STRUCTURAL DYNAMICS OF LIGANDED MYOGLOBIN

Hans Frauenfelder, *Department of Physics, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801*
Gregory A. Petsko, *Department of Chemistry, Massachusetts Institute of Technology , Cambridge, Massachuetts 02139 U.S.A.*

ABSTRACT X-ray crystallography can reveal the magnitudes and principal directions of the mean-square displacements of every atom in a protein. This structural information is complementary to the temporal information obtainable by spectroscopic techniques such as nuclear magnetic resonance. Determination of the temperature dependence of the mean-square displacements makes it possible to separate large conformational motions from simple thermal vibrations. The contribution of crystal lattice disorder to the overall apparent displacement can be estimated by Mössbauer spectroscopy. This technique has been applied to high resolution x-ray diffraction data from sperm whale myoglobin in its Met iron and oxy cobalt forms. Both crystal structures display regions of large conformational motions, particularly at the chain termini and in the region of the proximal histidine. Overall, the mean-square displacement increases with increasing distance from the center of gravity of the molecule. Some regions of the heme pocket in oxy cobalt myoglobin are more rigid than the corresponding regions in Met myoglobin.

INTRODUCTION

Proteins as Flexible Molecules

The most common picture of the structure of protein molecules appears in x-ray diffraction papers. The picture is computer-generated; chemical bonds are represented by straight lines and atomic positions by circles or the intersection of line segments. An example, one view of myoglobin, is shown in Fig. 1. Such illustrations, though necessary to show the path of chain folding, create the impression that proteins are rigid, open molecules. Computer-controlled raster displays generate pictures of proteins in which each atom is represented by a sphere of the appropriate van der Waals radius, centered at the position determined from x-ray diffraction. This construction is far more convenient than actual space-filling molecular models, which are quite susceptible to denaturation. An example of a computer-generated picture is shown in Fig. 2; this view of the small heme-containing protein myoglobin demonstrates that the structure is very tightly packed, with the atoms in the interior of the molecule not visible from the outside. Space-filling computer graphics representations of protein crystal structures have been pioneered by Feldmann and Porter (e.g., Feldmann, 1976), who have vividly illustrated the principle that close atomic contacts are a general feature of macromolecular architecture.

Although the tight atomic packing in proteins would seem to demand great rigidity in tertiary structure, a number of observations imply considerable flexibility. Hydrogen exchange studies indicate that nearly all the internal exchangeable protons in native proteins can be substituted, albeit slowly, requiring that solvent molecules gain access to protons in the center of the molecule (Englander et al., 1972; Woodward, 1979). At a minimum, this fact demands transient opening of channels ~4Å in diameter in the tightly packed structure. The

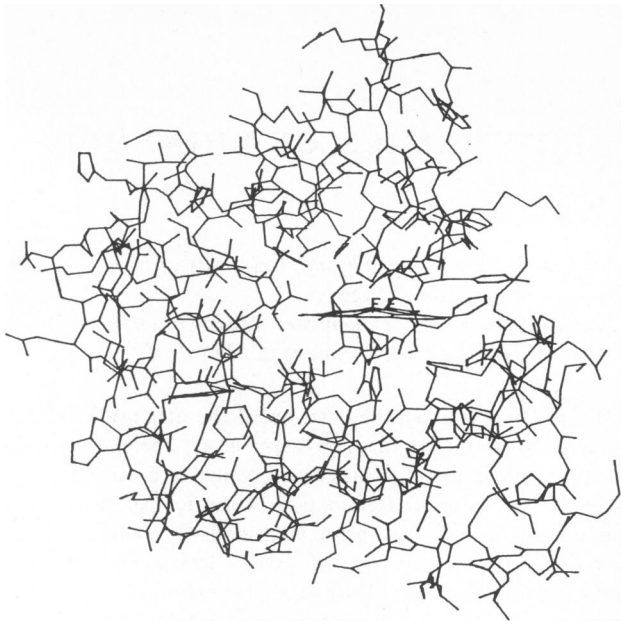

Figure 1 Computer-generated drawing of sperm whale Met myoglobin showing all nonhydrogen atoms. Every covalent bond is represented by a line segment. The heme is horizontal and slightly to the right of the center of the figure.

penetration of solvent into the interior of proteins has also been detected by the quenching of the fluorescence of buried side-chains by solvent (Eftink and Ghiron, 1975). Detailed information about the motions of specific groups has come from nuclear magnetic resonance studies of myoglobin, BPTI and hen egg white lysozyme in solution (Wittebort et. al., 1979; Campbell et al., 1975; Wüthrich, 1976). These studies have shown that tyrosine and phenylalanine side-chains flip by 180 degrees about their beta-gamma bonds. Flipping can occur in the interior of molecules with close packing such as shown in Fig. 2 only if neighboring atoms move out of the way. The chemical physics of this process has been illuminated by the influential work of Karplus and his coworkers, who have carried out molecular dynamics calculations on bovine pancreatic trypsin inhibitor (McCammon et al., 1977; Karplus and McCammon, 1979). They find that the protein is able to wander over a range of conformations which, though close to the x-ray structure, are not identical to it. The root-mean-square fluctuation of all non hydrogen atoms in the 100ps simulation is 0.75 Å. The motions are often anharmonic and diffusive in nature with the dominant factor being collisions with neighboring atoms. The backbone fluctuations are less than those for the side-chains, and the average motion increases with increasing distance from the center of the molecule.

