxation of the protein structure following deligation. Measurement of the transient anisotropy in the IR bleaching has allowed the equilibrium iron-carbonyl geometries to be assessed for these systems. All show distortion of the FeCO unit from the linear perpendicular orientation to the heme plane observed for crystals of model porphyrins. For myoglobin-CO, two distinct configurations are observed corresponding to the two bands in the IR absorption spectrum, and for protoheme-CO the degree of distortion from a linear perpendicular configuration is found to depend on the solvent.

This experimental technique has recently been extended to sub-picosecond time resolution. The output of a synchronously pumped cavity dumped dye laser is compressed to ~200 fs via optical fiber compression. The sub-picosecond pulses are subsequently amplified at 1kHz to a few μJ via a Q-switched ND:YAG pumped multipass amplifier. The output is then split to provide the photolyzing and upconverting pulses. Preliminary studies with this apparatus have revealed sub-picosecond upconverted intensities which approach those achieved with upconverting pulses of ~20 ps. We have shown that the ground state HbCO is <u>not</u> regenerated on the time scale 100 fs to 10 ps in contrast to earlier inferences based on the optical absorption kinetics. It is hoped to present at the Symposium an account of our on-going work on the evolution of HbCO to Hb and CO (free) using our femtosecond infrared methods.

Ligand Dynamics in the Photodissociation of Carboxyhemoglobin by Subpicosecond Transient Infrared Spectroscopy.

Lewis Rothberg and Tom Jedju, Bell Labs, Murray Hill
Bob Austin, Princeton University, Princeton, NJ

Time-resolved infrared spectroscopy with 0.5 ps resolution is used to track the evolution of the CO stretching vibration following visible photoexcitation of carboxyhemoglobin in deuterium oxide at room temperature. Polarization measurements determine the CO to be oriented nearly perpendicular to the porphyrin plane. The dissociation proceeds via a metastable excited state with approximately a 2 ps lifetime. The dissociated CO binds weakly in the heme pocket for at least 500 ps. This weakly bound state appears to correlate with the "B" state observed by Frauenfelder et al. at low temperatures in myoglobin.

Far Infrared Light at 51 cm^{-1} Enhances CO Recombination at Low Temperatures

Bob Austin and Mark Roberson, Princeton University, Princeton, NJ

We present evidence that the 2 millijoule, 2 microsecond FIR beam from the UCSB Free Electron Laser can influence the recombination rate of carbon monoxide with sperm whale myoglobin. We will discuss the control experiments that rule out simple heating effects in the observed *enhancement* of the recombination during the laser pulse. The temperature dependence of the signal is interesting and somewhat complex: warming from 10 K results first in a decrease in signal size, followed by a rise beginning at 100 K and increasing to 180 K. At 180 K, which is the glass transition temperature of the glycerol solvent, the signal disappears. We will briefly discuss theoretical options for the origin of the signal, stressing primarily nonadiabatic theories concerned with spin selection rules and/or phonon modes. As befits true Americans during this political season, we are patriotically prepared to defend our experiment physically and decisively.

PROTEIN FLUCTUATIONS, DISTRIBUTED COUPLING, AND THE BINDING OF LIGANDS TO HEME PROTEINS

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A general model for the binding of small molecules to heme proteins is presented. The model is based on a potential surface involving the iron-ligand binding coordinate, r, and an internal protein coordinate, Q (*e.g.* iron-porphyrin out-of-plane displacement). A protein fluctuation coordinate, x, is used to modulate the coupling (iron out-of-plane equilibrium position) of the unligated state of the system. A Gaussian distribution in the out-of-plane equilibrium position has been previously shown to account for the non-Gaussian inhomogeneous broadening of the deoxy myoglobin Soret band (Šrajer *et al.*, Phys. Rev. Lett. **57**, 1267, 1986). We propose that this distribution is driven by protein conformational fluctuations that are frozen into the ensemble at low temperature (quenched disorder) leading directly to the inhomogeneous distribution in the geminate rebinding kinetics observed by Austin *et al.*, Biochemistry **14**, 5355 (1975). Specific example calculations involving the low temperature geminate recombination of CO to myoglobin and leghemoglobin are discussed in detail and a simple intuitive picture is presented that separates the activation enthalpy into distal pocket, H_D, and proximal, H_P, terms. The proximal term involves the work needed to bring the iron atom to the in-plane transition state. At physiological temperatures, when the fluctuations are rapid with respect to the kinetic time scales, the observed single exponential rate and the corresponding Arrhenius barrier height are predicted from the low temperature kinetic parameters. These parameters, along with other experimental and theoretical constraints, are used to construct detailed potential energy surfaces that are useful in further investigations of ligand binding to heme proteins. As an example, we present an analysis of hemoglobin cooperativity. It is suggested that the protein conformations associated with the R and T states will couple differently to the unligated iron-porphyrin coordinate. Such differences in coupling can be envisioned as larger and more discrete versions of the fluctuations that drive the distributions in coupling found for the monomeric myoglobin system. It is shown that significant amounts of energy can easily be stored in the Stokes shift difference associated with the unligated T and R hemes. Such nuclear relaxation effects may have already been detected experimentally in magnetic susceptibility and kinetics experiments. The observed values for the relative "on" and "off" rates of the R and T states are in accord with the proposed potential surfaces.

"LIGAND BINDING TO METMYOGLOBIN: pH AND IONIC STRENGTH DEPENDENCE OF THE ASSOCIATION AND DISSOCIATION RATE CONSTANTS". James E. Erman, Josephine Y. Lin, Kevin C. Taylor, James Merryweather and Farrel E. Summers, Department of Chemistry, Northern Illinois University, DeKalb, IL 60115.

The rate of ligand association to and dissociation from horse heart metmyoglobin has been determined for a number of ligands over extended ranges of pH and ionic strength. The interaction between metmyoglobin and imidazole, 1-methylimidazole, and 5-nitroimidazole has been studied between pH 5 and 11.5, while the reactions with fluoride and azide have been investigated between pH 3.4 and 10 and between pH 3.5 and 11.5, respectively. The ionic strength dependence of the association and dissociation rate constants was determined at integral pH values between pH 5 and 10. The lowest ionic strength investigated varied with the concentrations of ligand required for accurate determination of the rate constant and ranged from 1 mM to 100 mM. The highest ionic strength used was generally 1.0 M.

Metmyoglobin clearly discriminates between the neutral and charged forms of the ligands. The major features in plots of the association rate constant versus pH can be accounted for on the basis of the pK_a's for the ligands and for the acid-alkaline transition in metmyoglobin. Postulating that a second ionizable group in metmyoglobin influences the ligand binding kinetics improves the agreement between experiment and theory for most ligands. The pK_a of the second protein group is near 5.

Strong evidence is obtained for reaction of ligands with both the acid and alkaline forms of metmyoglobin. The ionic strength dependence of ligand association indicates that the dominant ligand binding pathway involves the reaction between the neutral form of the ligand and metmyoglobin.

"The Effects of Solvent Viscosity on the Hemepocket Dynamics of Photolyzed Carbonmonoxy Hemoglobin"

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An increasing number of experimental and theoretical studies (for review, see 1, 2) have demonstrated that structural dynamics play a pivotal role in the functional processes of metalloproteins. These dynamics can range from local fluctuations of side chains about equilibrium positions to concerted motions of entire protein domains. The various levels of protein dynamics may exhibit different responses to solvent viscosity. Motions at the protein surface, or concerted motions of protein domains, would be expected to be quite sensitive to solution properties; whereas, local dynamics of the protein interior are influenced by viscosity only to the extent that they are coupled to more global dynamics. Recently, time-resolved resonance Raman spectroscopy has been used to trace the structural evolution of the heme itself and the relaxation of the hemepocket, subsequent to ligand photolysis, from a variety of hemoglobins. These dynamics at the active site have been directly correlated to the ligand binding properties of the proteins. This study examines the extent to which protein-solvent interactions directly affect the ligand binding dynamics at the heme active site of hemoglobin.

Time-resolved resonance Raman spectra of photolytic transients of HbCO were obtained from samples of varying viscosity via protocols described elsewhere.[3] Sample viscosity was regulated by adjusting the glycerol:water ratio of the solution. The data obtained in this study yield the following insights into the ligand binding dynamics of hemoglobin: 1) the species specific proximal hemepocket geometry of the photolytic transient species, generated within 10 ns of CO photolysis, is independent of solvent viscosity, but quite sensitive to pH and allosteric effectors. This clearly indicates that the initial evolution of the hemepocket is elastic and local in nature, with the 10 ns transient geometry determined by the solution sensitive disposition of the equilibrium ligated protein conformation. 2) Geminate ligand-heme rebinding does not appear to be influenced by solvent viscosity, while longer time rebinding is quite viscosity dependent. Apparently, the protein dynamics dictating the escape of the ligand from the distal pocket are local fluctuations that are insulated from the solvent, while the dynamics which modulate the ligand migration through the protein and across the protein-solvent interface are coupled to the solvent. 3) Subsequent relaxation of the metastable transient geometry is viscosity dependent, suggesting that it is predicated upon large-scale motions of the F-helix and, presumably, the entire "allosteric core" of the protein.

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Dynamics of Ligand Escape from the Heme Pocket of Myoglobin

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Molecular dynamics calculations are used to study the kinetics of the process by which a dioxygen ligand leaves the heme pocket of sperm whale myoglobin. Umbrella sampling techniques are used to generate free-energy profiles at several temperatures for escape along a path between the distal histidine and valine residues, and methods for assessing the statistical significance of such profiles are explored. The results are used to compute rate constants for ligand escape, both in the transition-state approximation and with full classical dynamics. Corrections to transition state theory rates (i.e., the transmission coefficients) are in the range 0.8 to 0.9 for this process, and the computed rate constants at room temperature are in good agreement with experiment. Near room temperature, the computed activation energy is less than 1 kcal/mol, but at lower temperatures (between 180 and 270 K) this rises to 5 kcal/mol. Potential physical origins of such non-Arrhenius temperature dependence are discussed in light of models of protein fluctuations that accompany ligand binding.

Dynamics of Myoglobin with the Proximal Histidine-Iron Bond Broken

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Ascenzi et al. have shown that in sperm whale myoglobin (Mb) the proximal histidine (F8) protonates, causing the histidine-iron bond to break with a pK of 3.45[1]. Brault and Rougee, using model compounds, showed that tetracoordinated species have a split Soret band[2]. We measured Mb in the tetracoordinated state by preparing a MbCO sample at pH3, lowering the temperature to 5K, and photolyzing the sample with bright white light. We saw a clearly split Soret, confirming that the proximal histidine-iron bond was broken.

We measured the rebinding kinetics of MbCO(pH3) following low temperature flash photolysis in the Soret in 75% glycerol/water from 10K to 280K and from 2 μs to 100 s. Below about 200K, the observed nonexponential kinetics are attributed to rebinding from the pocket, process I. The resulting distribution of enthalpic barriers for process I peaks at about 1 kJ/mol compared to 10 kJ/mol for Mb(pH7) and about 2 kJ/mol for protoheme. The pre-exponential for Mb(pH3) is closer to that of Mb(pH7), ~10^9 s^{-1}, than that of protoheme, ~10^{11} s^{-1}. Multiple laser pulses led to an increase in the amplitude of process I kinetics. This suggests the existence of a process parallel to process I which is faster than 2 μs. A similar process, called process I^*, has been observed in protoheme and horseradish peroxidase.

The CO infrared stretch bands of MbCO(pH3) measured from 10K to 300K in 75% glycerol/water reveal one broad peak centered at 1973 cm^{-1} and a shoulder at about 1966 cm^{-1}. The shoulder may be due to some proteins remaining in the unprotonated form. The photolyzed peak at 10K has an amplitude of about 40% of the unphotolyzed peak providing additional evidence for the fast process mentioned above. FTIR measurements of the linear dichroism of the CO stretch band at 1973 cm^{-1} following photoselective flash photolysis of MbCO(pH3) show that the bound CO is oriented with its axis at an angle of $\alpha = 34° \pm 9°$ with respect to the heme normal.

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Recombination of Myoglobin-CO after Photodissociation Studied by Mössbauer Spectroscopy at Low Temperatures

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Mössbauer spectroscopy is well suited for the study of ligand binding to myoglobin and hemoglobin over larger time scales because the Mössbauer parameters of the ligated and unligated species differ markedly due to the accompanying spin transition /1/. In the high-spin ferrous state of the photodissociated MbCO, which is commonly denoted by Mb*CO, the quadrupole splitting at 4.2 K is ΔE_Q = 2.32(2) mm/s and the isomer shift δ = 0.895(5) mm/s (with respect to α-Fe at RT), while for the low-spin ferrous state in MbCO ΔE_Q = 0.35(1) mm/s and δ = 0.27(1) mm/s.

We have followed the recombination of photodissociated MbCO in glycerol/water over about 8 days at temperatures 37 K, 47 K and 57 K. The intention was primarily to look for changes in the Mössbauer parameters due to the depopulation of shorter living conformational substates. The light source consisted of 2×23 LEDs (λ = 645 nm, 35 mW). It took approximately 3 hours to photodissociate the Mössbauer absorber up to 90% at 4.2 K. The recombination process after 24 h of illumination at the three quoted temperatures can be seen in Fig. 1. An interpretation in terms of a distribution of conformational substates /2/ will be discussed. No significant change of the Mössbauer parameters has been observed.

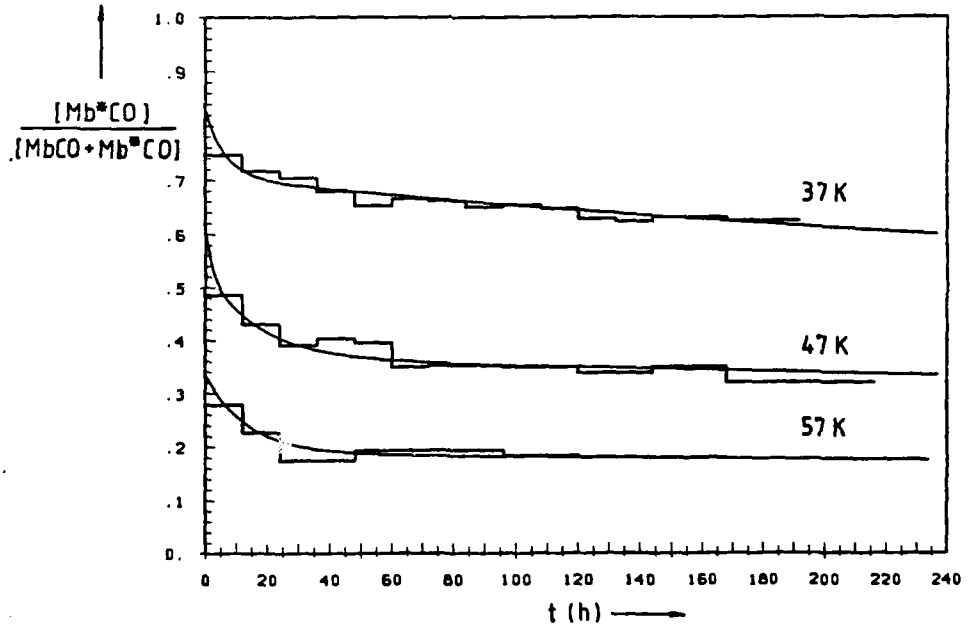

Fig. 1: Decay of Mb*CO in course of time after photodissociation.

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REACTIVE LINESHAPE NARROWING: MONITORING THE DISTRIBUTION OF PROTEIN CONFORMATIONS

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Low temperature measurements of geminate recombination of CO to Myoglobin (Mb) were intensively studied [1]. The non-exponential kinetics observed led to the assumption that at low temperatures (T) different frozen protein conformations modulate the heme affinity towards its ligand. At higher T, when relaxation is possible, kinetics becomes exponential.

We have previously suggested a simple but instructive model for the effect of protein conformation on the rebinding kinetics [2]. This model consists of a parabolic potential as a function of a conformational coordinate, x. The binding reaction is described by a coordinate-dependent rate-coefficient. Conformational relaxation is a diffusive motion of a distribution function, $p(x,t)$, in this potential. At low T, $p(x,t)$ decays and shifts simultaneously due to differential rebinding (Fig. 1). At higher temperatures, rebinding rate is expected to *decrease* with time due to relaxation into the less reactive equilibrium deoxy state.

One expects that if a spectral peak, which decays due to rebinding, is only inhomogeneously broadened "the observed line-shape should be a faithful monitor of the shape of $p(x,t)$" [2]. In practice, the relatively large homogeneous broadening makes the observation difficult, but not impossible. This is demonstrated by the near-IR band of Mb-CO [3], whose time dependence resembles Fig. 1.

The shift in the near-IR peak, at least below 60K, is not due to relaxation [4]. To demonstrate that its origin is differential rebinding, we plot [5] shift vs rebinding (Fig. 2). If reaction is fast on one side of the peak and slow on the other, the shift should correlate with the fraction of rebound heme irrespective of when rebinding occurs, hence independently of T. A second indication for a "reactive lineshape-narrowing" mechanism is the temporal decrease in the peak's width (Fig. 3). Our model fits semi-quantitatively both rebinding curves and spectral shifts [5]. The only new parameter, the ratio of protein coordinate to wavelength, gives an estimate of 15-20% for inhomogeneous broadening. Systems with less homogeneous broadening are preferable for future study.

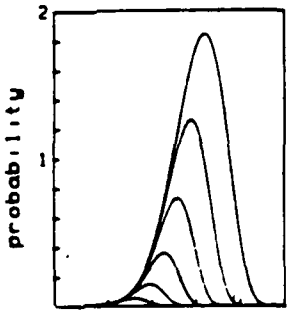

Fig. 1

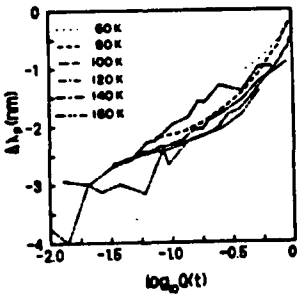

Fig. 2

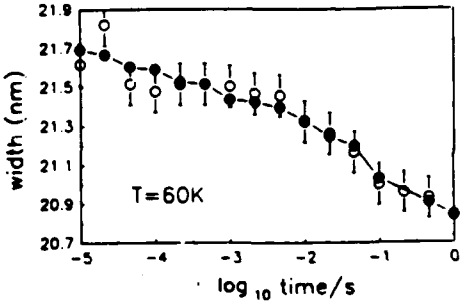

Fig. 3

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RELATIONSHIP BETWEEN SPIN STATE, CONFORMATION AND FUNCTIONAL PROPERTIES IN HUMAN HEMOGLOBIN.

