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13. ABSTRACT (Maximum 200 words)

Our goal is a quantitative understanding of the relation between protein structure, motions, and function. We attack four related objectives: (i) exploration of the structure and organization of the energy landscape of proteins; (ii) investigation of protein motions on the energy landscape; (iii) study of the dependence of the energy landscape and protein motions on external agents; (iv) elucidation of the connection between protein motions and biological function. We study protein dynamics and function by combining two techniques: flash photolysis and optical spectroscopy from the ultraviolet to the mid-infrared (including FTIR spectroscopy). We measure protein spectra, relaxations, and reactions over wide ranges in temperature (10-330K), time (ns-ks), pressure (0.2-400 MPa), solvent conditions and pH. Pressure is a crucial variable both as static parameter and perturbation to induce protein relaxations. Myoglobin is used as prototypical protein to explore concepts and laws that are the generalized to other more complicated heme protein. We then are able to extract protein specific behavior and refine the basic concepts and laws.

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PRESSURE STUDIES OF PROTEIN DYNAMICS

FINAL REPORT

Principal Investigators: Hans Frauenfelder and Robert D. Young

Institution: University of Illinois at Urbana-Champaign

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1 Introduction and Background.

1.1 General Concepts of Protein Dynamics. Recent work has shown that proteins are dynamic systems and that protein motions are essential to protein function and biological control [1-8]. It is becoming apparent that the problem of protein motions and function is part of a larger field, the science of complexity, which attempts to unify and understand fields ranging from glasses to neural networks [9-13].

We seek to obtain a quantitative understanding of the relations among structure, dynamics, and function of proteins. Progress towards this goal involves the following areas: (i) Dynamic Structure. The three-dimensional structure and mean-square displacements should be known with high resolution for all atoms of the proteins under investigation. (ii) Conformational Energy Landscape. A protein can assume a large number of slightly different structures called conformational substates (CS). Protein motions are transitions among the CS that depend on the arrangement (topology) of the of the conformational energy landscape. (iii) Dynamical Laws. To describe protein motions, knowledge of both the conformational energy landscape and the laws that govern transitions among the CS of this landscape is needed. (iv) Structure and Dynamics. The

spatial structure of the protein molecule completely specifies the conformational landscape. Our understanding of this connection is still at a beginning. (v) Function and Control. The motions that are relevant for the protein to perform a particular function should be known. The influence of external agents such as pressure, pH, solvent, viscosity, and solvent composition on the function of proteins also must be elucidated

Our work under this ONR grant is part of a broad program addressing each of the areas above. We firmly believe that all of the life sciences, from marine biology to medicine and pharmacology, will reap rewards from in-depth knowledge and understanding of the dynamic structure-function relation in biomolecules. This work also will lead to fundamental insights into the function of biological systems in extreme environments. In the next subsection we focus on the particular aspect of our work funded under this grant--the effects of pressure on protein dynamics.

1.2 Pressure Studies of Protein Dynamics. Studies of protein dynamics over wide ranges in pressure and temperature have contributed significantly to both biology and physics. We have described these contributions in our proposal and the published research papers which are appended.

Our group is unique in systematically performing studies over wide ranges in both temperature and pressure. We use pressure both as static variable and dynamic perturbation. As static variable pressure effects on ligand binding to myoglobins have conclusively demonstrated that conformational substates are crucial to controlling protein reactions [4]. We have also observed the transition to the glassy state in carbonmonoxy-myoglobin (MbCO) and have shown that the proteins have long-lived, nonequilibrium, metastable states [4,13]. As dynamic perturbation, pressure has allowed us to observe several nonequilibrium relaxation processes in MbCO [4,13-15]. Pressure has indeed proven to be a very powerful tool in studies of protein dynamics yielding unprecedented insights into conformational effects on protein reactions and the time- and temperature-dependence of protein relaxations. We append ten papers and a thesis which indicate the range and depth of the results of our work using pressure.