Complementary clarification of the nature of protein flexibility is provided by the work of Frauenfelder and colleagues on CO rebinding to myoglobin (Austin et al., 1975; Alberding et al., 1978). Low temperature flash photolysis revealed the existence of multiple barriers to ligand binding and nonexponential kinetics that could not be explained in terms of a unique protein structure. They proposed the existence of multiple conformational substates. Different substates represent different local configurations of the same overall protein structure. All substates perform the same biological function, but possibly with different rates. Transitions from one substate to another require the surmounting of potential energy barriers. If these

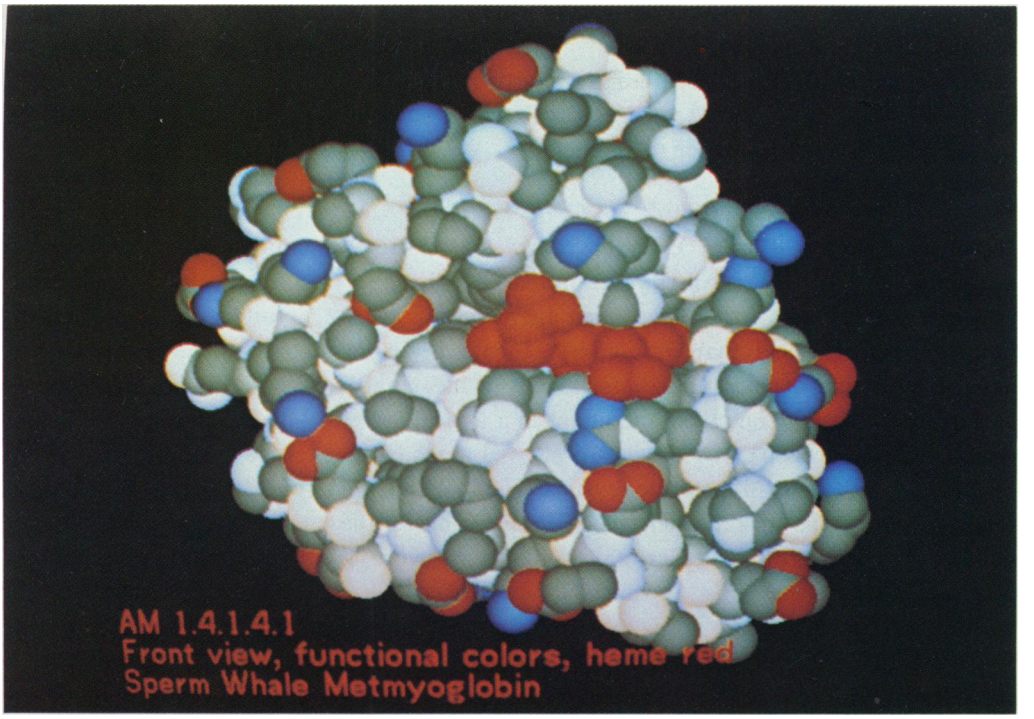

Figure 2 Computer-generated space-filling representation of sperm whale met myoglobin, in the same orientation as Fig. 1. Each atom is represented by a sphere of appropriate van der Waals radius. The edge of the heme is the red region in the right center. Each atom is colored according to its functional type: positive charged, blue; negative charged, red; hydrophobic, grey; neutral, white.

