

REPORT DOCUMENTATION PAGE			! Gpsn !Bqqspwfe!! !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!PN C!OP /!1815.1299!	
Qvcrjd!Sfqpsjoh!cvsefo!gslui jt!dpmfidupo!pg!jogpsn bupo!jt!ftjn bufe!up!bwfsbhf!2!i pvs!qfs!sftqpotf-ljodme!joh!ui f!jn f!gslsfwifx joh!jotusvdjupot-lt fbsdi joh!fyjtjoh!ebub!tpvsdft-! hbui fsjoh!boe!n bjobjoh!ui f!ebub!offefe-lboe!dpm qmijoh!boe!sfwifx joh!ui f!dpmfidupo!pg!jogpsn bupo!!Tfoe!dpm n foutsfhsbjoh!ui jt!cvsefo!ftjn buft!ps!boz!pui fs!bt qfdu!pg!ui jt!dpmfidupo! pg!jogpsn bupo-ljodme!joh!t!vhhftjupot!gslsfvdjoh!ui jt!cvsefo-lup!X bti johupo!I fber vbsufst!Tfswjdf!-!Ejsf!dpsb!f!gsl!jogpsn bupo!P qf!sbjupot!boe!Sfqpsu!-!2326!K!g!stpo!Ebwjt!I jhi x bz-! Tv!j!f!2315-l!Bsjoh!po-l!WB!33313.5413-l!boe!up!ui f!P!g!jdf!pg!N bobhfn foub!boe!Cvehfu!Qbqfsx psl!Sfevdjupo!Qspk!du!1815.1299-!X bti johupo-l!ED!31614/!				
1. AGENCY USE ONLY (Leave Blank)		2. REPORT DATE 11/13/03		3. REPORT TYPE AND DATES COVERED Final Report, May 01, 2001- April 30, 2003
4. TITLE AND SUBTITLE Advanced Developments of Electron Spin Labeling as High-Resolution Sensors of Protein Structure and Conformational Switching			5. FUNDING NUMBERS Grant#: DAAD19-01-0522	
6. AUTHOR(S) Wayne L. Hubbell				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Regents of the University of California, P.O. Box 951406 Los Angeles, CA 90095-1406			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U. S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211			10. SPONSORING / MONITORING AGENCY REPORT NUMBER 42652.1-MS	
11. SUPPLEMENTARY NOTES The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.				
12 a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited.			12 b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) The dynamic modes of a nitroxide side chain in a protein directly reflect both the local 3D structure and dynamics of the protein. If the structural and dynamical contributions can be resolved, a map of side chain mobility throughout a protein molecule can provide an image of protein structure and dynamic features related to function. To separate these contributions, libraries of spin-labeled mutants of T4 lysozyme (T4L), Myoglobin (Myb) and Cellular Retinol Binding Protein (CRBP) were prepared, and the corresponding EPR spectra analyzed by simulation techniques. In addition, x-ray structures were determined for select spin-labeled T4 mutants. The collective results of these efforts have provided an unprecedented level of sophistication in interpretation of the EPR spectra of labeled proteins, and establish the feasibility of separating structural and dynamical contributions. In addition, a "bridge project" to the DARPA MOSAIC project has been completed. In this venture, spin labeled Annexin XII and T4L were supported on planar surfaces and oriented in the external magnetic field of the EPR spectrometer. Such specimens provide an additional dimension (orientation) to extract structural information, and provide the basis for producing suitable specimens for the single spin microscope under construction at UCLA.				
14. SUBJECT TERMS site-directed spin labeling protein dynamics			15. NUMBER OF PAGES	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OR REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION ON THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED	20. LIMITATION OF ABSTRACT UL	

FINAL PROGRESS REPORT

Statement of the Problem Studied

Understanding the mechanisms of protein function is a major goal of contemporary biology. Over the past decade it has become evident that most protein function is mediated by molecular motions, and the static images of protein crystallography are insufficient to decipher the mechanisms. New technologies are needed to experimentally explore protein dynamics.

Site directed Spin Labeling (SDSL) has become a powerful method for the determination of structure in proteins of any degree of complexity. Since the beginning, it was realized that the EPR spectra of spin labeled proteins contain information about backbone dynamics of the protein as well as local structure, but to date the level of interpretation has been largely qualitative and it has not been possible to separate the roles of structure and dynamics. To do so requires a *quantitative* understanding of the structural origins of side chain motion as reflected in the EPR spectra, and how they are modulated by backbone motions. This must come from: (1) the generation of a database of spectra from proteins of known structure; (2) determination of the crystal structures of the spin labeled proteins, and (3) simulation of the EPR lineshapes from first principles to extract the dynamic modes of the nitroxide side chains. These are the basic goals of the project. In the long range, the outcome of this work will establish SDSL as a quantitative tool for determination of protein dynamics and provide essential information for interpretation of images from the single electron microscopes under development in the DARPA MOSAIC project.