1.3 Conformational Substates. Our work as well as that of others, has conclusively demonstrated the existence of conformational substates [8,16]. The significant problem is no longer to prove the existence of CS, but to explore their arrangement, characteristics, and dependence on protein structure. In part owing to our work under this ONR grant, we have found strong evidence that the CS are most likely arranged in a hierarchy as depicted in Fig. 1 [6,9,16].

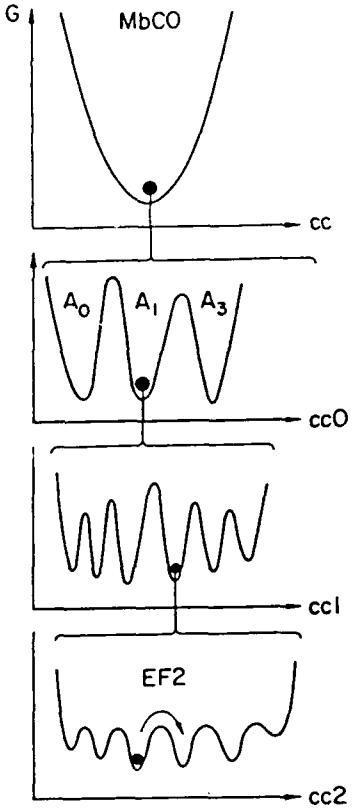


Fig. 1 Hierarchical arrangement of the conformational substates in MbCO, showing a one-dimensional cross-section through the multi-dimensional conformational energy surface. Three tiers of substates (CS^i, i = 0,1,2) are shown as functions of three conformational coordinates (cci, i = 0,1,2). •: represents a single protein molecule. EF2 represents equilibrium fluctuations in tier 2.

The highest tier of substates (CS0) has the highest barriers; substates of lower tiers are separated by barriers of decreasing height. Such a hierarchy of CS is not only important for biological control, but may also be essential for arriving at a quantitative model for other complex systems such as glasses and spin glasses [9,10,13,14].

1.4 Substates of Tier 0. The discovery of substates of tier 0 (CS0) in MbCO is of special importance. It was well known that the infrared spectrum of MbCO shows at least three different bands corresponding to three different CO stretch frequencies, but the physical meaning of the bands was not clear. We have used FTIR spectroscopy to show that the three stretch bands correspond to different substates of tier 0, denoted by A_0, A_1, and A_3 [16,17]: The A substates have different structures since the angle between the heme normal and the CO dipole differs for each [18]; the A substates bind CO at a different rate; they also have different energies, entropies, and volumes [4]. The populations of the different A substates depend strongly on temperature, pressure, pH, and environment. Thus, the three A substates of myoglobin can be considered as a simple model of isozymes and may have relevance to a quantitative understanding of control in enzymatic reactions.

1.5 Relaxation Phenomena in Proteins. Fig. 1 suggests that many different fluctuations and relaxation phenomena exist in proteins. At equilibrium the population of

proteins in the various CS is given by the Boltzmann distribution, but the protein molecules still fluctuate among the CS. The equilibrium fluctuations are denoted by EFi, i = 0,1,2,.... As temperature is lowered, successive EF will freeze out; first EF0, then EF1, and so on. The protein becomes glass-like. We have observed the transition of the protein to a glass-like state [4,13,16].

We have also observed nonequilibrium relaxation processes by establishing a nonequilibrium state through the application of pressure at high temperature and then lowering the temperature. After pressure release, the protein returns to the equilibrium state. Experiment shows that several relaxation processes exist, some of which are nonexponential in time and do not obey a standard Arrhenius relation for temperature [4,13,14]. At least two of the relaxation processes depend on solvent viscosity and consequently must involve large-scale protein motions. Clarifying the time, temperature, and viscosity dependence of protein motions is an important goal of current research in protein dynamics.