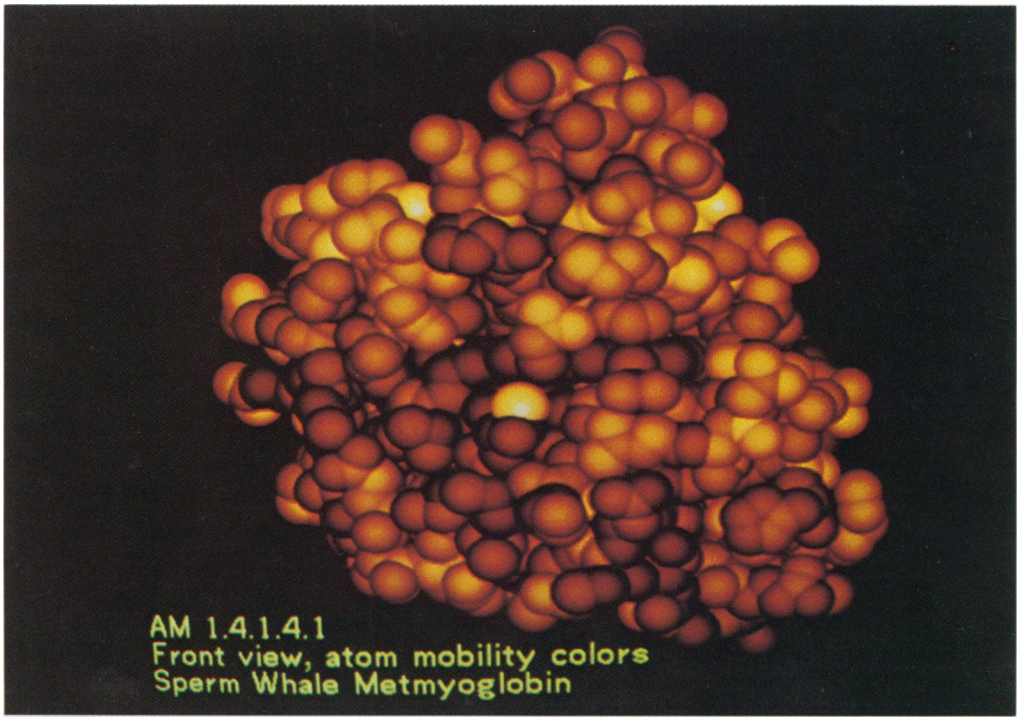

Figure 7 Computer-generated space-filling representation of Met iron myoglobin, in the same orientation as in Figs. 1 and 2. Each atom is shaded according to the magnitude of its $\langle x^2 \rangle_{cv}$. The darker the atom, the smaller the displacement.

barriers are large the distribution of conformations is essentially a static one at ordinary temperatures. This is equivalent to saying that the protein folds into many similar, but non identical, conformations on synthesis. If the barriers are of the same order as the kinetic energy available at ordinary temperatures the distribution is dynamic and any molecule can move rapidly from one substate to another.

Dynamic Information from Crystallography

It has been felt that x-ray crystallography is a static technique which cannot provide information about the flexibility of proteins. Although small molecule crystallography routinely yields detailed thermal motion analyses as part of structure refinement, there has been widespread belief that the quality of data available in a protein crystal structure determination did not permit the assignment of meaningful thermal motion parameters to each of the more than one thousand atoms in a typical protein. This belief was based mainly on the fact that protein crystals typically showed much larger average mean-square displacements, $\langle x^2 \rangle$, than small molecule crystals. The loose packing in protein crystals produces a static disorder in the crystal which could be responsible for the observed large $\langle x^2 \rangle$. However, neither efforts to grow better crystals nor observation at lower temperatures reduced the mean-square displacements to values typical for small-molecule crystals. One possible explanation for this failure is suggested by the flash photolysis experiments (Austin et al., 1975) and the computer simulations (McCammon et al., 1977) mentioned earlier: if proteins indeed exist in a large number of substates, a crystal will always be an ensemble of proteins in different states. Neither improved crystal growth nor cooling will significantly lower $\langle x^2 \rangle$; the large displacements are a fact of nature and not a feature of the experiment (Frauenfelder and Petsko, 1978). Meaningful information about protein dynamics can then be obtained from crystallography if the mean-square displacements of all nonhydrogen atoms in a protein are determined as a function of temperature and if an independent value for the lattice disorder contribution can be found. Frauenfelder, Petsko, and Tsernoglou (1979) indeed showed that x-ray diffraction data from myoglobin can permit the assignment of meaningful atomic displacement parameters and yield strong evidence for conformational substates . They determined $\langle x^2 \rangle$ for all 1261 nonhydrogen atoms in myoglobin at 220, 250, 275, and 300 K. To separate the contribution to $\langle x^2 \rangle$ from lattice disorder from those of simple vibrations and conformational substates, they used Mössbauer data obtained by Parak and collaborators (Parak and Formanek, 1971). Additional support for the existence of conformational substates comes from the work of Blake, Phillips and coworkers who showed that the temperature factors assigned to the atoms of lysozyme by refinement of the crystal structure at high resolution correlated in a sensible way with the various features of tertiary structure (Sternberg et al., 1979; Artymiuk et al., 1979). They also demonstrated that the correlations were the same for two different crystal forms of the enzyme, suggesting that lattice disorder was not dominant. Jensen and colleagues have reached similar conclusions concerning rubredoxin (Jensen, private communication).