Summary of the most important results

A. Libraries of spectra and lineshape analyses in T4 Lysozyme (T4L), Myoglobin (Myb) Annexin XII (An), Colicin E1 (ColE1) and Cellular Retinol binding Protein (CRBP).

As reported in interim reports I and II, libraries of EPR spectra of spin-labeled mutants in the α -helical proteins T4L and Myb were prepared and analyzed. In all results summarized here, the spin label side chain used is designated "R1", and the structure is shown in Figure 1.

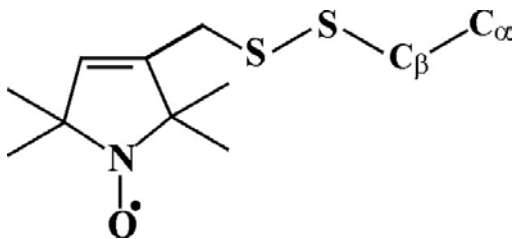


Figure 1

In T4L, R1 was "scanned" through a 23-residue helix-turn-helix motif that included all of helices G and H (114-135; Interim Report I). In Myb, a similar scan of 22 residues was prepared through helix H (128- 49; Interim Report II). Since Interim report II, additional mutants (62, 66, 67, 70, 74, 78) were obtained as part of a scan through helix E of Myb. These new spectra, combined with those previously obtained in other proteins provide a significant database for analysis. For many of the spectra, including those for 17 residues in 4 β -strands of the β -sheet protein CRBP, theoretical simulations were carried out to deduce the dynamic mode of the

nitroxide side chain. These studies revealed that there are apparently only four fundamental "dynamic modes" of the nitroxide side chain. These are designated: (1) "weakly ordered"; (2) "strongly ordered"; (3) "immobilized"; and (5) "complex". Example EPR spectra and their simulations are shown in Figure 2. Essentially all spectra observed so far belong to one of these classes. The next step was to determine the structural origins of these classifications. For this purpose, x-ray structures of selected mutants belonging to each class were determined in T4L.

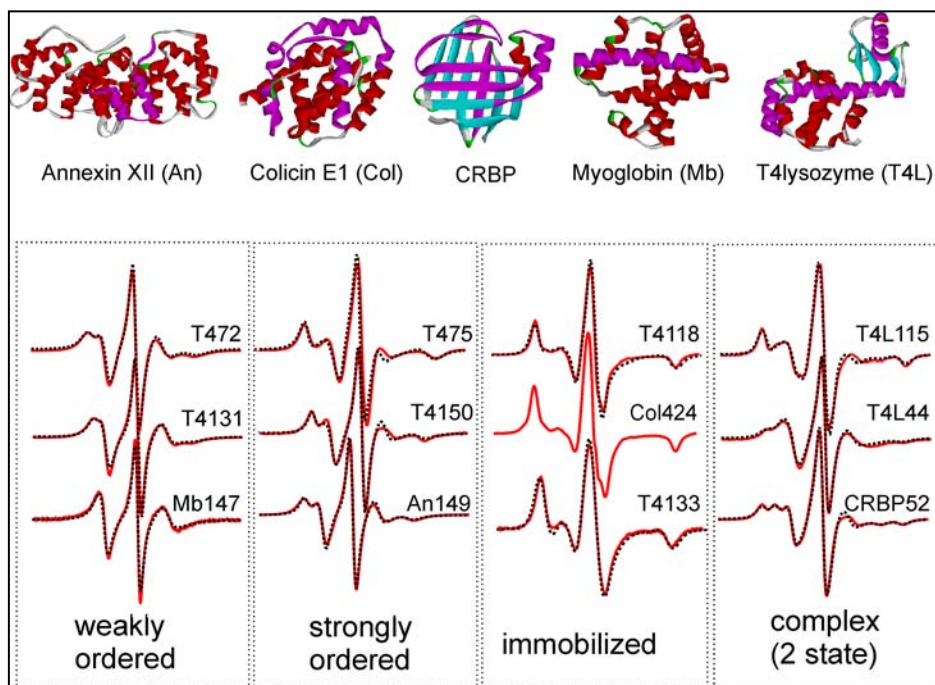


Figure 2 Proteins in the data base (Top panel). The magenta sequences are those for which R1 scans have been completed. Experimental (red solid) and simulated (black dotted) EPR spectra for examples of the four dynamic modes of R1 in a protein (Bottom panel).