1.6 Protein Dynamics and Function. Recently we have developed a fully dynamic model for the binding of small ligands like dioxygen and carbon monoxide to myoglobin and other heme proteins [6]. Our model makes predictions that can be tested. It implies that motions in at least three tiers (CSi, i=0,1,2) of the conformational energy landscape are essential to protein function and that the reaction energy landscape, consequently, is time- and temperature-dependent. We predict that the ligand binding reaction to heme proteins can be controlled in a variety of ways by conformational relaxation and fluctuations. For example, the rate of the binding reaction can be affected by shifting from a slower A substate to a faster one [19]. Thus control does not need a very specific pathway but can be exerted by a general dynamic mechanism. Insights into protein relaxations obtained from experiments using pressure as dynamic perturbation were crucial to developing the model [6].

2 Grant Results

2.1 Research Environment Prior to our ONR Grant "Pressure Studies of Protein Dynamics." The use of high pressure in studies of proteins was largely ignored by workers in the field for many years owing to the complexity of the effects of pressure on protein spectra and reactions. Part of the problem resulted from underlying assumptions which were made by those using pressure in protein studies:

(i) Kinetic experiments performed at ambient temperature or over a small temperature range provide all relevant information.

(ii) Protein reactions can be described by the Eyring equation, which expresses the rate coefficient by $k = \nu \exp[-G^*/RT]$ where the activation free energy is $G^* = E^* - TS^* + pV^*$ and V^* is the activation volume .

(iii) Conformational effects can be neglected. in particular the substates of tier 0 were not yet discovered nor were their effects known.

(iv) Protein relaxations are exponential in time and satisfy a standard Arrhenius relation in temperature.

(v) The effect of the environment can often be neglected.

As we intensified our work under the ONR grant we realized, sometimes after some difficulty, the inadequacy of these assumptions. In order to make additional progress we developed more sophisticated experimental, computational, and theoretical techniques. As a result, work under our ONR grant has led to a number of significant advances which have changed each of the 5 assumptions listed above and which have led to a fully dynamic picture of proteins and protein dynamics. We summarize advances in the most recent grant period and provide references to the original papers:

2.2 Progress and Results. We have made substantial progress toward all general objectives stated in our proposal submitted 06-29-88. These goals, stated on pp. 11-12, of our proposal included: (I) Protein dynamics. We planned to determine if the CS in proteins were arranged as in Fig 1. We also proposed to study relaxations and fluctuations in the conformational energy landscape. Our strategy was to begin with MbCO and then to move to more complicated heme proteins. (II) Physical principles. We proposed to investigate the physical and physico-chemical laws of protein dynamics and protein reactions. (III) Biological role of dynamics. We intended to test the functional importance of protein motions. We also continued our equipment development and designed a new, greatly improved, pressure cell which made low temperature, high pressure measurements essentially routine [4].

Below we summarize the most significant results of the past grant period.

I. Protein Dynamics. The history of physics demonstrates that quantitative theories were invented after the appropriate "energy landscape" was at least qualitatively explored. We can expect that protein science will be no different and that a correct theory of protein motions and function will only be possible after the conformational energy landscape of proteins is better known. Our work has made important steps in uncovering the arrangement of the energy landscape of proteins:

(i) Substates of Tier 0 (CS0) [4,15,16,17]: The infrared stretch bands of CO bound to heme proteins are a superb probe for investigating the CS0. The position and area of the CO stretch bands and angle between the CO dipole and the heme iron characterize the CS0. To study the A substates we measured the properties of the CO stretch bands as function of temperature, pressure, pH, and solvent composition. We extract the energies, entropies, and volumes of the substates by assuming that the entropy S(T,P) of the protein can be written as $S(T,P) = S(0) + sT + vP$, in analogy with the entropy of a glass. This relation is consistent with the temperature dependence of the specific heat of proteins. Standard thermodynamics yields the Gibbs free energy between any two substates of tier 0 as $\delta G(T,P) = \delta E(0) + P\delta V(0) - T\delta S(0) - \delta vPT - (1/2)\delta sT^2$. Fitting this relation to data for the relative ratios of the populations for the A substates results in the differences in internal energy, entropy, and volume between any two substates of tier 0 for MbCO. These results clearly demonstrate the limitations of experiments performed at a single temperature or within a narrow range of temperatures. We have also obtained preliminary data from experiments on CO bound to other heme proteins including P450, HRP, and CPO which are currently being evaluated [20]. We have observed CO stretch bands in HRP-C-CO corresponding to protein structures not previously resolved. P450 with substate bound has also yielded surprising results.