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It is now generally accepted that there is a thermodynamic linkage in carp hemoglobin between the spin equilibria of ferric derivatives and the quaternary state of the protein. The average free energy of this linkage is about 700 cal/mole of heme. Evidence that this relationship is a general one and also applies, for example, to the human protein has been lacking. Although liganded derivatives of human hemoglobin, HbA, cannot generally be converted to their T states, Perutz et al. (Biochemistry (1974) 13, 2174) presented evidence that IHP can produce such a transition with high spin ferric derivatives. Philo and Dreyer (Biochemistry (1985) 24, 2985) examined the effect of IHP on the magnetic susceptibilities of several high spin derivatives of HbA and concluded that the linkage free energy was much smaller than for the carp protein and in some cases virtually nil. However, an alternative explanation for their observations might be that IHP does not produce a complete transition to the T state in these derivatives of HbA. The properties of bezafibrate (2-[4-(2-p-chlorobenzamidoethyl)phenoxy]-2-methyl-propionic acid) have permitted us to explore this possibility. Perutz and Poyart (Lancet (1983) 2, 881) reported that bezafibrate lowers the oxygen affinity of human hemoglobin and its effects are roughly additive with those of organic phosphates. This is because it has a separate binding site. We examined the extent to which bezafibrate affects the spin state equilibria of ferric derivatives of human hemoglobin in the presence of IHP. Spin state changes have been assayed in three ways: by direct measurements of magnetic susceptibility, by determining the relative heights of spin marker lines in resonance Raman spectra, and by difference spectroscopy in the visible region (500-650 nm) of the heme absorption spectrum. In all, 6 derivatives of human ferric hemoglobin have been examined. In all but one case the combination of bezafibrate and IHP produces a larger shift to the high spin form than IHP alone. Only the thiocyanate derivative fails to display a convincing effect of bezafibrate in the presence of IHP. The average change in the free energy of the spin equilibrium produced by this combination of effectors, as estimated from the magnetic susceptibility measurements, is 635 cal/mole, similar to the average effect of the R to T state transition in carp hemoglobin on the spin state equilibrium of that molecule.

In human hemoglobin it is difficult to demonstrate the relevance of the linkage relationships observed with the ferric derivatives to the functional properties of the physiologically important ferrous form. Our ability to cross-link dimers of HbA between the Lys 99 residues of the two alpha chains to yield a functionally normal hemoglobin which cannot dissociate into dimers (Chatterjee et al. J. Biol. Chem. (1986) 261, 9929) has permitted the isolation of hybrid tetramers containing three ferric hemes and one ferrous heme, the latter on a single beta chain. With this hybrid the effect of the spin state of the ferric hemes on the functional properties of the ferrous heme has been explored. The substitution of cyanide ion for the water ligand on these ferric hemes produces a marked increased in the rate of CO combination with the ferrous heme in both the presence and absence of IHP. This effect is in accord with the thermodynamic linkage observed in the first part of this study.

Heme Orientational Disorder in a Monomeric Cyano-Met Hemoglobin Monitored at the Individual Heme Vinyls and Histidines by ^1H NMR .

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Introduction: The monomeric insect hemoglobin CTT IV exhibits a pH-dependent O_2 affinity (Bohr-effect). This hemoglobin is considered as the simplest allosteric model system. The O_2 affinity is controlled by the state of proton dissociation of His G2 (94) which is part of the Bohr site. In addition, the Bohr-effect is modulated by the heme orientational disorder (180°-rotation of the heme about the α,γ–meso axis). Hence, the actual O_2 affinity in solution is controlled by pH in two ways, via pH-induced conformational transition (Bohr-effect) and via pH-controlled distribution of two heme rotational components. The heme rotational components differ in O_2 affinity, pK value of the Bohr group and magnitude of the Bohr-effect. Therefore, heme orientational disorder should be monitored at the heme vinyls as well as the histidine which is part of the Bohr site.
Methods: ^1H NMR spectra were recorded at 25 °C with a 500 MHz NMR spectrometer (type AM 500, Bruker Analytische Meßtechnik, Karlsruhe, Germany). Titration experiments were performed with solutions of cyano-met CTT IV (20mg/0.3ml) in 0.2 M D_2O. Reconstitution of CTT IV was performed according to standard methods with 2-vinyl-d_3-4-vinyl protoheme-IX, pemptoheme, isopemptoheme and protoheme-III. pK values were calculated on the basis of chemical shift-versus-pH plots for histidine C-2 and heme vinyl protons according to the Henderson-Hasselbalch equation. In addition, the pK values of histidines were calculated on the basis of electrostatic interactions employing the Tanford-Kirkwood theory.
Results: In the three proteins containing a non-symmetric heme we observe heme orientational disorder with a major and a minor component. The replacement of the 4-vinyl group by hydrogen (isopempto) leads to a relative increase of the major component, whereas the replacement of the 2-vinyl group by hydrogen (pempto) has no effect on the proportion of heme rotational components. The hyperfine-shifted vinyl proton resonances are now assigned to individual vinyls: The 4-vinyl resonances (in isopempto CTT IV) are attributed to the major, the 2-vinyl resonances (in pempto and 2-vinyl-d_3-4-vinyl-proto-IX CTT IV) to the minor component. This assignment of vinyls is also supported by titration experiments. Isopempto CTT IV exhibits a higher pK value (7.4) than pempto CTT IV (pK = 7.1), as expected for the major and minor heme rotational component, respectively. The histidine C-2 proton resonance of His G2 is split indicating two heme rotational components. This evidence is based on titration experiments with proto-III CTT IV which shows no splitting of the histidine and vinyl proton resonances. The assignment of pK values to individual histidines was made by employing an electrostatic interaction model based on the Tanford-Kirkwood theory.The two components of the C-2 proton signal of His G2 differ in pK and intensity. The histidine C-2 proton resonances exhibit line-broadening (largest at the pK value) indicating slow exchange between the two conformations.

Kinetic Studies on Partially Liganded Species of Carboxyhemoglobin: $(\alpha_1^{CO}\beta_1^{CO})\alpha_2\beta_2$

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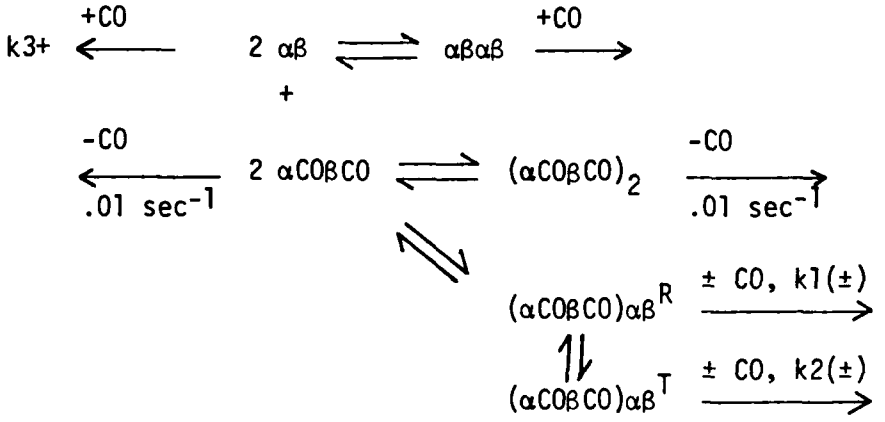
ABSTRACT

Kinetics of CO-combination with and dissociation from isomer III - $(\alpha_1^{CO}\beta_1^{CO})\alpha_2\beta_2$ and Hb Rothschild have been studied using the double mixing and microperoxidase methods. Isomer III was prepared in a manner so that it was the only reactive species in the reaction mixture. Both the reaction time course of CO-combination and CO-dissociation were biphasic. Detailed numerical analysis of the kinetic data on the basis of a reaction scheme that takes into account dimer-tetramer and R $\rightleftharpoons$ T equilibria indicated that in both of the proteins R $\rightleftharpoons$ T equilibria was slow. The effect of protein concentration, inositol hexaphosphate and wavelength on the reaction time course was consistent with the proposed reaction scheme. Isomer III: $k1+ = 6 \times 10^6$ M^{-1} sec^{-1}; $k3+ = 1.9 \times 10^6$ M^{-1} sec^{-1}; $k2+ = 2.1 \times 10^5$ M^{-1} sec^{-1}; $k2- = 0.13$ sec^{-1}, $k1- = 0.01$ sec^{-1}; Hb Rothschild: $kf = 2.8 \times 10^6$ M^{-1} sec^{-1}; $ks = 2.7 \times 10^5$ M^{-1} sec^{-1}.



ANION-LINKED PHENOMENA IN THE TETRAMERIC HEMOGLOBIN FROM THE MOLLUSC SCAPHARCA INAEQUIVALVIS

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The dimeric and tetrameric hemoglobins extracted from the mollusc Scapharca inaequivalvis are exceptional in that they assemble in an inside-out fashion relative to vertebrate hemoglobins. Thus, the heme-linked E and F helices are not exposed to solvent as in vertebrate hemoglobins, but form the intersubunit contact region in the homodimeric protein, HbI; in the tetrameric one, HbII, two heterodimers assembled in the same way give rise to the tetramer by contact between the A helices and the non helical segments preAA and GH. In the stabilization of these unique hemoglobin contacts hydrophobic residues appear to play a major role. However, ionic interactions are at the basis of another interesting property of HbII, namely its strong tendency to polymerize upon removal of oxygen. On the basis of chemical modification experiments and of the dependence of polymer formation on several experimental variables, anions have been suggested to have a specific role in the polymerization process. In turn, polymerization of the deoxygenated derivative results in a decrease of the oxygen affinity and in a slight increase in cooperativity of oxygen binding.

A deeper insight into these phenomena has been afforded by the combined use of ^{35}Cl NMR spectroscopy and sedimentation velocity experiments. HbII has high affinity chloride binding sites and binding of chloride to these sites brings about an oxygen-linked polymerization which manifests itself in an unusual dependence of the ^{35}Cl excess linewidth on the concentration of the anion. Polymer formation, as expected from previous knowledge of the system, is more pronounced in the deoxygenated derivative. The relaxation properties of ^{35}Cl allow one to detect polymerization readily also in the oxygenated derivative for which sedimentation velocity experiments provide evidence of polymer formation only in as much as the diffusion coefficients are indicative of heterogeneity. In contrast, in the deoxygenated protein the ultracentrifuge provides a sensitive measure of polymerization that goes through a maximum at around 20 mM chloride. Oxygen binding studies carried out at low chloride concentrations indicate that log p50 and cooperativity likewise go through a maximum in the same range of chloride concentrations and that anions also affect the size of the acid Bohr effect displayed by HbII.

THE PROPERTIES OF THREE MAJOR MONOMERIC HEMOGLOBIN COMPONENTS FROM THE MARINE ANNELID *GLYCERA DIBRANCHIATA*

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It has been established that the erythrocytes of the marine annelid *Glycera dibranchiata*, sometimes referred to as the common blood worm, contain three major monomeric hemoglobins [Kandler & Satterlee, *Comp. Biochem.* **75B** 499-503 (1983); Kandler, Constantinidis & Satterlee, *Biochem. J.* **226** 131-138 (1984); Cooke & Wright, *Biochim. Biophys. Acta* **832** 357-372 (1985)]. These proteins display homogeneous molecular weights on gel filtration columns and electrophoresis gels, however they can be separated by careful ion exchange chromatography.

Their importance tc structure-function studies of heme proteins lies in the fact that they have been shown to possess an exceptional amino acid substitution. In these proteins the E-7 position is naturally occupied by a leucine rather than the more common distal histidine. In fact, inspection of the published crystal structure [Padlan & Love, J. Biol. Chem. 249 4067-4078 (1974)] reveals that the E-helix presents four primary amino acids pointing toward the heme ligand binding site and all of these are either valine or leucine. This makes the heme ligand binding site in these proteins exceptionally nonpolar and, implicitly, should affect ligand binding dynamics.

Our work on these proteins has focused on ligand-binding kinetics studies, as well as sequencing & protein engineering, topics of other posters presented at this symposium. In this poster we illustrate data that is used in characterizing the purity and solution behavior of these proteins.

Prior to our work no high-resolution purity criterion existed for these proteins. Isoelectric focusing results showed that the individual protein components focused in multiple line patterns. However, this multiple line pattern does not indicate significant impurities in the monomer hemoglobin preparations. Isoelectric focusing the apo-protein components, high resolution proton NMR spectroscopy and amino acid sequencing all combine to provide evidence of the highly purified nature of these isolated component preparations.

STRUCTURE OF THE GIANT, INVERTEBRATE, HEXAGONAL BILAYER HEMOGLOBINS.

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The giant (ca. 60S), hexagonal bilayer hemoglobins(Hbs) and chlorocruorins (Chls) of annelids and vestimentiferans comprise one of the four groups of invertebrate, extracellular Hbs; the other three groups are the single-chain, the two-domain, multi-subunit and the multi-domain, multi-subunit Hbs(1). Their Fe content is 2/3 that of other Hbs. The molecular dimensions of several moleculesthe determined by STEM, vary from 25 to 30 nm in diameter and 16 to 20 nm in height. The M_r determined by STEM varies from 3,200 to 3,600 KD. The subunit structures of the hexagonal bilayer Hbs and Chls exhibit a common theme: two types of subunits, one comprising heme-containing chains of ca. 17 KD, and the other consisting of 34+3 KD chains containing little or no heme. The heme-containing chains form "functional" subunits and the heme-deficient chains are "structural" subunits. In most oligochaete and polychaete Hbs, the "functional" subunits are a monomeric subunit and a disulfide-bonded trimer of ca. 50 KD; in leech Hbs they are a monomeric subunit and a disulfide-bonded dimeric subunit. In the Chls, the "functional" subunit is a disulfide-bonded tetramer ca. 65 KD. Lumbricus Hb consists of 7 polypeptide chains of which 3 are "structural". The amino acid sequences of the "functional" chains of Lumbricus and Tylorrhynchus Hbs are known (2-4) and of one chain of Tubifex and Macrobdella Hbs (5). No data is available on the sequence of a "structural" subunit. The annelid globin sequences together with the the known invertebrate and vertebrate globins form a well-defined phylogenetic tree (6). The gene of one of the Lumbricus "functional" chains is known to have the typical vertebrate globin gene structure (7). A working model of Lumbricus Hb quaternary structure has been proposed, based on detailed studies of its dissociation at alkaline pH (8), at acid pH (9) and at neutral pH in the presence of dissociating agents such as urea, Gnd.HCl and NaSCN (10). The "structural" subunits form a scaffolding or "bracelet", decorated with twelve complexes of "functional" subunits. A corollary of this model is that the stoichiometries of the two types of subunits need not be related. The subunit stoichiometry of Lumbricus Hb is in agreement with the foregoing model: twelve complexes, each consisting of 3 copies of the "functional", M (17 KD) and T (51 KD) subunits (3x71 = 213 KD), decorating a "bracelet" formed by "structural" subunits, 20 D1 (31 KD) and 11 D2 (37 KD). The calculated M_r = 3,585 KD is in good agreement with M_r = 3,600 KD determined by STEM. In the hexagonal bilayer structure the M subunits occupy the periphery of the molecule (11), STEM images of circular structures, ca. 20 nm in outside diameter, formed by the unreduced subunits (ca. 30 KD) of Eudistylia Chl, have provided the first direct evidence for the self-aggregation of "structural" subunits. The quaternary structure of the Chl appears to be very similar to that of Lumbricus Hb (12) despite differences in dissociation. Supported by grants DK 38674 and DK 30382 (NIH) and U. S. Department of Energy.

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Aplysia limacina myoglobin: the molecular model at 1.6 Å resolution

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The three-dimensional structure of the ferric form of myoglobin from the mollusc *Aplysia limacina* has been refined by crystallographic methods at 1.6 Å resolution. The molecule is composed of 146 aminoacid residues, contains only one His, in the proximal position, and is rich in hydrophobic and aromatic residues. The tertiary structure, as expected, conforms closely to the common globin fold, with the eight α-helices surrounding the heme group. Several residues are found in the expected topological positions: among these Gly B6, Pro C2, Phe CD1, Ile E11, Phe F4, His F8. The distal position (E7) is occupied by Val 63, which is not directly in contact with the porphyrin. The two Trp's present in the structure are located on the A and H helices at positions A12 and H8. Despite the distance in primary structure, they are quite close in the folded protein. Helices A and G are visibly bent (approximately 30°) around residues Pro A14 and Pro G13. The crystallographic investigation has been conducted at pH 7.0, below the acid-alkaline transition point of *A. limacina* Mb. At this pH the sixth coordination position of the heme iron is not occupied by a water molecule. A solvent peak is found in the electron density maps at the entrance of the heme pocket, hydrogen bonded to the backbone carbonyl oxygen of residue 63 (distal), at 4.6 Å from the iron.

In parallel to the structural investigation on the native ferric form, the structures of *A. limacina* Mb at pH 9.0 (with a hydroxyl ion bound to the iron), and of its adducts with imidazole and azide (at pH 7.0) have been studied at 2.0 Å resolution. These derivatives show the binding of ligands to the distal site of a heme protein whose E7 residue is not histidine. Inspection of the three-dimensional structure of the molecule suggests a possible mechanism for the first stages of the reversible unfolding of *A. limacina* Mb induced at temperatures > 64°C, or by the addition of aliphatic alcohols.

CORRELATION OF ν(Fe-CO) WITH LIGAND DISSSOCIATION RATES FOR MONOMERIC *GLYCERA* HEMOGLOBINS.

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The monomeric hemoglobin fraction from *Glycera dibranchiata* can be separated reproducibly into three major and two minor components [1]. They present an interesting challenge in studies of structure-function relationships. Two of the major components , HbB and HbC, have the fastest oxygen dissociation rates thus far recorded and the association rates for oxygen and carbon monoxide are also extremely rapid relative to other hemoglobins [2].