B. Crystal Structures of T4 Lysozyme mutants bearing spin labeled side chains

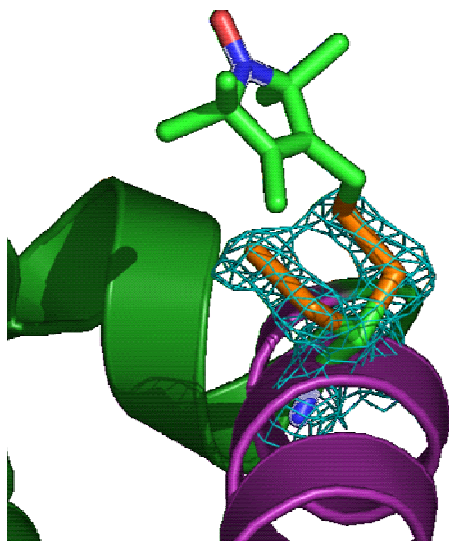


Figure 3 Electron density around the R1 side chain at site 131 in T4L. The nitroxide ring is modeled for only one of the rotamers.

(i) *131R1, a Weakly Ordered state.* Figure 3 shows the electron density map for 131R1, a helix surface site. The disulfide groups of two rotamers of the side chain are clearly resolved, but density for the nitroxide rings are missing due to spatial disorder. This suggests that the ring is mobile in solution, consistent with the spectral simulations that reveal an effective correlation time of ≈ 2 nsec with a weak ordering (order parameter $S \approx 0.4$; see spectrum in Figure 2). The weak ordering, due to the ordered disulfide, means that the internal motion of the side chain does not completely average the magnetic anisotropies of the nitroxide. Hence, the EPR spectrum is sensitive to additional motions due to backbone fluctuations of the protein. This result, together with spectral simulations, enables the determination of local protein backbone fluctuations in the nsec regime.

(ii) *150R1, a strongly ordered state.* The crystal structure of this site was just completed. The strongly ordered state (see spectrum in Figure 4; $S \approx 0.7$) arises from a combination of hydrophobic interactions with the disulfide, and an unusual H-bond between tyrosine 139 and the 4-H on the nitroxide ring. This is a similar situation to 75R1 that was previously reported (see original proposal). In the library of spectra produced during this project, at least 4 other strongly ordered states have been observed, and all are in the vicinity of groups capable of making H-bonds with the ring.

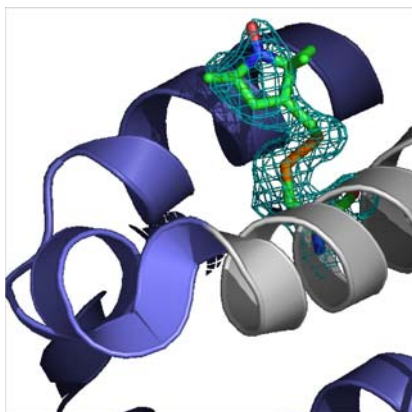


Figure 4 Electron density around the R1 side chain at 150 in T4L.

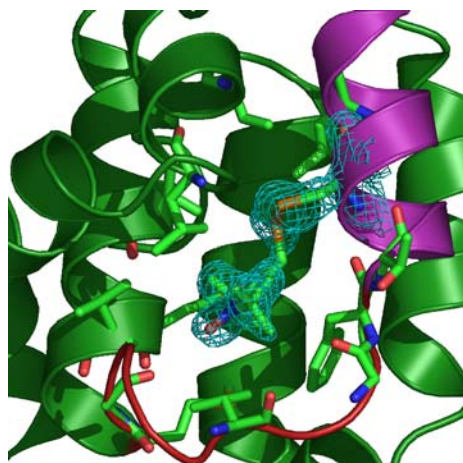


Figure 5 Electron density around the R1 side chain in 118 in T4L

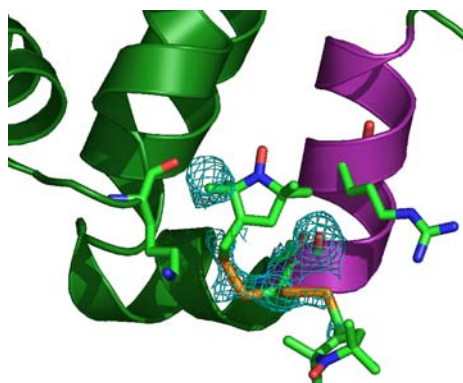


Figure 6 Electron density around the R1 side chain at 115R1.