(ii) Substates of Tier 1 [4,13,14]. We have measured separately the binding of CO to each A substate of tier 0 for sperm whale MbCO at low temperature from 10 μs to 1 s. We find that the time dependence of the kinetics is nonexponential and have determined the barrier distribution g(H) for the bond-formation step at the heme iron for each A substate. We therefore know that each CS0 must consist of a large number of CS of tier 1. We have also performed measurements of the kinetics for each A substate for several different pathways in the TP-plane using time-resolved FTIR spectroscopy at low temperature. These data show that the kinetics for A_0 and A_1 are affected very differently by pressure: The increase in binding rate of A_0 is mainly affected by a shift in population of the CS1, and the increase in A_1 mainly by a large activation volume.

(iii) Substates of tier 2 [6]. Evidence for the existence of CS2 comes from studies of the slowing of the rebinding of CO to Mb with increasing temperature above the glass transition region.

II. Physical principles. The experiments conducted under this grant using pressure as both static variable and dynamics perturbation have resulted in substantial progress in developing the underlying physical and chemical principles relevant to protein dynamics. The full range of the results are in the published papers which are appended. We focus on several important aspects:

(i) Pressure effects on spectral lines [4]. Spectral bands of proteins show elastic effects similar to that of crystals: The protein remains in the same substate and does not change conformation since the topology of atoms remains the same. However, we have shown that spectral bands of proteins also show a conformational effect: The CS of proteins can have slightly different volumes both within a tier and between different tiers of the hierarchy. Pressure-induced changes in substate populations not only shifts a spectral band but also causes changes in the shape of a band. We have demonstrated these effects in the Soret band and CO stretch bands of MbCO. The A_0 and A_1 substates behave very differently. A_1 shows very little or vanishing elastic and conformational shifts. A_0, in contrast, displays both elastic and conformational shifts. The difference in the elastic shift for A_0 and A_1 indicates that the environment of the heme-CO may be considerably different in the different substates. The conformational effect in A_0 implies that the substates of tier 1 within a given CS0 have properties different enough to produce a conformational shift.

(ii) Relaxation processes [13,14,15]. We use time-resolved FTIR spectrometry to measure protein relaxation near the transition temperature of the protein. In a relaxation experiment, the response of an observable is monitored after the system is perturbed by a pressure-jump (P-jump). The observable M can be the intensity, position, or width of a spectral band. Two different types of response are observed, elastic and conformational. The elastic shift is faster than our shortest measurement time (10s). The conformational shift which follows the elastic shift occurs since pressure changes the local or global arrangement of atoms so that the protein moves from one conformational substate to another.

The behavior of the observable is characterized by a relaxation function, $\Phi(t) = [M(t)-M(\infty)]/[M(0)-M(\infty)]$, where M(0) is the value of the observable after the elastic shift, and $M(\infty)$ is the new equilibrium value. The relaxation functions observed in glasses for the large-scale a relaxation and in spin glasses are not exponential in time, but can often be parameterized by the Kohlrausch-Williams-Watts law or stretched exponential [33]:

$$\Phi(t) = \exp\{-[\kappa(T)t]^{\beta}\} \qquad (1)$$

where $0 < \beta < 1$. The stretched exponential form implies that the relaxation involves a wide range of timescales. The exponent β determines the width of the distribution of relaxation times. The temperature dependence of the characteristic rate $\kappa(T)$ for the α relaxation in glasses often does not obey the Arrhenius law. We use a relation which is known to describe relaxation phenomena over many decades in glass-forming liquids and synthetic polymers:

$$\kappa(T) = A\exp[-(E/RT)^2]. \qquad (2)$$

This relation has been derived for a random walk of an excitation in one dimension within a Gaussian density of states.