Used as a direct probe of metal-ligand binding in the heme, resonance Raman (RR) spectra have been obtained at high resolution using difference cell techniques, for the CO and deoxy forms of the three major monomeric hemoglobins HbA, HbB, and HbC and a minor component HbD. Raman bands have been assigned using isotopic substitution and laser irradiation at 406.7 nm and 457.9 nm (Table 1). The wavenumbers of $\nu(Fe-N_\epsilon)$ for deoxy HbA, HbB and HbC are almost identical, suggesting very similar protein influence upon the heme through the iron-proximal histidine bond. This is consistent with the NMR data for the the corresponding carbonmonoxy derivatives [3]. The wavenumbers of the Fe-CO, and CO vibrations show variations which can be interpreted in terms of distal constraints, similar to those observed in ref. [3]. Inverse relationships between ν(Fe-CO) and the rates of CO association and dissociation reactions are suggested.

TABLE 1

Adduct	ν(Fe-CO)	δ(Fe-C-O)	ν(C-O)	$\nu(Fe-N_\epsilon)$
Hb(A)CO	501	573	1968	
Hb(A)^{13}CO	497	559	1923	221
Hb(A)^{13}C^{18}O	490	557	1877	
Hb(B)CO	494	572	1968	
Hb(B)^{13}CO	492	559	1922	222
Hb(B)^{13}C^{18}O	483	557	1877	
Hb(C)CO	494	574	1968	
Hb(C)^{13}CO	491	558	1923	221
Hb(C)^{13}C^{18}O	482	555	1877	
Hb(D)CO	496	574	1963	
Hb(D)^{13}CO	491	558	1919	219
Hb(D)^{13}C^{18}O	482	556	1874	

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A HEMOGLOBIN WHICH BINDS SULFIDE AND HEMOGLOBINS WHICH DO NOT

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Cytoplasmic hemoglobins in specialized cells (bacteriocytes) of the gill of clams living at hydrogen sulfide/oxygen interfaces assure a supply of oxygen and hydrogen sulfide from the environment to symbiotic bacteria in the gill. The bacteria oxidize sulfide (to sulfate?) and fix carbon dioxide into organic compounds ultimately consumed by the host.

Spectra of living gills of Solemya exposed to H_2S at low P_{O2} show about half the hemoglobin converted to $Hb^{III} \cdot HS$, the balance is an equilibrium mixture of Hb^{II} and HbO_2 with about 10% of the total as high spin Hb^{III} (Doeller, J.E., Kraus, D., Colacino, J.M., Wittenberg, J.B. 1988, Biol. Bull., in press).

Three hemoglobins were isolated from Lucina gill cytoplasm. None of them form sulfhemoglobin. Hb I is a typical monomeric clam hemoglobin, $P_{1/2,O2} = 0.2$ torr, $k_{off,O2} = 60\ s^{-1}$, $k_{on,O2} \gg 10^7\ M^{-1}\ s^{-1}$. This Hb when exposed to H_2S at low P_{O2} is converted to Hb^{III} HS. Ferric Hb I reacts very rapidly with sulfide or cyanide. It undergoes the familiar acid/alkaline transition with pK = 9.5.

Hb II and III are equilibrium monomer/tetramer mixtures, which remain oxygenated (or deoxygenated) when exposed to H_2S at low P_{O2}. Reactions of Hb II and III with oxygen are notably sluggish, K_{off} = 0.095 and 0.052 s^{-1}; K_{on} = 2 and 1.5 x $10^5\ M^{-1}\ s^{-1}$, respectively.

Ferric Hb II and III exhibit the familiar aquoferric optical spectrum at the acid limit, but are converted to mixtures of high and low spin hemoglobin at the alkaline limit, with pK for conversion = 6.5 and 5.7, respectively. EPR at the acid limit is that of a single high spin ferric hemoglobin species with an axial spectrum exhibiting features at g = 6 and g = 2. EPR at the alkaline limit suggests a roughly equal mixture of $Hb^{III} \cdot OH$ (g = 2.61, g = 2.20 and g = 1.82) and pentavalent high spin ferric hemoglobin exhibiting a rhombic spectrum with low field g values of 6.7 and 5.1.

Combination of ferric Hb II with sulfide is roughly 1000-fold (acid limit) or 300,000-fold (alkaline limit) slower than combination of ferric Hb I with sulfide. The rate constant for combination of ferric Hb II with sulfide increases 300-fold between pH 9.0 and pH 5.5. The pH-dependence fits the relation for a single ionization of pK 6.5 with limiting values of 17 and 5600 $M^{-1}\ s^{-1}$. The reaction follows 2nd order kinetics at all pH, suggesting that ligand binding per se is the rate limiting step.

We suggest that Hb I transports sulfide in the gill and that Hb II and III transport oxygen. Supported by NSF grant DMB 87-03328. JBW is a Career Awardee 5K06 HL00733 of the USPHS.

THE OXIDIZED FORMS OF THE THREE MAJOR *GLYCERA DIBRANCHIATA* MONOMER HEMOGLOBINS EXHIBIT ANOMALOUSLY SLOW CYANIDE BINDING RATES

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Compared to other monomeric heme proteins the *Glycera dibranchiata* monomer hemoglobin components I-III bind cyanide very slowly. This process has been studied under peseudo-first order conditions at pH=6.0, 7.0, 8.0, 9.0 using 100-250 fold excesses of potassium cyanide at each pH. At 20°, with micromolar protein concentrations, k_{obs} varies between7.08×10^{-5} sec^{-1} and 1.06×10^{-2} sec^{-1}, depending upon specific conditions of pH and cyanide mole excess.

The observed rate constants for each component can be separated into the bimolecular rate constants (k_{1app}) and these are shown in the table below. Comparison values of the bimolecular rate constants for cyanide binding to other heme proteins are shown in the table for comparison. It is clear that the *Glycera dibranchiata* monomer methemoglobins exhibit rate constants that are at least two orders of magnitude than most of the other monomeric heme proteins so far studied.

The bimolecular rate constant of each component can be separated into contributions from the binding of cyanide ion and HCN. Our results indicate that the ratio of these rate constants (k_{CN^-}/k_{HCN}) ranges from 106 to 220, thereby indicating a preference for binding cyanide ion over HCN.

These results reveal that cyanide binding is not instantaneous for these unique monomeric hemoglobins. It is not even as rapid as for metmyoglobin. What this infers is that previous efforts to measure the cyanide equilibrium binding constant were in error due to the method used. Efforts to estimate this equilibrium binding constant reveal that for each component it has a numerical lower limit that is at least two orders of magnitude larger than previously reported.

Comparison of Apparent Bimolecular Rate Constants, k_1^{app}, for Cyanide Binding to Various Ferric Heme Proteins.

Protein	Temp. °C	pH	μ (M)	k_1^{app} (M^{-1} s^{-1})	Reference
HRP=	25	7.05	0.11	9.8×10^{4}	Ellis and Dunford, 1968
CCP=	25	7.0	0.15	1.1	.rman, 1974
G.Pig Hb⁺	20	6.9	0.05	1..	Vega-Catlan et al., 1986
Human HbA⁺	20	7.0	0.05	200[b]	Vega-Catlan et al., 1986
S.W. Mb⁺	25	7.0	0.15	400[b]	Awad and Badro, 1967
Lba⁺	25	7.0	0.11	692[b]	Job et al., 1980
G.D.II Hb⁺	20	7.0	1.0	0.491	(Ref ?)
G.D.III Hb⁺	20	7.0	1.0	0.302	This work
G.D.IV Hb⁺	20	7.0	1.0	1.82	This work

=) Abbreviations: HRP=horseradish peroxidase; CCP=cytochrome c peroxidase; Hb⁺=methemoglobin; Mb⁺=metmyoglobin; Lba⁺=soybean met leghemoglobin a; G.D.=Glycera dibranchiata.

THE ENERGY SAVING OF REINDEER HEMOGLOBIN

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Previous results (1) have shown that hemoglobin from reindeer (Rangifer tarandus tarandus) is characterized by an overall heat of oxygenation which is two to three times lower than that of human hemoglobin A under the same experimental conditions (ΔH = -4 Kcal/mole O_2 vs -10 -12 Kcal/mole O_2). This particular aspect has been furtherly investigated in a set of precise oxygen equilibrium experiments which have clearly shown that an increase of temperature decreases the association constant for binding the first oxygen molecules without significantly affecting that for binding the last molecules. Thus, while ΔH of oxygen binding to the T state is strongly exothermic (ΔH = -15.6 Kcal/mole for oxygen in solution) that of the R state is very small being as much as -2.0 Kcal/mole of oxygen.
The different thermodynamics of the two conformational states of reindeer Hb results in a peculiar dependence of the temperature effect on the fractional saturation with oxygen which could be regarded as a very interesting case of molecular adaptation to extreme environmental conditions. In fact reindeer graze almost like their wild ancestors, beyond latitude 65°, showing a high degree of physical fitness (2) to the enviromental conditions. In our opinion a great part of this fitness could be related to the unusual thermodynamic properties of its hemoglobin which being characterized by a very little negative H in the upper part of the binding curve does not require much energy during its oxygenation-deoxygenation cycle. In other words at the level of the peripheral tissues i.e. legs, skin, etc. where temperature is $\sim 10°C$ lower than that of the lungs, oxygen delivery is not drastically impaired requiring 1/3 or less of the heat necessary for any other mammals up to now stucied (3).
The energy saving displayed by reindeer Hb is certainly one of the most elegant example of the different strategies which have been adopted during evolution to solve the problem of oxygen transport to the respiring tissues.

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High Resolution Structure of Glycera dibranchiata Hemoglobin

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The structures of both the deoxy and carbon monoxide forms of a monomeric hemoglobin from Glycera dibranchiata have been determined at 1.5A resolution by X-ray crystallography. Excellent agreement with ideal stereochemistry has been maintained while reducing the crystallographic R-value to 12.7% for deoxy and 14.7% for CO. The rms error for the structures (0.10A) is small, so a meaningful study can now be made of the molecular accomodations that accompany ligand binding in Glycera hemoglobin as well as comparisons with other high resolution monomeric globin structures.

STRUCTURE AND FUNCTION OF MINI-MYOGLOBIN

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The study of structural and functional properties of protein fragments, obtained by limited proteolysis, is of importance to investigate the folding pathway in monomeric or oligomeric proteins and to have information on the relation between exon-intron structure and function, in a protein encoded by a multi-exon gene. The hypothesis that exons encode discrete structural and functional units of a protein makes this study particularly interesting in the case of hemoproteins. Indeed, the constancy of the exon-intron mosaic structure among hemoglobins and myoglobins suggested that the basic functional property common to all these proteins (controlled ligand binding) is due to a common functional unit encoded by the central exon.

Several results - based on circular dichroism, fluorescence measurements and kinetics of ligand binding and dissociation - have now been collected, that show that mini-myoglobin (the fragment 32(B13) - 139 (H16) of horse heart myoglobin, reconstituted with the natural heme) acquires a conformation similar to that of the corresponding segment in the natural protein, which ensures the correct functional behaviour in ligand binding. However, the alpha-helical content of mini-myoglobin indicates that the terminal regions (the interrupted B and H helices) are unfolded; thus, it appears that the segment encoded by the central exon is able by itself to acquire the folding and the oxygen binding capacity of native myoglobin.

Electron Paramagnetic Resonance of Ni-Substituted Hemoglobin and Myoglobin.
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Recent interest in Ni-substituted hemoglobin and myoglobin has been related to the finding that these proteins do not bind oxygen or carbon monoxide. Ni hemoglobin is found to be in a stable "T-like" conformation. Beyond the value for studying a stable deoxy-like structure and preparing hybrids in a partially liganded state, d^8-Ni has unique properties related to the fact that the distribution of electron density between the $d(z^2)$ and $d(x^2-y^2)$ orbital is extremely sensitive to the strength of the axial ligand. Thus in NiHb the differences between the α- and β-chains are magnified to produce a difference in the spin state of these two chains, with the α-chain diamagnetic and the β-chain paramagnetic. Within the Fe-Ni hybrids where the α-chains are substituted with Ni, a shift from the T to the R state does produce a paramagnetic α-chain. Magnetic susceptibility and even visible spectroscopy are sensitive to those spin changes. However, the presence of paramagnetism in the $d(z^2)$ orbital of these high spin Ni complexes makes Electron Paramagnetic Resonance (EPR) a particularly sensitive method to detect even subtle changes in the electron distribution within the paramagnetic Ni-state.

The Ni(II) EPR for the S=1 state is spread out over a wide range of fields and is therefore difficult to detect. However, the proper interpretation of this spectrum contains a wealth of information regarding the coordination of the Ni. In addition to the g-values and the ability to actually detect even small anisotropy of the g-values, the zero field splitting parameters D and E are particularly sensitive to the distribution of electrons around the Ni. Finally, sharp double quantum transitions makes it possible to resolve the nitrogen hyperfine couplings of both the planar and axial nitrogens. These provide information regarding the number of nitrogen ligands and the strength of the interactions between these different nitrogens and the Ni. By this method the paramagnetic Ni in NiMb and NiHb, which are nearly identical in terms of visible spectroscopy show clearly distinct EPR spectra. This method should be able to detect even subtle changes in the Ni environment necessary to sort out the details of the various types of subunit interactions.

ALLOSTERIC ENERGY IN HEMOGLOBIN: LOCATION AND QUANTITATION BY H-EXCHANGE. G. Louie, S. G. Lee, J. J. Englander, S. W. Englander. Dept. of Biochemistry & Biophysics, University of Pennsylvania, Philadelphia, PA 19104.

When hemoglobin switches from the deoxy to the liganded state, some of its peptide group NH experience a great increase in their rate of exchange with water. These sites can be selectively labeled with tritium (functional labeling) and located by fragment separation methods. Two sets of allosterically sensitive peptide NH were identified and studied, one set at the beta chain C-terminus and another at the alpha chain N-terminus. Within each set, all the NH exchange at a similar rate in deoxy Hb and all are accelerated by a common factor when chemical or mutational modifications or allosteric events affect that segment. The concerted HX behavior shows that the exchange of these NH is governed by a local segmental equilibrium unfolding reaction. HX rate and changes in the HX rate can then be used to calculate segmental stabilization energy and changes therein.

Several conclusions can be drawn (conditions: pH 7.4, 0^{o}).

1. R to T transition: About 75% of the total allosteric energy change in hemoglobin is felt at the beta chain C-terminus, and about 20% at the alpha chain N-terminus.
2. Alpha chain αNH_3^+ to C-terminal Arg^+ (Cl^- - linked salt bridge): Salt bridge free energy is ~0.9 kcal, measured by removal of Arg-141 (des Arg Hb) and by removal of Cl^-.
3. Beta chain C-terminus:
   a. His-146 to Asp-94 salt link broken by the NES modification: 1.1 kcal per beta chain (experimentally identical to the decreased subunit dissociation energy in NES-Hb (Ackers et al.) and the bond energy calculated from the Bohr pK shift of His-146 (4 labs).
   b. DPG to His-143 link, by removal of DPG: worth 0.5 kcal.
   c. These two salt links (a & b) removed together: destabilization energy measured is 1.6 kcal, equal to a + b measured separately.
4. His^+-146 to Asp^--94 or His-146COO^- to αLys40: mutational changes.

Hb	Modification	From HX (at the C-term.)	From subunit dissoc.* (per alpha/beta dimer) 21.5oC ----> 0oC (meas.)	(calc.)
NES-Hb	break His146--Asp94	1.1 kcal	1.4 kcal	1.. kcal
Cowtown	His146--> Leu	0.0	0.25	~0.0
Barcelona	Asp94 --> His	0.4	0.25	~0.0
Bunbury	Asp94 --> Asn	0.0	0.2	~0.0
Kariya	αLys40 ->Glu	1.4	1.2	~0.9

*Ackers et al.

5. In oxy hemoglobin, the His-146 salt link does not appear to reform at low salt (cf. Russu & Ho; Perutz et al.).

X-Ray Diffraction Studies of Intermediate Hemoglobin Structures

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A complete description of ligand binding to hemoglobin must include analysis of the intermediate structural states of hemoglobin. These intermediate states include fully liganded hemoglobin in the quaternary structure normally associated with deoxyhemoglobin (the T-state), deoxyhemoglobin in the quaternary structure normally associated with fully liganded hemoglobin (the R-state), and the array of partially liganded hemoglobin tetramers in both the R- and T-states. We have applied single crystal X-ray diffraction analysis to study the structures of $[\alpha(Fe\text{-}CO)\beta(Mn)]_2$ and $[\alpha(Mn)\beta(Fe\text{-}CO)]_2$ (at resolutions of 3.0Å and 2.3Å, respectively) as examples of partially liganded T-state structures. Similarly, the structures of $[\beta(Fe)]_4$ and $[\beta(Fe\text{-}CO)]_4$ have been determined at 2.5Å resolution as a model system for studying unliganded R-state tetramers.

In both the $[\alpha(Fe\text{-}CO)\beta(Mn)]_2$ and $[\alpha(Mn)\beta(Fe\text{-}CO)]_2$ structures we find that CO binding induces small, localized changes in the T-state. Specifically, the iron atom is drawn toward the heme plane and the last turn of the F-helix moves slightly closer to the heme. In addition, the binding of CO induces a small rotation of the heme plane in the $[\alpha(Mn)\beta(Fe\text{-}CO)]_2$ tetramer, but not in the $[\alpha(Fe\text{-}CO)\beta(Mn)]_2$ tetramer. Comparing the R-state $[\beta(Fe)]_4$ and $[\beta(Fe\text{-}CO)]_4$ tetramers, we find ligand induced movements that are very similar to those seen in the T-state structures, but they are magnified by about a factor of 2 to 3. Taken together, these results support the concept that ligand-induced strain is at least part of the reason for the reduced ligand affinity of T-state tetramers.

THE STRUCTURAL BASIS OF LOW OXYGEN AFFINITY IN T STATE HAEMOGLOBIN DEDUCED FROM THE X-RAY STRUCTURES OF THE LIGANDED T STATE

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Since the oxygen affinity of the R state of haemoglobin is close to that of an isolated subunit, the crucial allosteric phenomena are i/ the low affinity of the T state and ii/ the kinetic pathway between the two quaternary structures. We have determined at high resolution (dmin=2.1A) the crystal structures of two liganded T state haemoglobins: one in which the alpha subunits are oxygenated and the beta oxygen-free, and a second in which all four haems are oxidized to the ferric form (methaemoglobin). We find the structural responses at the alpha and beta haems to be quite different. In the liganded T state alpha subunit, both the tight packing of the haem and the intersubunit contacts inhibit a conformational change at the end of the F helix which would allow the haem to become planar and the iron to assume the symmetrical octahedral coordination observed in the R state. In the beta subunit, by contrast, we find no strain on the proximal side, but the intersubunit contacts prevent the haem from tilting about an axis parallel to the F helix which would open up the binding site to oxygen. In both subunits, ligand binding in the T state induces structural changes towards the tertiary conformation of the R state.