(iii) *118R1, an immobilized site* Figure 5 shows the electron density map for 118R1. This site is buried in the protein interior, and electron density for the entire side chain is resolved. Remarkably, the backbone fold of the protein is essentially identical to the wt protein, except for rearrangement of a short loop connecting two helices. Because there are no cavities around the side chain, motion is only possible due to “breathing” modes of the protein; hence modulation of R1 motion is a measure of the frequency and amplitude of this protein dynamic mode.

(iv) *115R1, a complex state* Figure 6 shows the electron density map for 115R1. Two conformations of the side chain are resolved, but only atoms $C\alpha$ - $C\beta$ - $S\gamma$ - $S\delta$ have strong electron density; the rings are not resolved. These rotamers correspond to the two dynamic states of the side chain reflected in the EPR spectrum (see Figure 4). The lack of density corresponding to the nitroxide ring again reflects disorder in the crystal. In one rotamer, the disorder reflects the motion of a weakly ordered state. In the other, it is due to static spatial disorder due to strong but non-specific hydrophobic contacts with a neighboring helix. The complex dynamic modes thus represent conformational equilibria between ordered (either weak or strong) and immobilized states. Because the interactions giving rise to the immobilized state are tertiary in origin, the position of the equilibrium is sensitive measure of changes in tertiary structure of the protein.

In summary, structure determination of spin labeled proteins by x-ray crystallography and simulation of the corresponding EPR spectra has led to a quantum step forward in understanding the dynamic modes of the nitroxide side chain and their relationship to protein structure and global protein dynamics. This positions SDSL as one of the most powerful analytical tools now available for study of structure/function relationships in proteins.

Most of the analysis so far has been on α -helical proteins (Figs. 3-6). However, the structural origin of the striking complex spectrum of 52CRBP in a β -strand is of great interest (See Figure 2). Although no crystal structure is available, modeling and spectral simulations of R1 along 4 strands in CRBP suggest the origin of the unusual dynamic mode. Results of this work are contained in a manuscript recently submitted for publication (M.A. Lietzow and W.L.Hubbell, “Motion of spin labeled side chains in cellular retinol binding protein: correlation with structure and nearest neighbor interactions in an antiparallel beta sheet. In preparation for *Biochemistry*).

C. Analysis of spin labeled Annexin XII and T4L bound to a planar lipid bilayers

In the solution state of proteins, information regarding the net orientation of nitroxide side chains with respect to the protein structure (a helix axis, for example) is lost due to the random distribution of molecules with respect to the magnetic field. This is unfortunate, because such information can provide another level of structural detail. However, the information can be recovered if the protein is attached to a suitable planar surface. To explore this possibility, and to bridge the current efforts to the MOSAIC project, methods were developed to orient spin-labeled Annexin XII on planar phospholipids bilayers. The results, obtained in part with support from this grant, showed the feasibility of using immobilized arrays of spin-labeled proteins to determine the relative orientation of secondary structural elements [Risse, T., Isas, M., Haigler, H. and Hubbell, W.L. "Structure and dynamics of Annexin 12 bound to a planar lipid bilayer", *Phys. Rev. Letters* 91:188101 (2003)]. Similarly oriented arrays of T4L have now been produced using His-tag labeled T4L immobilized on Ni(II) containing lipid bilayers. This strategy should be quite general.

In addition to providing a new level of structural information, the planar arrays of spin-labeled proteins are anticipated to provide substrates for the single spin microscopes being developed under the DARPA MOSAIC project.

Publications and Presentations

Papers published in peer-reviewed journals

Structure and dynamics of annexin 12 bound to a planar lipid bilayer. T. Risse, W. L. Hubbell, J. M. Isas and H. T. Haigler. *Phys Rev Lett* (2003) 91:188101.

Papers published in conference proceedings

A new spin on protein dynamics. W. Hubbell and L. Columbus, 25th International EPR Symposium, Denver, July, 2002.

Interactions of spin labeled side chains in proteins: implications for structure and dynamics. Z. Guo and W. L. Hubbell. Forty-sixth Annual Meeting of the Biophysical Society, San Francisco, CA, February, 2002.

Papers presented at meetings and not published in proceedings

Determination of structure and dynamics in proteins. Myoglobin as a model system. S. Oga and W. Hubbell, JSEI Lake Arrowhead Conference, September 2002.

Identification of specific sidechain-sidechain interactions using site-directed spin labeling. M. Fleissner, L. Columbus, W. Hubbell, JSEI Lake Arrowhead Conference, September 2002.

Characterization of nitroxide side chains in helical proteins by X-ray crystallography. Z. Guo, D. Cascio, W. L. Hubbell, 47th Annual Meeting of the Biophysical Society, San Antonio, February, 2003.

Scientific Personnel Supported by the Project

Shirley Oga, B.S., Staff Research Associate, was supported by the project.