In a typical P-jump experiment, pressure is released from 200 to 40 MPa in a few seconds. Infrared spectra are measured from 10-10^4 s over a range of about 50 K in the transition region. Fig. 2 displays two relaxation functions for MbCO in 95% glycerol/water. For Φ_0 the observable is the relative area of the A_0 band, and for Φ_1 the observable is the peak of the A_0 band. Φ_0 describes nonequilibrium motions within tier 0 (FIM 0), corresponding to the transition $A_0 \rightarrow (A_1, A_3)$. Φ_1 describes nonequilibrium motions in tier 1 of the A_0 substate (FIM 1).

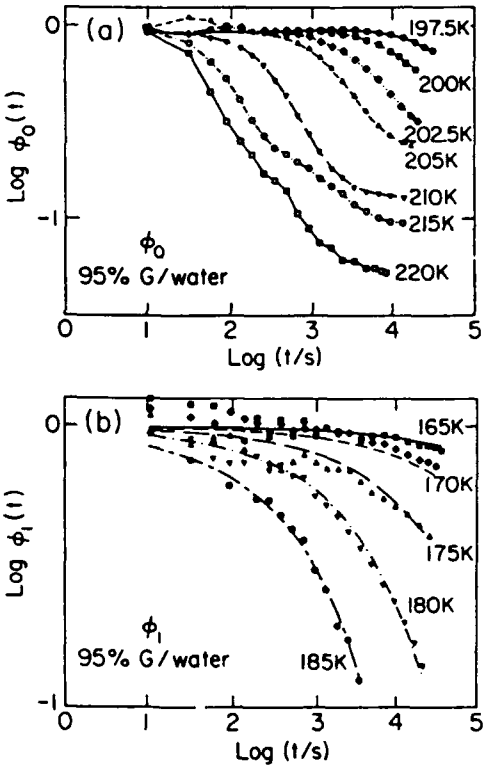


Fig. 2 Relaxation functions (a) $\Phi_0(t)$ and (b) $\Phi_1(t)$ at various temperatures for MbCO in 95% glycerol-water (v/v), pH 7. $\Phi_0(t)$ was obtained from the area of the A_0 substate and describes transitions ($A_0 \rightarrow (A_1, A_3)$ in tier 0. $\Phi_1(t)$ was calculated from the peak position of A_0 and describes relaxation in tier 1.

The time dependence of Φ_1 is well described by the stretched exponential, Eq. (1), and the temperature dependence of the rate coefficient κ_1 can be described by Eq. (2). The relaxation function Φ_0 is more complicated and shows at least two components, the significance of which is not yet clear. We fit the faster component to an exponential and find that the rate coefficient κ_0 is also well described by Eq. (2).

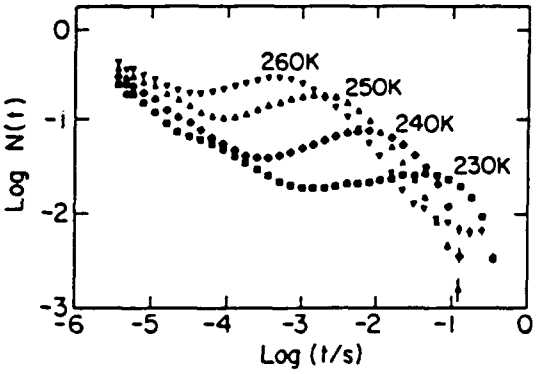
(iii) Spectrum of Glass Transitions in Myoglobin [13,14,15]. The relaxation data in Fig. 2 show that a protein experiences at least two glass transitions as temperature is lowered. FIM 1 freezes out about 30 K lower in temperature than does FIM 0. As

temperature increases, FIM 1 is essentially completed before FIM 0 begins. In addition, measurements in 75% and 95% glycerol/water show that they are strongly dependent on solvent viscosity. FIM 0 and FIM 1 each freeze out about 10 K lower in 75% than in 95% glycerol/water.