Crystallographic Studies of Intermediate State Hemoglobins

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The X-ray structures of several analogues of hemoglobin allosteric intermediates have been determined and refined: human Hb in the T state with carbon monoxide at the α heme to 2.9 Å, human Hb in the T state with carbon monoxide at the β heme to 2.6 Å (which was studied in collaboration with R.C. Liddington and N. Shibayama), and horse Hb in the R state with no ligand to 2.0 Å resolution. The partially liganded crystals were prepared from the symmetric, mixed-metal hybrids $\alpha^{Fe(II)}CO\beta^{Co(II)}$ and $\alpha^{Ni(II)}\beta^{Fe(II)}CO$, and the R-state deoxy crystals were prepared from (bis-maleimidomethyl ether)-Hb. The intermediate state structures were compared with those of oxy-Hb, HbCO and deoxy-Hb. The R state globin is flexible and will accommodate an unliganded heme having similar stereochemistry to that found in the deoxy T state. Ligand binding at the T state α subunit results in structural change which is small in comparison with the tertiary changes associated with the T->R transition. With respect to the F helix axis, the plane of the α heme is parallel in the T structure and is tilted roughly 10^{o} in the R structure, regardless of the ligation state. In the parallel orientation, the Fe cannot be drawn into the heme plane without causing steric clashes with residues in the proximal heme pocket. The T state globin may lower the affinity of the α heme by restraining heme tilt through the contacts made with the FG corner. The conformation of this corner, which makes important interfacial contacts in both the α and β subunits, is a function of quaternary structure and not ligation state. Both the FG corner and the E helix play important regulatory roles in the β subunit. In the T->R transition, the β heme plane rotates and translates into the heme pocket and the E helix is displaced away from the ligand. The FG corner and the E helix of the liganded β subunit in the T state undergo small displacements in the same direction as the T->R transition. The movement in the E helix is required to accommodate the ligand, and the FG corner contacts restrict heme rotation and translation. Hence, an important component to the regulation of the T state β heme affinity may be the restriction the globin places on the movement of the E helix and FG corner.

Linkage Thermodynamics and Information Theory

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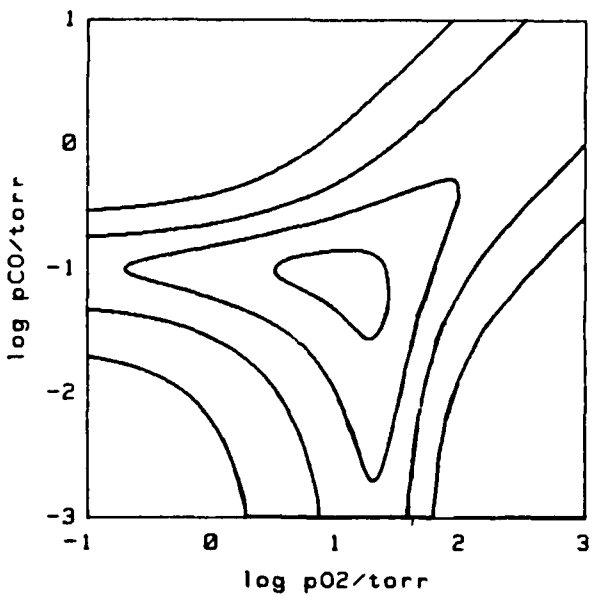
The principles of information theory are used in connection with those of linkage thermodynamics to devise suitable experimental strategies in the study of ligand binding phenomena. The results are discussed in relation to: 1) the identical linkage of oxygen and carbon monoxide to human hemoglobin; 2) the oxygen linked dissociation of human hemoglobin into dimers; 3) individual site binding phenomena.



Isoentropic levels for O_2 and CO binding to human hemoglobin

SYMPOSIUM ON OXYGEN BINDING HEME PROTEINS:
STRUCTURE, DYNAMICS, FUNCTION AND GENETICS
October 9-13, 1988
Asilomar Conference Grounds, Pacific Grove, California

UNCERTAINTIES IN THE VALUES OF EXPERIMENTAL PARAMETERS. THE SUM-OF-SQUARES PROFILE AND MONTE CARLO ANALYSIS.

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Binding of oxygen to hemoglobin is a function of the concentration (activities) of hemoglobin, protons, and anions, as well as oxygen. The shape of the oxygenation curve as well as the oxygen activity for 1/2 saturation are both changed when one of these three variables is studied with the other two held constant. To formulate a model for the binding of oxygen to hemoglobin, taking into account the effects of aggregation, proton binding, and anion binding, requires about eleven parameters--all interdependent. These complex parameters may be lumped into four stepwise apparent or experimental parameters, K_1, K_2, K_3, and K_4, where the relationship between these parameters and the four Adair constants A_i is $A_i = \prod_{j=1}^{i} K_j$.

While evaluation of these four stepwise parameters is a fairly straightforward procedure involving nonlinear curve-fitting techniques, the determination of the uncertainties in these parameters has relied on approximate extrapolations from linear methods.

The sum-of-squares profile provides a qualitative graphical description of the interdependence of the fitting parameters and the nature of their uncertainties. This procedure is derived from an extension of the grid search method used in nonlinear curve-fitting.

The Monte Carlo method provides a technique for the propagation of the errors in the raw data through the nonlinear fitting procedure to develop a distribution of the values of the experimental parameters. With this distribution defined, uncertainties for any level of confidence may be evaluated.

Physiological model of Hemoglobin: The Two State Model, Mean Field Theory and the Thermodynamics of Cooperativity.

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We present a simple quantitative formulation of the thermodynamic states of hemoglobin in contact with its five major ligands, O_2, CO_2, Cl^-, 2,3-diphosphoglycerate and H^+. Our treatment derives essentially from the two state concerted transition model of Monod et al. (1965), X-ray crystallographic structure of Perutz et al. (1980) and from the mean field formalism for treatment of electrostatic interactions (Linderstrøm-Lang, 1924; Edsall, J. T. and Wyman, J., 1958; and Tanford, 1962). We present an algorithm for the efficient computation of observable quantities such as the occupancy of various ligand binding sites. We also give a method for determining the intrinsic thermodynamic parameters of hemoglobin based on a few critical experiments and we carry out this determination. Furthermore, we compare theoretical computations with other relevant experimental observations and analyze the discrepancies. Our results support the validity of the two state concerted transition model of hemoglobin over a wide range of conditions.

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INTERMEDIATE COMPOUNDS BETWEEN HEMOGLOBIN AND CO UNDER EQUILIBRIUM AND KINETIC CONDITIONS

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Fig.A shows the distribution of species at intermediate states of ligation in solutions of human hemoglobin Ao (3mM heme concentration in 0.1M KCl) equilibrated at 20°C, pH 6.99±0.01 with gas phases of known carbon monoxide concentration as determined by a previously described method[1,2]. In agreement with previous results obtained at 50% carbon monoxide saturation, we confirm that:
a) the hemoglobin-CO interaction is highly cooperative (Hill n = 3.0);
b) mono-, di-, and triligated species are significantly represented at equilibrium;
c)chain heterogeneity is very slight between species in the same state of ligation;
d) the only detectable diligated species are of the asymmetric type.
Analysis of the data of fractional saturation vs. CO concentration is in progress to assess which of the various proposed models of hemoglobin function[3,4,5] better describe the equilibrium data and predict the observed distribution of intermediate species.

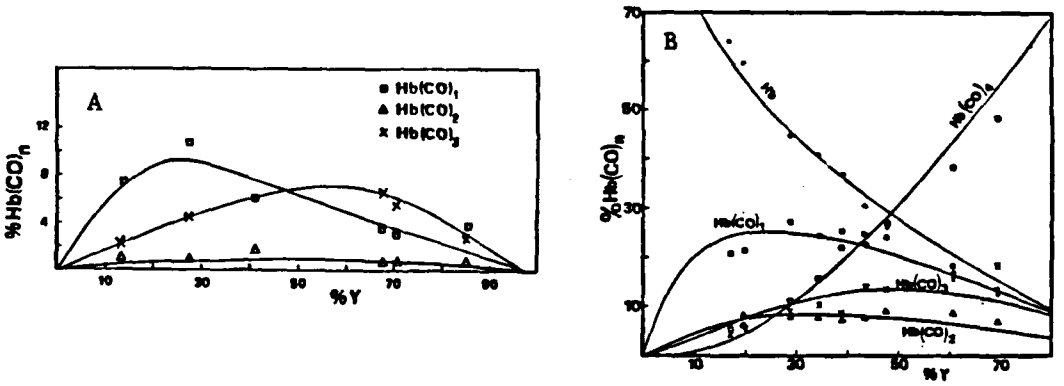

Fig.B shows the preliminary data on the isolation of the intermediate compounds in the course of the "on" reaction between human hemoglobin and CO (22°C, pH 6.9 in 0.1M KCl).

The mono-, di-, and triligated species are all represented. The only diligated species present in significant amount are of the asymmetric type and are found in lower concentration than predicted by previous reports[6]. Analysis of the data according to the usual scheme of four consecutive reactions, assuming negligible chain heterogeneity and $l'_4 = 6.0 \mu M^{-1} s^{-1}$,[6] yields the following values of the kinetic constants (intrinsic): $l'_1 = 0.46$, $l'_2 = 1.2$, $l'_3 = 5.3$. These values were used to calculate the curves in Fig.B. Data are preliminary since some systematic errors due to the isolation procedure spoil the accuracy of the measurements at low and high values of fractional CO bound.

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CHLORIDE ION AND PROTON BINDING TO HUMAN HEMOGLOBIN: LOCATION OF BINDING SITES AND QUANTITATION OF THE ENERGETICS OF BINDING

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Chloride ions and protons are important effectors of the hemoglobin molecule. Extensive experimental studies have demonstrated that differences in chloride and proton binding properties of the oxygenated and deoxygenated forms of hemoglobin modulate the oxygenation-linked structural transition between these two forms of the molecule. To assess further the atomic mechanisms through which the differential binding effects arise we would like to know WHERE in each form of hemoglobin do these ions bind, HOW MANY ions are bound (at a variety of pH values and ion concentrations), and WHY they are bound.

Our approach entails the theoretical modelling of proton and chloride ion binding properties of oxyhemoglobin and deoxyhemoglobin through the use of the static accessibility-modified Tanford-Kirkwood (SA-TK) algorithm to quantitate the energetics of electrostatic interactions. To overcome the lack of detailed structural information on the location of chloride binding sites (needed for the calculation), an algorithm was developed that allowed the identification of ion binding sites directly from the magnitude of the electrostatic potentials at the surface of a macromolecular structure. The procedure entails the calculation of the surface electrostatic potential over a range of pH values and ionic strengths. Sites of putative anion or cation binding are identified as local extremes in the magnitude of the potential. Coordinates for the sites are obtained from maps of electrostatic potentials calculated at a resolution of 0.20 A. The dielectric model used currently in the calculation of the electrostatic potential is consistent with the dielectric model of the SA-TK algorithm. However, it has been demonstrated that the predicted location of ion binding sites is independent of the dielectric model employed.

The chloride ions are incorporated into the calculation as point charges and the extent of binding at each site is calculated as a function of pH and Cl concentration with a formalism built into the SA-TK algorithm, which takes into consideration explicitly the linkage between proton binding and the binding of other small ions. The algorithm has been tested extensively and successfully on sperm whale myoglobin, where it predicted correctly the location and the extent of ion binding, as well as its dependence on pH and ion concentration. The availability of independent crystallographic structures for the deoxygenated and the oxygenated state of the molecule has allowed us to characterize the proton and chloride binding properties of each structure over a large range of pH values and Cl concentrations. The energetics of the contribution by the differential ion binding properties of the two structures to the oxygenation-linked structural transition between them has been calculated by difference of the calculations in the individual structures.

ANIONS EFFECT IN BOVINE HEMOGLOBIN. C. Fronticelli, E. Bucci & A. Razynska
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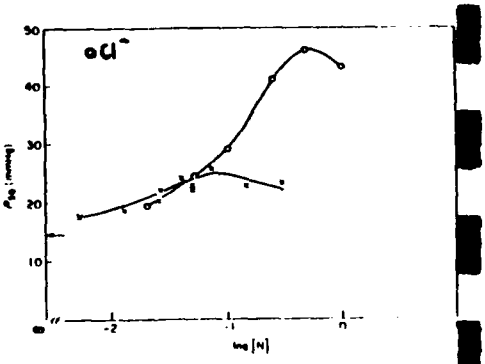
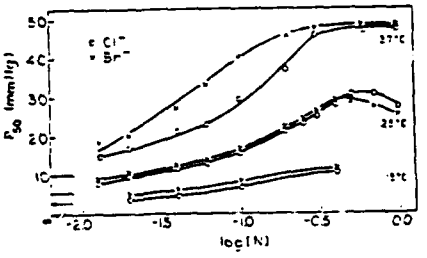
Mammalian hemoglobins can be devided into two groups, those with a high oxygen affinity and those with a low oxygen affinity. In physiological concentration of Cl^- (0.1M) the oxygen affinity of the low affinity hemoglobins is not sensitive to 2,3-DPG (Bunn, H.F.,Science 172:1049, 1971). Bovine hemoglobin (HbBv) belongs to this group.



In our laboratory we have seen that the oxygen affinities of human hemoglobin (HbA) and HbBv in the presence of 2,3-DPG were identical, provided that Cl^- anions were not present in the medium. Measurements of oxygen affinity in the presence of increasing Cl^- concentrations indicated that in HbBv there are high affinity sites equally accessible to Cl^- and 2,3-DPG and low affinity sites accessible only to Cl^- which are responsable for the low oxygen affinity of HbBv.



Oxygen affinity measurements in the presence of increasing concentration of Br^- or Cl^- indicated that their effect on oxygen binding parallel the molecular size and density charge of the anions (Fig 2). This effect, while large at 37°C, tend to vanish at lower temperatures and is absent in HbA. Competition experiments indicate that Br^- and Cl^- bind to the same binding sites in the HbBv molecule.

The values of the heat of oxygenation of HbBv calculated from the van't Hoff isochore were: ΔH=7.9 kcal/mol O_2 in the presence of 0.1 M Cl^- and ΔH=8.3 kcal/mol O_2 in the presence of 0.1 M Br^-, confirming the higher binding affinity of the hemoglobin molecule for the Br^- (charge density 0.08 $Å^{-2}$) than for Cl^- (charge density 0.10 $Å^{-2}$).

From the oxygen binding curves at 37°C the following Adair's overall binding constant were calculated:

Anionns	$\beta 1 (torr^{-1})$	$\beta 2 (torr^{-2})$	$\beta 3 (torr^{-3})$	$\beta 4 (torr^{-4})$
0.1 M Cl	0.59	1.1	0.0	3.1E-8
0.1 M Br	1.9	0.16	0.0	6.1E-7

The binding constants have different values for the two anions, consistent with presence in the HbBv molecule, of binding sites where the preferential binding is affected by the charge density of the anions.

A principal difference between the amino acid composition of HbA and HbBv is at the amino terminus of the β chains where in HbBv a methionine replaces valine β1 and histidine β2. It is relevant to stress that the β2 histidine is either replaced by hydrophobic amino acids or is deleted in all hemoglobins whose oxygen affinity is low and not regulated by 2,3-DPG. This may indicate a correlation between oxygen affinity and hydrophobic characteristics of the amino terminus amino acids of the β chains as a general phenomenon in hemoglobin.

Kinetic Studies of "Quaternary Enhancement" and Alpha/Beta Differences in Binding the Last Oxygen to Hemoglobin Tetramers and Dimers

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Analysis of O_2 binding equilibria by two independent groups has suggested that the affinity for binding the fourth O_2 to Hb tetramers is very high, about 800-1200 cal/mol (3-7 fold) higher than that of dimers.[1,2] This "quaternary enhancement" effect requires that assembly of a triply-liganded tetramer from dimers raises the affinity of the fourth site. Quaternary enhancement is incompatible with the 2-state allosteric model, and the belief that dimers and HbO_2 have a common "R-state" tertiary structure and affinity.

Recently, Gibson & Edelstein challenged the reality of the quaternary enhancement effect, based on kinetic data, and suggested that equilibrium O_2 binding data can be adequately fitted without invoking quaternary enhancement.[3] However, the statistical validity of quaternary enhancement has been re-asserted.[4]

We agree with Gibson & Edelstein that it is important to constrain model fitting with independently determined parameters whenever possible, and that kinetic data may be one important route for doing so. However, the Gibson & Edelstein studies failed to directly address the key issue of the relative affinities of dimers and $Hb_4(O_2)_3$. Furthermore, the extent to which alpha/beta differences need to be incorporated into models (and their interpretation) remains an open question.

We therefore have undertaken further kinetic studies, under solution conditions identical to those used by Ackers and coworkers. Using partial laser photolysis and O_2/CO replacement techniques, we have, for the first time, resolved the rates of O_2 association and dissociation to both alpha and beta subunits within "R-state" tetramers and dimers. We find that the β subunits are faster than α for both O_2 binding (~2-fold) and release (2-3-fold).

The kinetically-determined O_2 binding constants derived from these data are essentially identical for dimers and $Hb_4(O_2)_3$, and are significantly lower than those derived from fitting the equilibrium data. That is, the data do not support the reality of significant quaternary enhancement. These data also show that in dimers the α subunit has a slightly higher affinity than the β (~300 cal/mole), but that this difference essentially disappears in $Hb_4(O_2)_3$. The kinetic data imply that the quaternary enhancement seen in the equilibrium studies results from both an overestimate of the tetramer affinity and an underestimate of the dimer affinity.

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Searching Conformational States in Biomolecular Systems

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Polypeptides and proteins can exist in a variety of metastable conformational states, corresponding to the minimum of some postulated potential energy function. Structural transitions among these minima, along with vibrational motion within each minimum, determine to a large extent biological structure and activity, and are therefore of extreme importance in fields like catalysis and drug design. Understanding the dynamics of these systems is essential to understanding their functions. The dynamics of structural fluctuations has been implicated in binding of ligands, such as oxygen or carbon monoxide, to heme portions of hemoglobin and in enzyme-substrate reactivity. In addition to these small structural fluctuations, the problem of large structural rearrangement in computer modeling needs to be addressed. For example, a drug-ligand system may be capable of binding in two orientations, with little difference in free energy between orientations. If mutating a residue significantly increases the thermodynamic preference for one of the orientations, and a sizable free energy barrier to structural rearrangement exists, this preferred orientation may not be realized in the simulation. This potential problem is handled in computer simulation by assuming that mutation does not cause large structural rearrangement, an assumption not valid in general.

An important goal, therefore, is to determine the native conformation of these systems, as well as to investigate the kinetic processes responsible for conformational transitions between nearby free energy minima. This involves, ideally, elucidation of the entire free energy surface for the peptide or protein of interest. Consequently,the ability to evaluate absolute free energies for particular configurations is an important theoretical goal, an ability that would allow construction of free energy surfaces in these systems.

The initial research has developed and tested a method to evaluate absolute free energies of any mechanically stable conformation of a biomolecule, regardless of the thermodynamic stability of the configuration, based on expansion of the free energy about an Einstein solid with the same underlying structure. Our work has considered the reference state to be an Einstein solid having the same structure as the biomolecular system whose free energy is being analyzed. The reference state is then reached from the biomolecule by "switching-on" harmonic springs that tend to fix atoms to their fiducial sites. The parameterized Hamiltonian of the system to be simulated, $H(\lambda)$, is basically formed by perturbing the full interaction potential of the biopolymer, V_0, with a harmonic site potential. The free energies obtained will include configurational entropy contributions, even from highly anharmonic potentials. This method will allow one to include contributions to the absolute free energy from undulations and modulations in an otherwise smooth potential surface, since at the particular temperature of the simulation, the system will naturally sample all intricacies and facets of the energy surface. In addition, the present proposal addresses valid criticisms made about the coupling parameter and perturbation methods of free energy evaluation. The problems that are addressed are: (a) a reference system is required, (b) the value of the reference free energy must be known, and (c) the reference system must be similar in structure to the system under study. In our method, the reference system has the same structure as the biomolecule being studied, and the free energy of this reference state is known analytically. In addition, because the reference state and system of interest will be structurally similar, we expect the method to be fast and accurate, with few intermediate states required for convergence. The absolute free energy algorithm will allow a transparent and general evaluation of free energies of stable states, regardless of energy. In addition, bond angle and bond length fluctuations can be trivially included.

DIRECT DETERMINATION OF THE FREE ENERGY COUPLINGS BETWEEN LIGAND BINDING AND SUBUNIT ASSOCIATION IN OLIGOMERIC PROTEINS

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Experiments by many workers over a long time have been required to demonstrate the stringent free energy coupling between ligand binding and subunit association in stripped hemoglobin, and we still lack data about the complexes with DPG and other effectors. A general method to determine the sign and magnitude of these couplings would be invaluable and is in fact provided by the study of the dissociation of oligomers under hydrostatic pressure [1-3]. The coupling is revealed by the differences in dissociation pressure respectively in the absence and in the presence of saturating amounts of ligands. Experiments with yeast hexokinase[4] and yeast glyceraldehyde-phosphate dehydrogenase [5] with substrates, pseudosubstrates and allosteric effectors shows couplings, some positive others negative, of 0.5-4 kcal/mol. The simultaneous binding of two ligands produces effects that are not additive: the subunit boundaries appear to act as non-linear "summing junctions" for the transmitted effects. The spread of values of the couplings in sign and magnitude precludes the assignment to the protein of a small number of energetic states with a unique structural counterpart.

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Structural Changes Associated with the Cooperative Ligation of Hemoglobin

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During the past several years, we have investigated the structural changes associated with the cooperative ligation of hemoglobin (Hb). We have approached this problem from two directions. First, we have investigated the structure and properties of Hb as a function of oxygenation by ^{1}H-NMR spectroscopy. Our results can be summarized as follows: (i) by monitoring the intensities of the α–heme resonance at 12 ppm from H_2O and of the β-heme resonance at 18 ppm, we have concluded that, in the absence of organic phosphate, α and β chains of Hb have similar affinity for O_2; (ii) in the presence of organic phosphate, α chains have a higher affinity for O_2 as compared to the β chains; (iii) on oxygenation, the intensities of the α-heme resonance at 12 ppm and of the β-heme resonance at 18 ppm decrease more than the total number of deoxy chains available as measured by the degree of O_2 saturation of Hb, thus suggesting the sensitivity of these hyperfine-shifted proton resonances to structural changes which are believed to occur in the unligated subunits upon the ligation of their neighbors in an intact Hb molecule; (iv) a comparison of these ^{1}H-NMR data with the populations of partially oxygenated Hb tetramers leads to the conclusion that in the presence of organic phosphate, the Hb molecule with one O_2 bound maintains the β-heme resonance at 18 ppm but not the α-heme resonance at 12 ppm, thus suggesting that some cooperativity must occur in the deoxy quaternary structure of the Hb molecule during the oxygenation process; and (v) the variation of the exchangeable proton resonances as a function of oxygenation strongly suggests that the breaking of one or more inter- or intrasubunit linkages of a ligated subunit can affect similar linkages in unligated subunits within a tetrameric Hb molecule. Second, we have investigated the structural and ligand binding properties of valency hybrid Hbs such as Hb M Milwaukee (β67E11Val→Glu), $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL, $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL, $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_C$XL, and $(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_C$XL, and the structural properties of $[\alpha(\text{des-Arg})\beta]_A$ $[\alpha\beta]_C$XL, $[\alpha(\text{des-Arg-Tyr})\beta]_A[\alpha\beta]_C$XL, $[\alpha(\text{des–Arg})\beta(\text{NES})]_A[\alpha\beta]_C$XL, and $[\alpha(\text{des-Arg})\beta]_A$ $[\alpha\beta(\text{NES})]_C$XL. Our results can be summarized as follows: (i) ^{1}H-NMR results on Hb M Milwaukee, $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL, and $(\alpha\beta^{+CN})_A$ $(\alpha\beta)_C$XL as a function of ligation show that the structural changes of these Hbs are not concerted; (ii) the $\alpha_1\beta_2$ subunit interfaces in $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL and $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL are different from those of deoxy-Hb and oxy-Hb; (iii) the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ subunit interfaces in deoxy $[\alpha(\text{des-Arg})\beta]_A[\alpha\beta]_C$XL and deoxy $[\alpha(\text{des-Arg-Tyr})\beta]_A$ $[\alpha\beta]_C$XL are different and two different types of intersubunit interfaces can coexist in these cross-linked, asymmetrically modified Hbs; (iv) the ^{1}H-NMR spectra of deoxy $[\alpha(\text{des-Arg})\beta(\text{NES})]_A$ $[\alpha\beta]_C$XL and deoxy $[\alpha(\text{des-Arg})\beta]_A(\alpha\beta(\text{NES})]_C$XL at low pH cannot be explained simply as a sum of the spectral features specific for the deoxy-like and the oxy-like quaternary structures; (v) the O_2 binding properties of cross-linked asymmetrical valency hybrid Hbs, $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL, $(\alpha\beta^{+CN})_A(\alpha\beta)_C$ XL, $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_C$XL, and $(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_C$XL, support our ^{1}H-NMR results that there are at least three functional and energetically important structures of Hb in going from the deoxy to the ligated state. The relationship between these results and those of the recent X-ray crystallographic data on human deoxy-Hb, $(\alpha_2{}^{O}2\beta_2)$, and met-Hb in the T and R states recently reported by Dodson and coworkers [R. Liddingston, Z. Derewenda, G. Dodson, and D. Harris, Nature 331, 725-728 (1988)] will be discussed.

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Dynamics of Deuterium Exchange Effects on Protein Secondary Structure in Hemoglobin, Myoglobin, and Red Cells Monitored by Infrared Spectroscopy.

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We have developed infrared methods to measure the components of the secondary structure of proteins in aqueous and D_2O media. Changes in infrared spectra for solutions of extensively purified human HbA and bovine heart Mb and suspensions of human red cells have been observed following exposure to D_2O. With carbonyl hemeprotein species, the effect of D_2O on ligand carbon monoxide stretch bands is immediate and only ca. $1cm^{-1}$ red shifts are observed. However, marked changes in Amide I, II and III regions near 1650, 1550, and $1300cm^{-1}$, respectively, do occur.

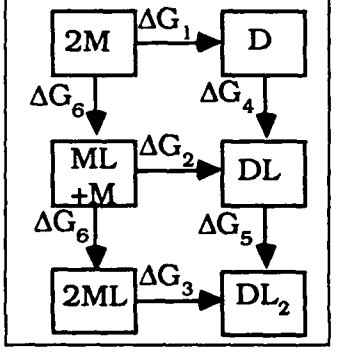
Changes in the Amide I region, due mainly to the C-O stretch vibration of peptide bonds, occur less rapidly than the changes in Amide II region where the vibrations receive a major contribution from peptide N-H. An Amide I band for α-helix is found at $1656cm^{-1}$ in H_2O media; D_2O promotes a shift to $1650cm^{-1}$. The rate of shift depends upon temperature, the D_2O/H_2O ratio, and protein structure. The shifts occur much faster for MbCO than HbCO in solutions at pH6.5 and 4 to 6mM in heme. CO saturated red cells undergo shifts much more rapidly than do HbCO solutions. Furthermore, the extent of α-helix band shift may be maximal at intermediate times and decrease on further standing. For example, following exposure to 2:1 H_2O/D_2O saline at 23°C red cells exhibit a partial shift from $1656cm^{-1}$ to $1650cm^{-1}$ which becomes maximal at 24 hours but after one week the spectrum returns to the original water spectrum.

Questions raised by these findings include: (1) What is the structural basis for the shifts in α-helix spectrum? (2) What causes the Mb> red cells >>Hb order in rate of D_2O-induced shifts? (3) How can the reversal of D_2O-induced shifts in H_2O/D_2O mixtures be explained? (4) How does D_2O exchange modify hemoglobin structure to not only alter the secondary structure but to also affect cooperativity?

GENERAL PURPOSE NUMERICAL METHOD FOR ANALYZING COMPLEX MULTI-COMPONENT PROTEIN-LIGAND ASSEMBLIES. Catherine A. Royer and Joseph M. Beechem, University of Illinois at Urbana-Champaign, Department of Physics, Laboratory for Fluorescence Dynamics, 1110 W. Green St., Urbana, IL 61801.

Human hemoglobin has long been used as a model system for the study of the allosteric regulation of biological activity. Various generalized "Adair-type" of equations have often been utilized to analyze the observed hemoglobin oxygen binding isotherms. We propose here a numerical method which directly solves for the concentrations of all the possible equilibrium species of such multicomponent systems. While under any given set of conditions, some of these "micro-species" may not be highly populated, the coupling free energy which links them to the rest of the protein states of the system may still play an important role in determining the concentrations of the more populated states. To perform this analysis, all of the possible subunit and ligand binding equilibria are utilized, each equilibrium having a particular Gibbs free energy. Given this set of simultaneous non-linear Gibbs free energy equations, we use a modified iterative Monte Carlo algorithm to solve for the concentrations of all the microscopic equilibrium states, x_i. From the set of concentrations, x_i, all of the various individual and complex binding site isotherms can be constructed. This technique was first used to examine a monomer-dimer equilibrium system, with one ligand bound per monomer. The system chosen was one of cooperative ligand binding and ligand enhanced monomer-dimer association (i.e., second order free energy coupling). The multiple equilibria of such systems can be graphically represented as in figure 1a. This system requires that a set of six simultaneous non-linear equations in six unknowns (x_i) be solved. Target free energies in this simulation were chosen from the values of the free-energy coupling determined from the Neurophysin II-oxytocin system.

Figure 1a



M = Monomer; D = Dimer
L = Ligand; T = Tetramer

Figure 1b

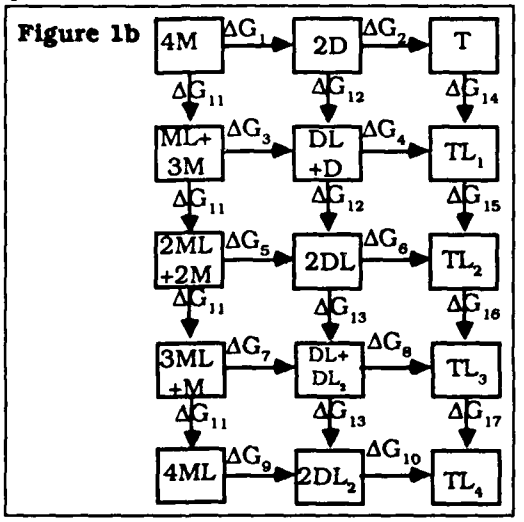
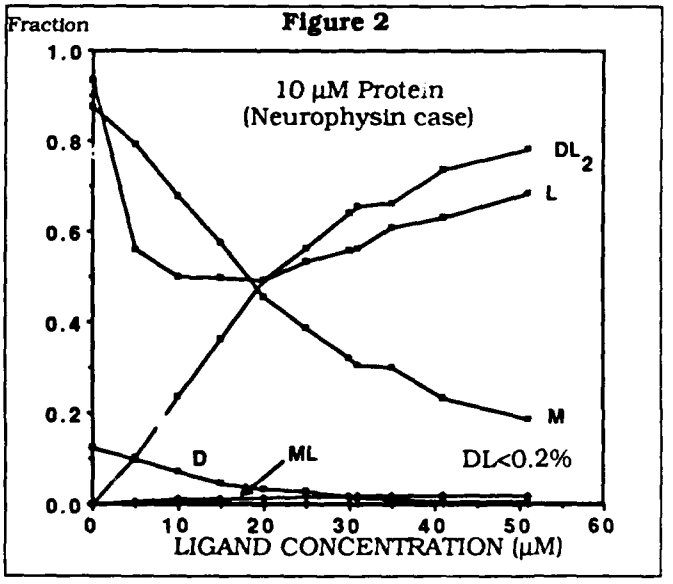


Figure 2 shows a typical example of the Monte-Carlo solution to these non-linear equations. These types of systems can be solved very quickly by the Monte-Carlo technique (less than 5 minutes on a standard IBM/AT or equivalent). The errors between the input set of free energies and those predicted from the various microspecies differ by less than 0.03 Kcal per species. The algorithm has now been expanded to solve the multicomponent equilibria of a monomer-dimer-tetramer system binding four total ligands (17 simultaneous non-linear equations in 11 unknowns, figure 1b). To test the solutions from this case, target free energies were chosen (where possible) from the literature for human hemoglobin A. The Monte-Carlo algorithm currently in use will solve the system to within approximately 0.3 Kcal/mole per species. We expect that certain fine-tuning algorithms will eventually improve this result.

Figure 2



This Monte-Carlo solution of complex multi-equilibria processes is being incorporated into a general purpose non-linear analysis program designed to fit various types of spectroscopic data (steady-state absorbance, steady-state and time-resolved fluorescence, etc.) using multiple data sets which vary both ligand/protein concentrations as well as temperature and pressure. Such overdeterminations should aid in unraveling the complex energetics involved in multi-subunit macromolecular assemblies.

Molecular Dynamics Study of CO diffusion in leghemoglobin

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The diffusion of a small ligand (carbon monooxide) in leghemoglobin was simulated [1] using the LES methodology (Locally Enhanced Sampling) [2].

According to X-ray data [3] which supply the average structure of the protein, the ligand is locked in the heme cavity of leghemoglobin (and other globins [4-6]) with no simple access to the solvent. In this study we search for the exit route(s), including the effects of protein flexibility in the calculation.

In LES we run several tens of CO trajectories in a single moving frame of the protein. The different CO's represent a probability density of a single carbon monoxide. They do not interact with each other and the protein "feels" the force averaged over all the ligands. The approximation is expected to be reasonable if the ligand does not push the protein significantly. The reduction in the numerical effort compared to the usual molecular dynamics is between one to two orders of magnitude. The method enables the search for the diffusion path in a fluctuating flexible protein for the first time.

Several LES simulations of a flexible protein which blocks the ligand escape were performed.

The results are significantly different compared to a previous LES study for myoglobin [2] and are the following:

(a) Only a single reaction coordinate was found in contrast to myoglobin in which five were observed. The observed path is close to the contact between the B and G helices.

(b) The energy barrier is small and the process is fast. Even at room temperature the ligand(s) escape at a rate of ~100 ps. The above point suggests that direct simulation of the ligand escape using LES may be possible.

(c) While in myoglobin the ligand can access a large portion of the protein, in leghemoglobin the diffusion is confined to the close proximity of the heme group.

Several different calculation protocols were employed in order to investigate the sensitivity of the final conclusions to the simulation procedure. We varied the average kinetic energy of the ligand from 5.4 to 0.6 kcal/mole (the last corresponds to room temperature). We further run trajectories with different initial velocities (maintaining the temperature constant) and different number of CO replica (sixty or one hundred and twenty). In another test we examined the effects of the different equilibration procedures of the protein on the results. Equilibrated protein structures which are slightly different from each other were employed at several simulations. The above mentioned results remained the same in all the above tests.

The faster diffusion from leghemoglobin compared to myoglobin is consisted with the experimental finding of F. Stetzkowski et al [7].

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AUTOCATALYTIC LIGAND BINDING TO HEMOGLOBIN AT HIGH TEMPERATURE.

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The exploration of the thermodynamic parameters of ligand binding to proteins, and to human hemoglobin in particular, is hindered by poor thermal stability of the samples. We have prepared and purified a derivative of human hemoglobin crosslinked twice: between the α chains (presumably 99 Lys - 99 Lys) and the β chains (82 Lys - 1 Val); this protein proved to be stable for more than 30 min. at 80° C.

Human hemoglobin was crosslinked through the β chains first, using the reagent 2 nor - 2 formyl pyridoxal phosphate (1) and the desired derivative was purified chromatographically; this material was used to start the reaction with bis 3, 5 dibromo salycyl fumarate (2), yielding the desired twice crosslinked derivative.

Optical spectra of the Soret region of deoxygenated and CO saturated crosslinked hemoglobin were collected after 15 min. incubation at temperatures between 15° and 90° C; both derivatives display a reversible optical transition with an estimated ΔH of 8 Kcal/mol of heme; an analogous optical transition is observed in the Soret and near U.V. regions of the C.D. spectrum.

Carbon monoxide binding was followed by means of a flash photolysis apparatus as a function of temperature, as already described in the case of trout Hb I (3); crosslinked hemoglobin maintains cooperativity even at very high temperature (maximum explored = 85° C), as indicated by the 50 times greater rate of CO recombination after partial photolysis (15 %) with respect to complete photolysis.