(iv) Reaction theory and conformation volume [4]. We have performed flash photolysis studies CO binding to horse (hMb) and sperm whale (swMB) at low temperature in each pathway in the TP-plane. We have shown that the simple Eyring equation for the binding rate is not adequate for proteins and must be generalized to include the effects of pressure on different conformational substates. This is done by defining a conformation volume V_C which expresses the direction and effect of conformational changes on the binding rate. Previous kinetics data on proteins must be reexamined in light of the importance of pressure-induced conformational effects on binding rates of proteins.

(v) Time- and temperature dependence of large-scale conformational transitions [15]. We have measured separately the binding of CO to each A substate of tier 0 for sperm whale MbCO above 200K from 10 μs to 1 s. Fig. 3a shows the rebinding kinetics for the A_0 substate from 230 to 260 K. The unusual rebinding kinetics for the A substate are a result of the FIM 0 transition $A_0 \rightarrow (A_1, A_3)$. We extract the rate coefficient κ_0 for this transition using the maximum entropy technique and find that is well described by Eq. (2). Fig. 3b plots $\log(\kappa_0)$ versus $(1000/T)^2$ from two entirely different experiments; the P-jump (release) experiments and the A substate rebinding experiments. The agreement is excellent and proves that both experiments probe the same FIM 0.

Fig. 3a Flash-photolysis kinetics of MBCO in 75% glycerol/water (v/v), pH 5.7, monitored in the A_0 band (≈ 1966 cm^{-1}) in the infrared. The fraction N(t) of molecules that is yet to rebind to the substate A_0 is plotted as as function of log t. (a) 60-160 K. Lines are fits using eqs. (1) and (2) and the parameters in table 1. (b) 230-260 K.



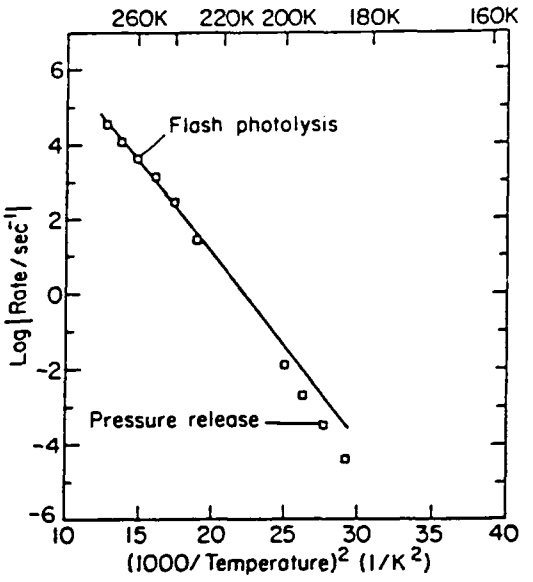


Fig. 3b Logarithmic plot of the characteristic rates for the transition $A_0 \rightarrow (A_1, A_3)$, as measured by flash-photolysis and pressure release, given as a function of $(1000\ K/T)^2$. The straight line is the result of a fit to the flash-photolysis data.