Our data confirm that, contrary to the case of trout Hb I, the equilibrium between the high and low oxygen affinity conformations of human hemoglobin (after chemical crosslinking) is scarcely influenced by temperature and that an additional, probably tertiary, transition is present for both conformations , is spectroscopically evident and shows a midpoint at around 50° C (in 0.1 M Na/K phosphate buffer pH 7.0).

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CHARACTERIZATION OF FUNCTIONAL INTERMEDIATES FOR THE REACTION OF HUMAN HEMOGLOBIN WITH CARBON MONOXIDE.

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The kinetics of CO binding to human hemoglobin has been the object of a very large number of investigations for at least two decades (see ref. (1) and references quoted therein). However, quantitative details on the mechanism of this reaction have been collected only in the last few years, taking advantage of the use of very fast lasers (see for example ref. 2,3,4). Additional information has been coming also from experiments using the rapid-mixing technique and several investigations have led to unravel an unexpected complexity in the CO binding pathway to human hemoglobin (5,6).

First observations about this complexity arise from a work by Gray and Gibson (7), who failed to find a unique isosbestic point for the CO binding process, which appears to display a biphasic rise-and-fall behaviour at wavelengths close to the equilibrium isosbestic value. This feature, which has been confirmed also by us in this investigation, refers to two consecutive bimolecular processes as demonstrated by (i) the concentration-dependence of the two phases: and (ii) the isolation of the two phases if (a) deoxyHb is mixed substoichiometric amounts of ligand ($[CO]/[Heme] < 0.5$, where only the fast portion is observed) or else (b) partially CO-saturated hemoglobin ($\bar{Y} \simeq 0.5$) is mixed with a saturating concentration of carbon monoxide (only the slow phase is present). Moreover, the investigation has been extended to modified human hemoglobin (e.g. des-His Hb, CPA-reacted Hb, NEM-reacted Hb) and to the effect of IHP. Altogether, these results allow to formulate a hypothesis of the likely sequence of events which characterize the CO binding process.

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Spectroscopic Studies of Conformational Disorder and Reactivity - Hemoglobins and Myoblobins

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An especially promising spectroscopic probe of hemoglobins and myoglobins is absorption band III at ~760 nm. This absorption band is present in the spectrum of equilibrium and transient deoxy forms of these hemeproteins and is conveniently absent in all liganded forms. Studies on this band revealed that low temperature photoproducts of liganded hemoglobins and myoglobins have different absorption maxima, compared to the corresponding equilibrium deoxy forms.[1-4] These studies all indicate that the wavelength of band III is sensitive to structural elements that are responsive to ligation induced changes in the protein. A direct connection between these structural elements and functionality was exposed in kinetic hole burning experiments[5] which indicated that, in the MbCO photoproduct, the functionality band III at cryogenic temperatures is inhomogeneously broadened. The ensemble of frozen protein conformations is mapped onto the absorption envelope so that the faster rebinding structures correspond to the lower energy segments of band III. Thus, as photolyzed ligands rebind, the net effect is a progressive line narrowing, or hole burning, as a function of rebinding. We have recently extended these studies on MbCO to include other ligands and proteins.

Investigations were conducted on deoxy Hb, and photoproducts of COHbA, COHb (bluefin tuna) and half liganded carboxy derivatives of Fe-Mn hybrids. The hybrids were particularly useful for distinguishing subunit heterogeneity from substate heterogeneity within both the R and T quaternary states. These studies clearly show that the behavior observed in the COMb photoproduct extends to hemoglobins. However, there are substantial chain specific differences as a function of species, state of ligation and quaternary structure. The general response of band III to changes in protein tertiary and quaternary structure suggests that the wavelength of band III is modulated by the same parameters that determine the ν(Fe-his) behavior in room temperature and cryogenic phototransient species.

A comparison of the kinetic hole burning phenomena for COMb and O_2Mb revealed similar patterns for the photodissociated populations. However, while both MbCO and HbCO are easily and fully photolyzed even at 2K, the corresponding oxy samples yield only 50 to 70% photoproduct. Using picosecond transient absorption techniques, we have found that the "unphotolyzable" fraction of the population originates from photophysical processes occurring faster than ~10 ps. Furthermore, the temperature dependence of the picosecond quantum yield for photodissociation suggests that this yield controlling process is distributed in a fashion similar to the rebinding kinetics. Thus, it appears that a given frozen conformational substate has not only a well defined rebinding rate, but also a well defined intrinsic quantum yield for photodissociation.

This work supported by the NSF (DMB-8604435).

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Infrared and Spin-State Properties of Anion Complexes of Human Hb, Whale Mb and Glycera Hb. R. Kassner, A. Jain, and D. Mallikarachchi, Department of Chemistry, University of Illinois at Chicago, Chicago, IL 60607

The effect of interactions between the distal histidine and heme bound ligands on the infrared and spin-state properties was investigated by comparing the properties of human methemoglobin and whale metmyoglobin to those of Glycera methemoglobin, which lacks the distal histidine, and a model heme complex. The infrared spectra of NCS^- and N_3^- complexes were measured. The infrared spectra of NCS^- and N_3^- complexes of human Hb and whale Mb are characterized by two distinct bands associated with high- and low-spin complexes. The NCS^- complexes of Glycera Hb and a hemeoctapeptide exhibit a similar broad peak which appears to be a composite of two peaks arising from high- and low-spin forms of the complexes. The azide complex of Glycera Hb exhibits two distinct peaks, one of which is similar to the model, and each of which appears to be the resultant of two peaks corresponding to high- and low-spin complexes. Shifts of 10-30 cm^{-1} towards lower frequency are observed for high-spin NCS^- and low-spin N_3^- complexes of Hb and Mb relative to Glycera Hb and the model heme complex. The shifts are consistent with a donor-acceptor interaction between the distal histidine and bound triatomic ligands.

Spin-state properties of N_3^-, NCO^-, NCS^-, and HO^- complexes were determined by measuring magnetic susceptibilities of solutions by Evan's method. The spin-state equilibria and thermodynamic values for the Glycera Hb are similar to those for human Hb and whale Mb, suggesting that interactions between the distal histidine and the bound ligands have little effect on the spin-state properties of these proteins.

MODULATED EXCITATION STUDIES OF THE RATE OF ALLOSTERIC CHANGE IN HEMOGLOBIN WITH 3 LIGANDS BOUND

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The method of modulated excitation uses weak photolysis of hemoglobin to perturb the conformational equilibrium betwee allosteric states (R and T). The rate of crossing between the states can be referenced to the population which has been excited, thereby obviating the need to account for the rate of ligand binding. Previously this method has been used with success to follow the rate of allosteric change with three CO molecules bound, with observation of the Soret difference spectra which are assigned to the alternate quaternary structures.

We have now successfully applied this method to the study of oxyhemoglobin, and find that, at pH 7 in 0.1 M phosphate buffer, the species with three oxygen molecules bound has a greater relative T state stability than the molecule with three CO molecules bound. The allosteric constant L_3 is greater than unity (approx. 1.2 versus 0.3 for CO). This implies that the allosteric constant c is ligand specific. The differences between ligands are reflected primarily in changes in the the R→T rate. The structure sensitive absorption seen in HbCO which we have ascribed to liganded hemes is absent for oxygen.

We have also incorporated into the modulation method the fluorescent DPG-analog PTS (8-hydroxy-1-3-6 pyrene trisulfonate) used as a probe to monitor the structural kinetics. This dye's fluorescence is quenched upon binding. We find the fluorescent probe and the absorption spectra give essentially equivalent results for the allosteric kinetics. In addition, we can resolve the rate of binding and release of the fluorophore to both the R and T structures. We find that binding rate to the T state is close to diffusion limited; the weaker binding to the R state is accomplished through changes in the association rate constant. While R state binding is approximately ninefold weaker (dissociation constant 0.9 mM versus 0.1 mM at pH 7, bis-tris), it is necessary to account for the kinetic data.

SPECTRAL ANALYSIS OF SULFMYOGLOBIN ISOMERS AND OF THE EXTRACTED FE(III)S_C-CHLORIN: PYRROLINE SUBSTITUENT EFFECTS

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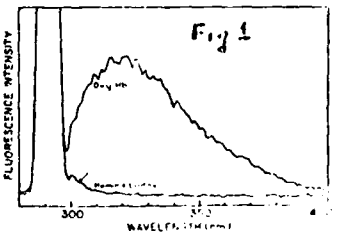
Metaquo- and metcyano-complexes of isomeric sulfmyoglobins [S_A-Mb, S_B-Mb, and S_C-Mb], and ClFe(III)-, $(DMSO)_2$Fe(III)-, and $(CN^-)_2$Fe(III)-complexes of the extracted S_C-chlorin were studied by resonance Raman (RR) spectroscopy. Q_y (red) excitation RR spectra of the ClFe(III)S_C-chlorin have increased intensity at ~690 and ~340 cm^{-1}, relative to all previous chlorins studied. These frequencies are typical of thioether C-S stretching and deformation modes; confirmation will require isotopic labeling. RR spectra of S_A-, S_B-, and S_C-metaquoMb differ from one another, as do those of S_A-, S_B-, and S_C-metcyanoMb, although only the structure at sulfur-modified pyrroline ring E varies. Given our previous reports of RR (and IR) spectral variations between model chlorins differing only in pyrroline structure, it is clear that "local" pyrroline modifications are transmitted throughout the chlorin macrocycle. RR spectra of S_A- and S_B-metaquoMb have a single ~1612-cm^{-1} band (the "ν_{10}-equivalent"), whereas for S_C-metaquoMb an additional band is present at ~1640 cm^{-1}. The extra band shifts to ~1650 cm^{-1} for the $(DMSO)_2$Fe(III)S_C-chlorin. The ν_{10}-equivalent of S_A- and S_B-metcyanoMb is at ~1642 cm^{-1}, as expected for ferric low-spin systems, but for S_C-metcyanoMb this band shifts to ~1648 cm^{-1} and is more than doubled in intensity (suggesting the superposition of two RR bands). For the $(CN^-)_2$Fe(III)S_C-chlorin, two RR bands are present at ~1640 and ~1655 cm^{-1}. The novel RR band of S_C-Mb or the extracted S_C-chlorin is absent from RR spectra of all previous model and biological metallochlorins studied, and is thus likely to derive from the thiolene isocyclic ring of the S_C-chlorin prosthetic group. This macrocycle is well-characterized as a result of detailed NMR studies by La Mar and coworkers [J. Am. Chem. Soc. 1986, 108, 7108]. The RR frequency of the novel band is most typical of an isolated double bond. If it arises from the double bond of the thiolene isocyclic ring of the S_C-chlorin, then the thiolene ring (and presumably also pyrroline ring B) are most likely out of the plane of the macrocycle. Furthermore, the frequency shifts observed for the novel band between S_C-metaquoMb and Fe(III)S_C-$(DMSO)_2$, and between S_C-metcyanoMb and Fe(III)S_C-$(CN^-)_2$, demonstrate modulation of the chlorin vibrational modes by the protein or solvent environment, and even suggest control of the extent of thiolene non-planarity by the active-site amino acids.

TIME RESOLVED EMISSION SPECTRA OF LIGANDED HEMOGLOBIN. Henryk Malak, Enrico Bucci, Clara Fronticelli, Joseph R Lakowicz. Dept of Biochemistry, University of Maryland Med.Sch.,660 W.Redwood St, Baltimore MD 21201.

We used front-face illumination to examine the steady state and time-resolved emission from the intrinsic tryptophan emission of human hemoglobin. Experimental conditions were identified which eliminated all contributions of scattered light as shown in Fig 1, where the emission of oxyhemoglobin (Tris-HCl pH 8.0, 2 g/dl) excited at 290 nm is compared to the scattering of a suspension of ludox in a hemin solution.

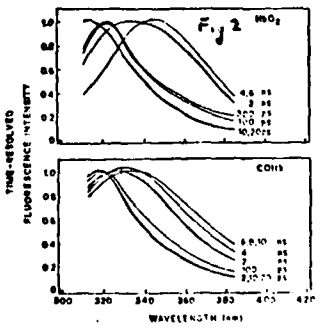


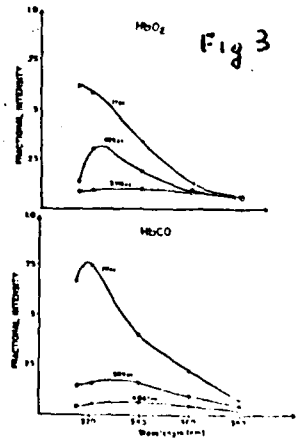
The sensitivity obtained using front-face optics was adequate to allow measurement of the wavelength-dependent frequency response of the emission to 2 GHz. The intensity decays displayed ps and ns components in the emission at all wavelength from 315 to 380 nm. As expected, the table below shows that in COHb the contribution of the ps components decreased from 78 to 52% over this range of wavelengths. In the table only the ps lifetime components are reported with the respective preexponentials (α) and fractional intensity (f).

nm	315	322	340	360	380
τ(ps)	29	28	27	27	26
α	0.992	0.989	0.981	0.976	0.970
f	0.78	0.76	0.64	0.59	0.52

Consistent with these data the static anisotropy of either oxy or CO hemoglobin showed a high value near 0.3 at 305 nm, where the contribution of the picosecond emission is predominant. The static anisotropy decreased at long wavelength where the nanosecond components were more significant.

The frequency-domain were used to calculate the time-resolved and decay-associated emission spectra. These spectra indicate that the ps components of the emission display maxima near 320 nm, whereas the ns components are centered at longer wavelengths near and above 335 nm. Fig 2,3.





The time evolution of the spectra shown in fig 2 suggests that the ps and ns components belong to molecular species not relaxing into each other during the time of observation.

STOPPED-FLOW FRONT-FACE FLUOROMETER: A PROTOTYPE DESIGN TO MEASURE HEMOGLOBIN R->T TRANSITION KINETICS

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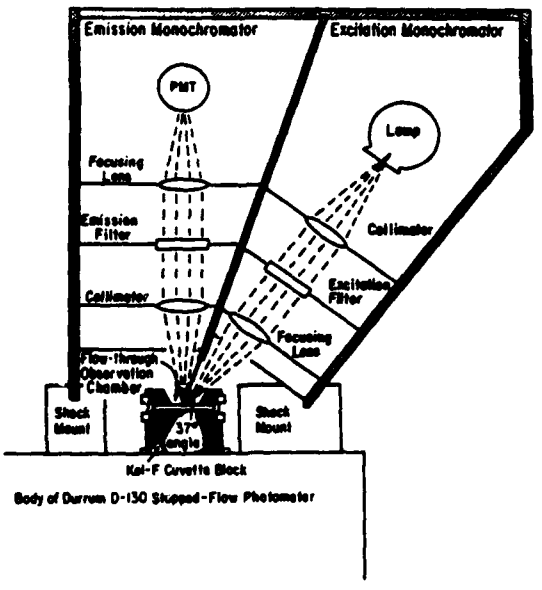
Stopped-flow techniques are successfully used to study the kinetics of the R->T transition of hemoglobin (Hb). We have previously used front-face fluorometry to demonstrate that (1) the intrinsic fluorescence of Hb primarily originates from β37 Trp; (2) the intrinsic fluorescence is sensitive to the R->T transition; and that (3) the emission of the fluorescent probes bound to specific sites on the Hb molecule (β93 Cys) is sensitive to the R->T transition. These findings suggested that a stopped-flow front-face fluorometer could probe R->T transitions at specific sites, such as the aromatic amino acids and sites selectively binding extrinsic fluorophores. We have developed a prototype instrument using as the core a Gibson-Durrum stopped-flow apparatus on line with a digital data analysis system using a modified Marquardt algorithm. Excitation (470 nm) and emission light (520 nm) were selected by narrow band pass filters. To study the R->T transition, a solution of purified oxy HbA covalently bound to the fluorescent probe 5-iodoacetamidofluorescein (HbA-AF) (1.0 g%) was mixed rapidly with deoxygenated buffer (pH 7.35, .05M potassium phosphate) containing 2 mg/ml of sodium dithionite. The hemoglobin, at a final concentration of 0.5g% after mixing, is essentially completely tetrameric. A first order reaction was observed with a rate constant near 8 sec^{-1}, similar to the oxygen dissociation rate reported for oxy Hb A. In conclusion, (1) the stopped-flow front-face fluorometer is capable of obtaining significant and reproducible data, and (2) the extrinsic fluorophore, 5-IAF covalently bound to β93 of Hb (Hb A-AF), may be used as a reporter group of R->T transition kinetics.



Interaction of Heme with the Substituted Tyrosine in Hemoglobins M and Its Relation to Function

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In hemoglobin (Hb), the proximal and distal histidines play an important role for preventing the ferrous heme from oxidation and for cooperative ligand binding. Hbs M are mutant hemoglobins in which one of these histidines is replaced by tyrosine and its heme is stabilized usually in ferric state. Although patients with Hbs M show obvious cyanosis, only individuals with Hb M Saskatoon (β E7 His→Tyr) have less cyanosis than those of the other Hbs M (Hb M Iwate, α F8 His→Tyr; Hb M Boston, α E7 His→Tyr; Hb M Hyde Park, β F8 His→Tyr). In order to know why the sympton for individuals with Hb M Saskatoon differs from those with the other Hbs M, we have investigated the reducibility of abnormal chains by erythrocyte methemoglobin reductases and the interaction of heme with the substituted tyrosine by resonance Raman (RR) spectroscopy.

Under anaerobic conditions, abnormal β chain of Hb M Saskatoon was reduced by methemoglobin reductases purified from human erythrocytes at almost the same rate as was metHb A. However, abnormal chains of the other Hbs M were scarcely reduced by these enzymes. In fact, we have found recently that more than half of the abnormal chains exist in ferrous state in the fresh blood from an individual with Hb M Saskatoon.

In the RR spectra, all of four Hbs M exhibited the fingerprint bands for the Fe-tyrosinate proteins around 1600, 1500 and 1270 cm^{-1} besides the usual RR bands of an iron porphyrin. However, only the abnormal β subunit of Hb M Saskatoon displayed the Raman spectral pattern of a six-coordinate heme while other abnormal subunits displayed that of a five-coordinate heme, and its Fe-tyrosinate stretching frequency was the lowest among them. From these observations we deduce that the characteristics in the heme structure of the abnormal β chain of Hb M Saskatoon are present in the weak Fe-tyrosinate bond, which makes the strain for pulling the heme iron toward the tyrosine weaker and thus allows weak coordination of the proximal histidine at its trans position.

Transmission of Distal Interactions Between Hemoglobin Subunits. Joseph M. Rifkind, Lu Zhang, Vijay S. Sharma,† and Abraham Levy. National Institute on Aging, National Institutes of Health, Gerontology Research Center, Baltimore, Maryland 21224, and †Department of Medicine, University of California - San Diego, La Jolla, California 92093.

Previous Mössbauer and Electron Spin Resonance studies indicate that both deoxyhemoglobin and methemoglobin undergo reversible complex formation with the distal histidine in the temperature regime above 200 K. Freeze quenching experiments imply that limited concentrations of these bis-histidine complexes are present even at room temperature, presumably in dynamic equilibrium with the usual heme states with the distal histidine $\geq$ 4Å from the iron. The relatively large energy barrier (> 60 KJ mole^{-1} for deoxyhemoglobin) associated with the formation of these complexes can not be attributed solely to the heme rearrangement expected for the formation of a low spin complex. Instead, a major contribution must originate on the distal side of the heme pocket and reflect the necessary flexibility required to decrease the iron-histidine distance to that required for bond formation.

A number of studies have investigated the effect of distal interactions in regulating ligand binding. However, the transmission of distal interactions between subunits and thereby a contribution to cooperative interactions, have for the most part been neglected. For the purpose of distinguishing distal interactions which are not an integral part of the quaternary T $\rightarrow$ R conformational changes, we have compared oxygen and carbon monoxide. Both HbO_2 and HbCO exist in very closely related quaternary R structures, however, numerous studies indicate that the tendency for a more linear CO bond produces distortions of the distal ligand pocket. We find that partially liganded hemoglobin enhances the formation of the bis-histidine complex of the still unliganded hemoglobin subunits. This enhancement was, however, found to be greater for CO than O_2.

In order to further investigate the transmission of these distal interactions between subunits we have used the valency hybrids $(\alpha^{III}\beta^{II})_2$ and $(\alpha^{II}\beta^{III})_2$. The formation of bis-histidine complexes in the oxidized chains was followed with the reduced chains in different liganded states, e.g. in the deoxygenated state or in the liganded state with either oxygen or carbon monoxide bound. These results indicate very similar results for the deoxy and oxygenated hybrids, with changes in both the type and formation of bis-histidine complexes when CO is bound to the reduced chains.

The pathway for transmission of these distal effects was investigated by comparing the results of these hybrids where either both α-chains or both β-chains are oxidized with the hybrid $(\alpha^{III}\beta^{III})(\alpha^{II}\beta^{II})$, where one of the $\alpha_1\beta_1$ dimers are oxidized. With this hybrid no difference was found between O_2 or CO bound to the reduced chains. Subunit interactions involving the $\alpha_1\beta_1$ interface are lacking in this hybrid. These results therefore imply that distal interactions can be transmitted across the $\alpha_1\beta_1$ interface as opposed to the $\alpha_1\beta_2$ interface primarily responsible for the T $\rightarrow$ R quaternary change.

FUNCTIONAL PROPERTIES OF HEMOGLOBIN IMMOBILIZED IN COACERVATES PREPARED FROM GELATIN A AND POLYANIONIC POLYCARBOHYDRATES

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Abstract

Complex coacervation is a phenomenon of phase separation that may occur in a solution of positively and negatively charged poly-ions. The resulting two phases are distinguished by the total concentration of both poly-ions, with the concentrated phase often containing vesicular structures composed of the two polyelectrolytes. We have used this phenomenon in an attempt to prepare a hemoglobin-based synthetic blood substitute.

Coacervates were prepared from gelatin A and acacia, a branched polymer of neutral sugars and glucuronic acid, in the presence of hemoglobin (HbA). HbA seems to be contained in the wall of the spherical coacervate vesicles (2-20 μm in diameter, 2.5 g HbA/100 ml), which bind oxygen reversibly (p50=2.8 mm of Hg at pH 7.5, 20°C) and cooperatively (Hill coefficient n=1.6). Inositol hexaphosphate (IHP), an effector which modulates hemoglobin's function by binding to its anion-binding site, prevents incorporation of HbA in the coacervate phase, but does not interfere with the coacervation process. In addition, IHP can extract HbA from the gelatin/acacia vesicles. This strongly suggests that the carboxylate groups of the glucuronic acid residues of acacia anchor HbA into the vesicle wall by binding to the anion-binding site of HbA.

Coacervates prepared, in the presence of HbA, from gelatin A and pectin, a polygalacturonic acid, are spherical particles, with a diameter of 1-2 μm, containing 6.9 g HbA/100 ml. Oxygen binding by HbA in these coacervates is reversible and cooperative (p50=6 mm of Hg, n=1.9 at pH 6.6 20°C). Kinetic studies on CO binding show that the CO "on" rates for HbA in coacervates and red blood cells are similar (around 5×10^4 M^{-1} sec^{-1}) and much slower than for HbA in solution.

HbA-containing (6.2 g of HbA/100 ml) coacervates prepared from gelatin A and dextran sulfate have a diameter of about 1 μm, and p50 and n values of 24.3 and 1.6, respectively, at pH 6.6, 20°C.

The HbA-containing coacervates slowly dissolve in isotonic salt solutions (145 mM NaCl pH 7.4), but they can be stabilized by treatment with glutaraldehyde. Oxygen binding by HbA incorporated into the stabilized coacervates derived from dextran sulfate is very similar to oxygen binding by human red blood cells: p50=26 mm of Hg and n=1.97 at 37°C in isotonic salt. These results show how a novel approach, based on an old concept, has led to the preparation of immobilized HbA, with functional properties similar to those of intraerythrocytic HbA.

DEVELOPMENT OF BLOOD SUBSTITUTES: MECHANISMS OF PROTECTION AGAINST HEMOGLOBIN OXIDATION BY PLASMA AND ERYTHROCYTES

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Hemoglobin autooxidation rates in buffer, whole blood and plasma were studied under physiological conditions using a modified human hemoglobin, HbXL99α, that is potentially useful as a blood substitute. The rate of autooxidation in plasma was decreased by 3 fold compared to the rate observed in physiological buffer. The major protective mechanism was shown to be scavenging of H_2O_2 produced in the reaction of hemoglobin with molecular oxygen. An amperometric assay for catalase revealed an activity of 10-12 U/ml normally present in plasma. This amount of catalase, however, could not account for the observed protection against hemoglobin autooxidation. Alternate, saturable mechanisms of H_2O_2 removal appear to be important. MetHb is reduced when transfused in the rat (Snyder, S. R. et. al (1987) Proc. Natl. Acad. Sci. USA **84**, 7280-7284). A net reduction of metHbXL99α was observed during incubations in human plasma, whole blood and washed erythrocytes. The extent of reduction could be accounted for by the available ascorbic acid. The reduction of metHb by ascorbate in simple buffers reaches a limiting value of about 50% due to the generation of H_2O_2 formed in a reaction between reduced ascorbate and oxyhemoglobin. Further reduction occurs in whole blood, but not plasma, due to efficient scavenging of extracellular H_2O_2 by erythrocyte catalase. H_2O_2 diffuses rapidly across the red cell membrane. Catalase activity measured in erythrocyte suspensions (10,200 U/g Hb) is only 12 fold less than the free enzyme. Except under conditions of extreme hemodilution, reduction of metHb by ascorbate and scavenging of H_2O_2 by red cell catalase effectively suppress autooxidation of extracellular hemoglobin in blood both in vitro and in vivo.

THE INTRAVASCULAR PERSISTENCE OF DBBF-HEMOGLOBIN

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The use of cell-free hemoglobin as a red cell substitute is limited because of its high oxygen affinity, rapid clearance from the circulation, and high colloid osmotic pressure. Modification of hemoglobin using crosslinking reagents can potentially overcome these limitations. We studied the intravascular persistence of human hemoglobin (14 g/dl) crosslinked between α chains at 99 Lysine with bis-(3,5-dibromosalicyl)-fumarate (DBBFHb) in a variety of animals and infusion protocols. Comparisons were made with isolated human hemoglobin Ao. Plasma hemoglobin concentration was measured spectrophotometrically. Half times for disappearance from the circulation were determined from empirical fits of the data to single exponential functions.

	Hemoglobin Ao		DBBF-Hemoglobin	
Animal	dose (ml/kg)	T1/2 (hr)	dose (ml/kg)	T1/2(hr)
Rat	7	1.1	7	4.4
			30	24
Rabbit	7	1.9	7	12.5
Monkey			7	16
Pig			7	6.8
			21	12.4
			35	21.7

Isovolumic exchanges of whole blood for DBBFHb (35 to 70 ml/kg) were also done in pigs. Because of the high colloid osmotic pressure, the maximal plasma hemoglobin concentration achieved was 7 g/dl, falling rapidly to 4 g/dl by 5 to 10 hours after the procedure, and then the remainder of plasma hemoglobin disappeared with a T1/2 of about 36 hours. Renal excretion accounted for less than 1% of administered DBBFHb in the first 7 hours. Calculations based on the volume of red cells removed and subsequent hematocrit measurements suggest that the plasma volume may increase by a factor of 1.8 during a 70 ml/kg isovolumic exchange in the pig. This dilution also complicates the analysis of the early kinetics of disappearance from the circulation. These studies show 1) that polymerization of hemoglobin tetramers is not necessary to prolong plasma retention, 2) that the T1/2 is dose and species dependent, 3) that modified hemoglobin is removed from the circulation by predominantly extrarenal mechanisms, 4) that colloid osmotic pressure limits the plasma concentration to about 7 g/dl due to a large increase in intravascular volume. The clinical consequences of these findings are not known.

TWO FUNCTIONS OF INTRACELLULAR MYOGLOBIN IN CARDIAC MYOCYTES

by BEATRICE A. WITTENBERG, JEANNETTE E. DOELLER AND JONATHAN B. WITTENBERG, Dept. of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, N.Y. 10461.

We have studied myoglobin function in freshly isolated adult cardiac myocytes. When the oxygen supply is limiting, facilitated diffusion dominates the experimental results. Here we report experiments carried out with unlimited oxygen supply, so that facilitated diffusion vanishes to reveal a second function of intracellular myoglobin. Myoglobin oxygenation is selectively inactivated either by carbon monoxide, which binds to deoxymyoglobin with high affinity, or by sodium nitrite which converts intracellular myoglobin to a ferric derivative which no longer binds oxygen. With both these inhibitors, both the steady state respiratory oxygen uptake and the rate of ATP synthesis by oxidative phosphorylation are significantly reduced at the superabundant extracellular oxygen pressure reported here. We measure decreased phosphocreatine/ATP as well as decreased ATP/ADP ratios in myoglobin inactivated cells. Assuming a constant rate of ATP utilization, these data demonstrate that functional myoglobin enhances the rate of ATP synthesis by oxidative phosphorylation. We conclude that myoglobin functions both by enhancing the flow of dissolved oxygen to cytochrome oxidase (facilitated diffusion) and by enhancing electron flow through the mitochondrial electron transport chain. This implies delivery of myoglobin-bound oxygen to an undefined mitochondrial terminus (myoglobin mediated oxygen delivery). The myoglobin mediated oxygen delivery is sensitive to inhibitors of oxidative phosphorylation including cyanide, antimycin, myxothiazol and oligomycin, but is not affected by the glycolytic inhibitor iodoacetate. This work was supported in part by a Participating Laboratory Award from the New York Heart Association, and by NIH HL19299 (BAW).

CRYSTAL STRUCTURE OF BEZAFIBRATE AND A COMPARISON WITH BEZAFIBRATE BOUND TO HEMOGLOBIN.

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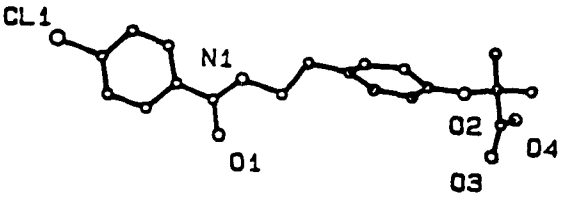
The crystal structure of Bezafibrate (BZF), 2-[p-(2-(p-chlorobenzamido)ethyl)-phenoxy]-methyl-propionic acid, was undertaken to compare the conformation of the antilipidemic drug bound to hemoglobin (Hb) [1] with the conformation of the small molecule crystal structure of the drug.

BZF crystallized in the orthorhombic space group $P2_12_12_1$ with cell dimensions a=17.823(2)Å, b=19.842(1)Å, c=10.319(1)Å and Z=8, two unique molecules per asymmetric unit. Data was collected on a Rigaku AFC5 diffractometer, with a rotating anode Cu source. The structure was solved by direct methods and refined to an R value of 0.056 for 1768 independent reflections.

The BZF molecule adopts a conformation with the two phenyl rings fully extended, but not coplanar, in the two independent molecules of the small molecule structure and the Hb-bound structure. The angles between the normals to the two phenyl rings of BZF are -67.9°, 60.4° and 33.5° for the two unique molecules in the small molecule crystal structure and the Hb-bound molecule respectively. The overall shape of the BZF molecule in the three conformations (one bound to Hb and two from the crystal structure) is similar. The main difference between the conformations is the degree of twist of the aromatic rings with respect to each other. The packing diagram of the small molecule crystal structure shows a "herringbone" type of intermolecular interaction between the aromatic rings.



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CHEMICAL PROPERTIES OF HUMAN ERYTHROCYTES TREATED WITH GLUTARALDEHYDE.

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The functional properties of human red blood cells treated with glutaraldehyde have been studied with the aim of investigating the quantitative relevance of oxygen diffusion to the ligand binding and release; possible applications of modified erythrocytes as blood substitutes may be foreseen.

The reaction was carried out on red blood cell suspensions (4 mg/ml of hemoglobin) either in the presence or in the absence of oxygen by incubation in 2.5 mM glutaraldehyde in PBS for 20 min. at room temperature (see also ref.1).

Treated cells became resistant to hemolysis and no hemoglobin was released after sonication or treatment with distilled water, organic solvents (e.g. toluene, diethyl ether) or mechanical homogeneizers.

Ligand binding to modified erythrocytes is reversible and non cooperative; an apparent oxygen binding constant could be measured for cells reacted in the presence of oxygen ($p1/2 = 0.4$ mmHg). Erythrocytes reacted with glutaraldehyde in the absence of hemoglobin ligands bind oxygen reversibly but a marked functional heterogeneity is evident ($p1/2 \simeq 10$ mmHg, $n1/2 \leqslant 0.7$).

Carbon monoxide combination kinetics was studied by means of a stopped flow apparatus on cell suspensions or by means of a microspectrophotometer on single erythrocytes (in this case the ligand is present in the cell suspension and is photodissociated using a 150 W xenon mercury lamp). Both techniques showed only minor differences between treated and native erythrocytes and almost no difference at all between cells reacted in the presence and in the absence of oxygen.

These data substantially confirm the minor relevance of hemoglobin diffusion to the ligand binding process, as already suggested (2, 3 and references therein), and indicate that polymerization of erythrocytes with bifunctional aldehydes can be used to produce sterile preparations of stable oxygen carrying corpuscles with great mechanical and osmotic resistance, of possible interest as blood substitutes (4).

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EVIDENCE FOR THE PRESENCE OF A "MEMORY" OF PREVIOUS SICKLING CYCLES BY MICROSPECTROSCOPY IN SINGLE SICKLE RED BLOOD CELLS.

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The polymerization of intracellular HbS ($\alpha_2\beta_2$6 Glu ⟶ Val) is the key event in triggering the cell deformation, and thus the occlusion crisis, which represents the main pathological event characterizing the sickle cell anemia. In this respect, the use of microspectroscopy turned out to be very useful in the investigation on the mechanism of HbS polymerization both in solution (1,2) and in erythrocyte (3), allowing at the same time to study the process in individual cells and to have an overview of the functional distribution in a population from a single patient.

Similarly, it has been used here to investigate the properties of cells which have undergone several cycles of sickling and unsickling as it occurs "in vivo". Thus, exploiting the photosensitivity of the HbCO complex it is possible to quickly "create" deoxy Hb photolyzing a cell saturated with carbon monoxide and to follow repeatedly cell deformation which gives an optical signal clearly delayed with respect to that of photolysis (related to the change in the thickness of the cell and thus on the optical pathway). Moreover, it was possible to follow the kinetics of ligand uptake in the same cell under deformed conditions or else before deformation occurs decreasing the time of photolysis. Two main results seem to come out from these studies, namely (i) after the first sickling event the cell displays a preferential axis of deformation and (ii) a given erythrocyte becomes saturated by ligand at a significantly slower rate when it is deformed than when it keeps its native shape.

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Symposium on Oxygen Binding Heme Proteins

ABSTRACT

OXYGEN DELIVERY TO THE PERFUSED HEART BY HEMOGLOBIN Ao AND DIASPIRIN CROSS-LINKED HUMAN HEMOGLOBIN. V.W. Macdonald and R. Winslow. Division of Blood Research, Letterman Army Institute of Research, San Francisco, CA 94129-6800.

Cell-free solutions of chemically modified human hemoglobin are currently being developed for use as oxygen-carrying blood substitutes in man. It has been assumed that the affinity of unmodified human hemoglobin for oxygen is too high for release of the ligand in large enough quantities to effectively sustain aerobic metabolism and tissue function during its brief passage through the microcirculation. A major goal of recent chemical modification efforts has therefore been to lower the oxygen affinity of the native molecule into a "normal" physiological range. The present work examines this issue in a live tissue model of oxygen delivery. Isolated rabbit hearts were perfused with either HPLC-purified hemoglobin Ao (HbAo) or hemoglobin cross-linked between alpha chains with 3,5-bis-dibromosalicyl-fumarate (HbXLDBBF) in Krebs-Henseleit bicarbonate (K-H) buffer at 37° C. P50s of HbAo and HbXLDBBF under these conditions were 12 mmHg and 30 mmHg, respectively. Hemoglobin concentrations were adjusted to 1.5 g/dl (0.93 mM heme). Assuming an oxygen-binding coefficient of 4 moles O_2/mole Hb tetramer, this provides a hemoglobin-bound oxygen content equal to that of oxygen dissolved in K-H buffer fully equilibrated with 95%/5% O_2/CO_2 at 760 mmHg. Myocardial performance was determined using an isovolumic Langendorff preparation in which PaO_2 and PvO_2 were continually monitored by electrodes placed in the perfusion line, each in series with a spectral flow cell from which hemoglobin spectra were collected in real time. Hearts were perfused under constant flow conditions both with and without hemoglobin and made hypoxic by progressively lowering the buffer PO_2. In K-H buffer alone or with 1.5 g/dl BSA, left ventricular developed pressure (LVDP) continually decreased to 30% of initial values at the lowest PaO_2 (80 mmHg). However, when either HbAo or HbXLDBBF was present, these hearts only exhibited a 10-15% loss of LVDP over the course of the titrations. Spectral data demonstrate progressive deoxygenation of both HbAo and HBXLDBBF during the hypoxic titrations. These data demonstrate that despite its relatively high affinity for oxygen, HbAo is as capable as HbXLDBBF, with its lowered oxygen affinity, of delivering oxygen to an otherwise hypoxic heart. The relationship between the measured oxygen affinity of cell-free hemoglobin solutions and their ability to deliver oxygen across the microcirculation is not as simple as previously assumed and requires more detailed investigations before a "normal" physiological range of ligand affinities can be defined.