III. Biological role of dynamics. Experiments performed under our ONR grant have led to several new and significant insights into the biological role of dynamics. Most of the results are contained in the enclosed publications. Here we focus on three main areas:

(i) Function and dynamics in horse and sperm whale myoglobin [4]. The effect of pressure on the binding of CO and O_2 to swMb and hemoglobin at physiological temperatures has long been a puzzle: Hasinoff showed in 1974 that the application of pressure at high temperature speeded up the binding of CO to Mb indicating activation volume V^* for CO is negative, but slowed the binding of O_2 to Mb indicating that the activation volume for O_2 is positive. We have measured the rebinding of CO and O_2 to both horse and sperm whale myoglobin at low temperature from 1 μs to 100 s with monitoring in the Soret band. We have also measured rebinding of CO to swMb at low temperature from 1 s to 10 ks with monitoring in the CO stretch bands. These measurements were performed using each of the pathways in the TP-plane. They provide substantial insight into the importance of structure and conformational effects for protein function. We describe the main results below.

Soret band. The rebinding curves for hMbCO and swMbCO monitored in the Soret band appear similar after cooling to low temperature at atmospheric pressure. However, the two proteins behave very differently under pressure. In both proteins the activation volume V^* for MbCO is negative and about the magnitude expected for bond formation (~ -10 cm^3/mol) so pressure speeds up binding. The activation volume for O_2 is also negative for both proteins, but small in magnitude. The conformational effects, which are characterized by the conformation volume V_C, are large and negative for swMbCO and $hMbO_2$, large and positive for $swMbO_2$, and small and positive for hMbCO. The large

difference between the conformation volumes for swMbCO (-9 cm^3/mol) and swMbCO (+7 to +13 cm^3/mol) obtained from the Soret band suggests a strong interplay between ligand and protein.

CO infrared stretch bands. The rebinding curves monitored in the infrared show that the speed up of CO binding to Mb with increasing pressure has two causes: Pressure shifts the population from the slower A_1 substate to the faster A_0 substate, and the rebinding of both CS0 speeds up. The A_0 and A_1 substates behave differently under pressure. The increased rebinding rate of A_0 is caused by a large conformation volume, while the increase in A_1 is the result of a large activation volume.

O_2 binding to Mb. We have not yet measured the infrared stretch spectrum of O_2 bound to Mb. However, the Soret kinetics for CO rebinding to Mb together with the infrared data for MbCO rebinding allow some indirect conclusions regarding the binding of O_2. The conformation volume V_c of binding to swMb is large and positive. Pressure therefore slows down O_2 binding and the main cause of the slowdown is a conformational shift not an activation volume. In contrast the binding of O_2 to hMb is more weakly affected by pressure, and the conformation volume has the opposite sign from swMb. This difference may be relevant to the fact that whales are marine mammals and dive while the horse does not.

(ii) FIM 2: $Mb^* \rightarrow Mb$ [6]. Our studies of the binding of CO and O_2 to myoglobin and other monomeric heme proteins have resulted in a model of the binding process in which protein relaxation and fluctuations are essential for control and specificity. Our model unifies a large body of experimental results and makes predictions which can be tested. The pressure studies of protein relaxations were essential to development of the model. These studies show that Mb has a third tier of conformational substates (tier 2) and motions (FIM 2) which are local and probably involve the structure of the proximal histidine, iron, and porphyrin. Immediately after photodissociation of MbCO, the iron moves out of the heme plane and the myoglobin molecule, denoted Mb^*, is not yet in the deoxy structure. The iron then slowly relaxes further out of plane with concomitant changes in the Mb structure. This process is denoted by $Mb^* \rightarrow Mb$. This relaxation process is nonexponential in time, non-Arrhenius in temperature, and well described by Eqs. (1) and (2).

(iii) Myoglobin as Allosteric Protein [15,17]. Myoglobin is dependent on external agents such as pressure, pH, and solvent viscosity and composition. In addition the rebinding kinetics of the A substates for MbCO exhibit features seen in ligand binding to hemoglobin including: bond formation, ligand motion involving exit and entry from the protein, and a large-scale structural transition or "switch,". Thus, myoglobin may be an

excellent model for insights into allosteric effects, in general, and hemoglobin, in particular.

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