MODIFICATION OF HUMAN HEMOGLOBIN WITH MONO(3,5-DIBROMOSALICYL)FUMARATE

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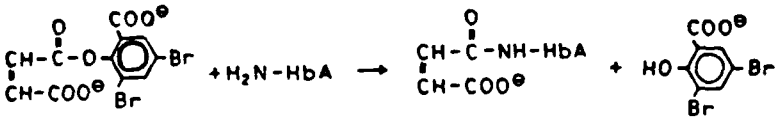
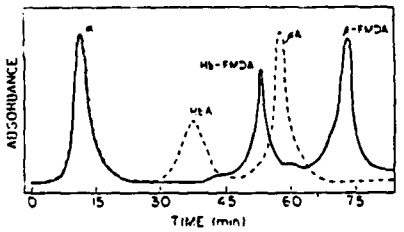
In our laboratory we are exploring site specific chemical modification of hemoglobin molecule, for the modulation of the physico chemical properties of the protein. Human oxyhemoglobin was reacted with mono(3,5-dibromosalicyl)-fumarate (FMDA).

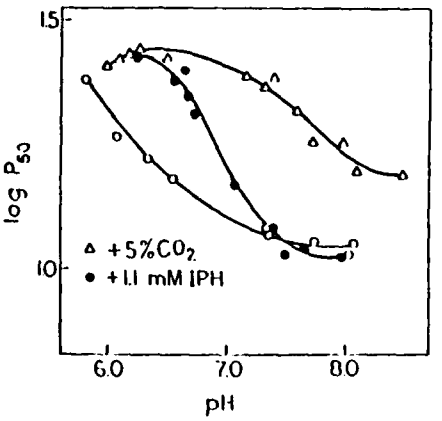


The hydrophobic character of the salicylic acid which carries two atoms of Br in position 3 and 5, associated to the presence of a carboxyl group in position ortho, introduces in this derivative selectivity for specific groups in the β cleft of the hemoglobin molecule (I.Klotz, D. Haney & L. Wood. J. Biol. Che. 260:16215,1985)

Chromatographic resolution of the modified hemoglobin (Hb-FMDA) gives a symmetric main peak representing 65 to 70% of the total protein. Comparison of the chromatograms, obtained using an anion exchange column (5PW), of HbA and Hb-FMDA previously reacted with p-hydroxymercurybenzoate, shows that the chemical modification was specific for the β chains which had a different elution times. The unmodified and the chemical modified β chains are eluted in single symmetric peaks as for homogeneous proteins.



Oxygen equilibrium measurements indicated that Hb-FMDA is very responsive to the presence of 5% CO_2, with a P1/2 = 24mmHg at 37°C in 0.1 M Tris-Cl buffer at pH 7.4. The oxygen affinity is lowered to 12 mmHg in the absence of CO_2. Addition of 1.1 mM IHP, did not modify the oxygen affinity at pH 7.4, however it interacted with the protein at lower pH, producing a decrease in affinity and an increase in the slope of the Bohr effect.



Sedimentation velocity measurements show that at neutral pH the macromolecular properties of Hb-FMDA were those characteristic of a tetramer at all protein concentration. At pH 5.5, where the sedimentation velocity of HbA decreases due to the dissociation of the molecule in αβ dimers, Hb-FMDA still retain the sedimentation velocity of a protein largely tetrameric even at a concentration of 0.36 mg/ml.

These data stress the relevance of the stereochemistry and charge distribution in the β cleft to the modulation of oxygen affinity and to stability of the tetrameric form of oxyhemoglobin.

METHYL ACETYL PHOSPHATE: A NEW CHEMICAL MODIFIER FOR HEMOGLOBIN.
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Methyl acetyl phosphate (MAP) is an acetylating agent specific for some anion binding sites in the hemoglobin (Hb) molecule. With human Hb, the acetylation is competitive with 2,3-diphosphoglycerate (DPG). Three amino groups on the β-chain Val-1, Lys-82, and Lys-144 are acetylated. These amino acid residues are located at or near the DPG binding pocket. No acetylation was observed with any amino acid residues on the α-chain. Our results indicate that the binding of MAP to the target anion binding site is the prerequisite for acetylation by MAP.

With bovine Hb, the acetylation is competitive with chloride ion. The sites of acetylation in oxy bovine Hb are Met-1(β) and Lys-81(β), whereas in deoxy bovine Hb they are Val-1(α) and Lys-81(β). These could be the chloride binding sites in bovine Hb. MAP modified Hb (both human and bovine) have a lowered oxygen affinity. After the acetylation, Hb no longer responds to its allosteric regulators, DPG and chloride. Thus, MAP is a covalent labeling agent for these binding sites.

The site specific acetylation by MAP was applied to sickle cell Hb. We found that MAP inhibits erythrocyte sickling in vitro. Acetylation by MAP increases the solubility of deoxy HbS and prevents changes in the cell density distribution upon deoxygenation. Together with other properties of MAP, such as its permeability towards the red cell membrane without lysis even at high concentrations (up to 50 mM), MAP could be a novel anti-sickling agent. (Supported in part by NIH Grant HL-18819.)

Kinetics of Chloride-Sulfate Exchange in Young and Old Human Red Blood Cells using ^{35}Cl NMR

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It has been shown that the rate of anion transport in human red cells increases as the cells approach the 120 day limit of their lifespan [Zanner & Galey, (1985) BBA **818**, 310]. Efflux in the old cells was found to be 20% greater than that of young cells. These studies were done by measuring the rate of the radioisotope $^{35}SO_4^{=}$ efflux from cells in exchange for Cl^-. Under the conditions of the radiotracer experiment, hematocrit was <5%, and Cl^- was present in both the intracellular and extracellular environments. Due to these conditions, and the fact that Cl^- has a much greater affinity for the exchange protein than $SO_4^{=}$, not all of exchange sites were available for interaction with sulfate. Anion exchange in unseparated red blood cells has also been measured using ^{35}Cl NMR and phosphate as the exchange ion [M. Brauer et al. (1985) J. Biol. Chem. **260**, 11643]. In these experiments, the Cl^- efflux from the cell into a Cl^--free environment was measured, using a 50% hematocrit.

We have used ^{35}Cl NMR to study the kinetics of the band 3 anion exchanger in young and old red cells, using $SO_4^{=}$ as the exchange ion. We used a 50% hematocrit, in order to maximize the NMR signal, and measured Cl^- efflux from the cell. The NMR experiment measures only changes seen in extracellular $[Cl^-]$. Intracellular Cl^- is invisible due to the large quadrapolar moment of the chloride ion and its interaction with other intracellular components. Due to the initial absence of extracellular Cl^-, all outward facing transporters are available for sulfate/chloride exchange at the beginning of the experiment. With the increase of $[Cl^-]_o$ over the time course of the NMR experiment, there is an increase in competition for the transporter, and therefore an effective decrease in the number of carriers available for $SO_4^{=}/Cl^-$ exchange. This complicates the kinetics of the process. However, initial rates of the sulfate/chloride exchange compare favorably with the $^{35}SO_4^{=}$ efflux experiments. The NMR experiment shows a 33% increase in the rate of anion transport in old cells when compared with young cells. This is in good agreement with the radiotracer study of the differences in anion transport in young and old red cells. Hence, ^{35}Cl NMR appears to be a valid technique for assessing changes in anion transport in the aging red cell.

THE EFFECT OF 3-(DIETHYLAMINO)PROPYL ISOTHIOCYANATE ON THE FUNCTIONAL PROPERTIES OF HUMAN HEMOGLOBIN

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The effects of chemical modification on the functional properties of hemoglobin should depend on the charge of the modifying reagent. Hemoglobin reacted with a positively charged compound, 3-(diethylamino)propyl isothiocyanate, shows an increase in oxygen affinity over a wide pH range and a decrease in the alkaline Bohr effect of 50% both in the presence and the absence of chloride ions. The dependence of the oxygen affinity of the modified hemoglobin on chloride concentration was normal below 0.1M Cl^- but absent above 0.1M Cl^- indicating disruption of the low affinity binding sites. These results suggest that the observed reduction in Bohr effect is due to disruption of both the chloride dependent and the chloride independent Bohr groups.

The increase in the oxygen affinity of red cells after 30 min. treatment with the compound indicates penetration of the cell membrane (probably by the unprotonated form). These results suggest possible applications in the treatment of sickle cell anemia.

Equilibrium Oxygen Binding to an αα-Cross-Linked Hemoglobin

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Oxygen equilibrium curves of purified human hemoglobin Ao and hemoglobin cross-linked at the α chains by bis(3,5-dibromosalicyl) fumarate (ααHb) were measured by the method of Imai(1). The alkaline Bohr effect and oxygen-linked chloride effect were determined at 0.2mM heme in 50mM bis-Tris or Tris buffers. The ααHb had a lower affinity for oxygen under all conditions, with P50's 2-5 fold higher than for HbAo, depending on pH and [Cl^-]. The Bohr effect was 60% less in the ααHb than in HbAo (0.21 H^+ compared to 0.49 H^+ released per heme for ααHb and HbAo, respectively). Hill coefficients for ααHb did not change with pH and were slightly but consistently less than for HbAo (n = 2.4-2.5). The oxygen-linked chloride effect was also less for ααHb (0.18 Cl^- released per heme for ααHb compared to 0.42 Cl^- for HbAo), suggesting a link between these altered heterotropic effects.

Mathematical fits of the binding curves to the Adair model gave zero values for the third equilibrium constant (K3) of HbAo under every condition, whereas K3 was nonzero for ααHb in all cases. The two-state Monod-Wyman-Changeux (MWC) model fit the HbAo curves poorly and the ααHb curves only slightly better. Neither model offers a mechanistic interpretation for the low oxygen affinity of ααHb. The α-cooperon model(2) specifies two quaternary states like the MWC model but also allows an interaction between binding in the T state (i.e., the T state is cooperative). This model fit all of the HbAo and ααHb curves very well, with less systematic error in the residuals than for the Adair model. The affinity of the T state (KT) was 2-5 fold lower for ααHb than for HbAo, and varied less with pH. The interaction factor in the cooperon model, which represents the extent of cooperativity in the T state, was >1 for O_2 binding to HbAo but was <1 for ααHb at physiological pH. If the cooperon model is correct and there is cooperativity in the T state of HbAo, then these results suggest that O_2 binding to T-state ααHb is not enhanced and possibly inhibited. In addition, equilibrium constants for the R state (KR) of ααHb are >10-fold lower than for HbAo, and L, where $L = [T_o]/[R_o]$, is independent of pH and reduced by 10-1000 fold compared to HbAo.

Comparing ααHb to HbAo, we find that the alkaline Bohr and O_2-linked chloride effects are reduced, indicating less conformational change on O_2 binding. In ααHb there appears to be a triply-ligated species, the T-quaternary state has a slightly lower O_2 affinity, there is no or perhaps a negative interaction between binding in the T state, the quaternary equilibrium is shifted towards the R state and uninfluenced by pH, and the affinity for O_2 in the R state is much lower, resulting in a 2-fold smaller difference in cooperative free energy for ααHb than for HbAo.

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STABILIZATION OF HEMOGLOBIN BY CROSSLINKING. Kenneth W. Olsen, Thao Yang, Thomas D. Corso, Elisa A. Hofmann, Carol A. Podlasek, Susan M. Bauer, Carlos A. Orihuela, and Alicia J. West, Department of Chemistry, Loyola University of Chicago, 6525 N. Sheridan Rd., Chicago, IL 60626, USA.

Human hemoglobin can be stabilized by a variety of crosslinking reagents, including bis(3,5-dibromosalicyl)fumarate (diaspirin), diimidates, diisothiocyanostilbene disulfonate (DIDS) and difluorodinitrobenzene (DFDNB). The stabilizing effects of these reagents were demonstrated by thermal denaturation done in 0.01 M MOPS, pH 7, containing 0.9 M guanidine. The changes in absorbance between 200 - 650 nm were monitored by a diode array spectrophotometer while the sample was heated at a rate of 0.3°C/min from 25 to 70°C. The diaspirin crosslinking reagent, which introduces a fumarate crosslink between the two Lys β82's in oxy Hb (Walder et al. (1980) J. Mol. Biol. 141:195), produced a markedly higher transition temperature (T_m) of 57°C, while that of normal hemoglobin was 42. Similar results were obtained when the Hb was crosslinked between the two Lys α99's by reacting deoxy Hb with the diaspirin (Chatterjee et al. (1986) J. Biol. Chem. 26:9929). Crosslinking of HbA with a series of different length diimidates showed that these reagents could also stabilize the molecule, but the stabilization was quite dependent on the degree of modification and the number of inter-chain crosslinks. With diimidate crosslinking, the polymeric hemoglobin produced was the most stable product. Difluorodinitrobenzene can also be used to crosslink between lysines, but the crosslinking distance is much less. Depending on the reaction conditions, this reagent can produce a protein with a single inter-chain crosslink (T_m = 58°) or a less-stable polymeric hemoglobin. DIDS reacted with deoxyhemoglobin gave several products, one of which contained a single crosslink between the β-chain amino termini (Kavanaugh et al. (1988) Biochem. 27:1804). This single crosslink raised the T_m of cyanomethemoglobin by 7°C. Hb A multiply crosslinked by this same reagent showed a similar increase in its T_m. Purified β82 diaspirin crosslinked hemoglobin was reacted with dimethylpimelimidate (DMP) to produce a double crosslinked hemoglobin. Denaturation of this modified protein demonstrated T_m's of 52 and 68°C for methemoglobin. The lower T_m is less than that of β82 crosslinked hemoglobin and probably represents protein that has reacted with DMP but not been crosslinked by it. The higher T_m represents the successfully double crosslinked hemoglobin. These results should improve the design of crosslinked hemoglobins as blood substitutes.

Energetics of Hemoglobin Subunit Interactions - Application to Thalassemia

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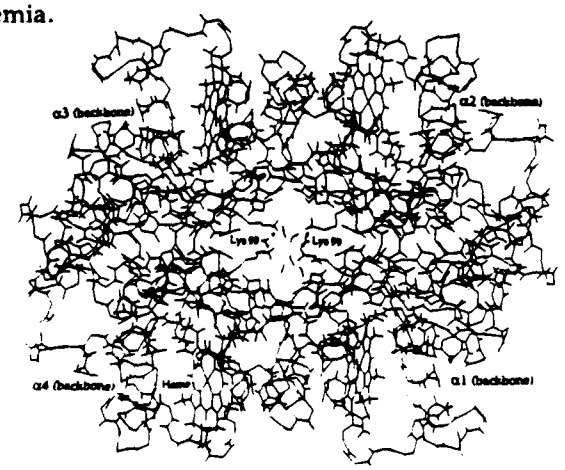
Hemoglobin consists of a tetrahedral arrangement of four distinct polypeptide chains that associate into a singular macromolecular assembly. Normally, two of these chains are of type alpha (α), and the other two are of a similar but distinct type called beta (β), yeilding a tetramer of $\alpha_2\beta_2$. A significant deficiency of β-hemoglobin subunit leads to β-thalassemia, while a deficiency of α-chain leads to α-thalassemia. In the latter case a stable β_4 hemoglobin tetramer forms, but the coresponding α_4 (β-thalassemia) does not form. Thus, β-thalassemia, or Cooley's anemia results in a more serious clinical condition, resulting in 100,000 child deaths per year. Although Cooley's anemia was discovered in 1925, and the structure of hemoglobin was determined in 1961 by Dr. Perutz and his colleagues, there is no cure and little treatment for this orphan disease.

The aims of this research are (1) to determine which subunit-subunit energetic interactions are critical for subunit assembly (or for preventing assembly), (2) to determine which subunit-subunit interactions are critical for the cooperative quaternary conformational change, and (3) to devise novel compounds which will facilitate the formation of biologically stable oxygen-transporting α-hemoglobin tetramers.

We have studied intersubunit interactions for four tetramers, α_4, $\alpha_2\beta_2$, β_4, and $\alpha_2\alpha^{(99\ Lys->Glu)}{}_2$, using molecular mechanics, and graphics. The $\alpha_2\beta_2$ hemoglobin (from crystal coordinates) has a strong attractive interaction in the central cavity near α-Lys 99 due to close contact on different subunits between counter ions: (1) α2-Lys 99 with β2-Glu 101, β2-Asp 99, and α1–Asp 94, and (2) β2-Glu 101 with β1-Arg 104., thus attracting the four subunits into a single molecule. The β_4 hemoglobin (from crystal coordinates) has a moderately repulsive interaction in this same region due to the clashing of like ions from different subunits in the residues β-Glu 101, β-Asp 99, and β-Arg 104 (β1–β3 and β2-β4). However, the α_4 hemoglobin (simulated by transposing α-chains into β-chain positions) has a more repulsive interaction for the same region due to clashing Lys 99 ions (α-Val vs β-Glu 101), resulting in a net positive energy for the tetramer. The β_4 hemoglobin has other strongly attractive intermolecular interactions on the periphery of the protein that might be responsible for its stability (and rigidity), resulting in a large net negative energy (2.5 times the $\alpha_2\beta_2$). Replacing two of the Lys 99 residues with Glu in the α_4 tetramer changes the intermolecular interactions dramatically, yielding a strong net attraction in the central cavity.

These preliminary results suggest that an important cause of the lack of association among α-chains into tetramers may be the packing of four repulsive charges (Lys 99) in close contact without sufficient counter charges. This packing may prevent other important hydrophobic interactions between chains. These data combined with literature data on α-chain crosslinking agents suggest that the Lys 99 residues might be crosslinked in order to yield a functional α_4 hemoglobin and provide a viable therapy for β-thalassemia.



α4 hemoglobin, looking into the "central cavity," with Lys 99 highlighted.

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