METHODS

Data Collection and Analysis

Large single crystals of both Met iron and oxy cobalt sperm whale myoglobin were a generous gift of Professor Takashi Yonetani of the University of Pennsylvania. In both cases the protein was prepared in the desired oxidation state before crystallization, to avoid any question of the crystal lattice inhibiting a necessary conformational change during ligandation. For most measurements the Met iron myoglobin

crystals were maintained in contact with a mother liquor composed of 75% saturated ammonium sulfate, 0.1 M phosphate, pH 6.1. This solution will supercool to ~250 K under the conditions of data collection; work below this temperature demands use of a cryoprotective mother liquor (Petsko, 1975). For protein crystals grown from high salt solution the most generally useful mixture is 2-methyl-2,4-pentanediol:water, and this mother liquor was used for myoglobin. A mixture of 70% pentanediol:30% water, pH 6.1 with 0.02 M cacodylate buffer prevented crystal destruction at 220 K.

X-ray diffraction data were measured on Met myoglobin at 300 K, 275K, 250 K and 220 K and on oxy cobalt myoglobin at 250 K on a Nicolet $P2_1$ automatic diffractometer (Nicolet Instrument Corp., Madison, Wisc.) equipped with a cold gas stream low temperature device. Measurements were made to a resolution of 1.5Å on the crystals, which were monoclinic, space group $P2_1$, with unit cell dimensions a = 64.3Å, b = 30.8Å, c = 34.8Å, β = 105.85° for Met iron and a = 64.5Å, b = 31.0Å, c = 34.8Å, β = 105.84° for oxy cobalt myoglobin (Petsko et al., 1978). To prevent radiation damage from interfering with the accuracy of the measurements, two steps were taken. Data collection was performed very rapidly, at a rate of 4,000 reflections/ day, using a limited ω step scan to assure adequate precision (Wyckoff et al., 1967), and data were taken at the highest temperature first to insure that an increase in $<x^2>$ on going to 300 K was not simulated by radiation damage from earlier data collection.

The mother liquor for oxy cobalt myoglobin was 90% saturated ammonium sulfate, which permitted measurements at 250 K. The oxy form is unstable in the x-ray beam at elevated temperatures, so only one data set was collected. The optical absorption spectrum, taken after irradiation and subsequent dissolution of the crystal, showed no significant oxidation to the Met form.

The solution of the oxy colbalt myoglobin crystal structure has been reported previously (Petsko et al., 1978). The structure was refined by restrained least squares (Konnert, 1976) using all observed reflections between 10 and 1.54Å resolution. The current crystallographic R-factor is 0.19. Individual isotropic "thermal motion parameters" were assigned to each atom by this refinement method, which restrains atoms from wandering too far from positions that maintain ideal bond lengths and angles.

The Met iron structures were refined independently by the same procedure. All refinements were terminated at an R of 0.18 to make the standard deviations of the thermal parameters comparable for each structure. The behavior of the 220 K structure during refinement suggested that it was the poorest quality data set and suffered most from changes in equilibrium atomic positions. This feature is discussed by Frauenfelder et al. (1979).

Determination of Mean Square Displacements

Mean-square displacements were calculated for every atom in each structure from the individual isotropic thermal parameters. The theory of this method will now be discussed in detail:

During refinement, the electron density of each atom is modeled by $f_i \exp(-B \sin^2\theta/\lambda^2)$, where f_i is the atomic scattering factor of the atom at rest. The exponential term is used to fit the "smearing out" of the electron density over that expected for a stationary atom of finite radius. $\mathrm{Sin}^2\theta/\lambda^2$ is a vector in reciprocal space, and B is the temperature (displacement) factor. B is related to the mean-square displacement of the atom from its average position by the expression $B = 8\pi^2 <x^2>$. The single isotropic B value can be replaced by a tensor whose six parameters are related to the axial lengths and orientation of an ellipsoid of displacement. Use of anisotropic displacement factors enables crystallographers to analyze both the magnitude and the principal directions of atomic motion, but demands a larger amount of data. In this study we have restricted ourselves to isotropic B values, but note that the full potential of the crystallographic approach to protein dynamics will be achieved only when anisotropic displacement parameters are used.

The mean-square displacement of atoms in a small, rigid molecule like benzene is due to thermal vibration and overall molecular diffusion or tumbling. In a crystal the molecule as a whole is held rigid and only vibration is important. Protein molecules are much larger and contain many single bonds around which free rotation is possible. Further, although the forces along the polypeptide backbone are strong, the interactions between different regions of the molecule that hold the secondary and tertiary structures together are weak and can be deformed with the energies available at ordinary temperatures. As a result, protein molecules contain atoms and groups of atoms that can undergo conformational motions, namely transitions between substates. Examples of such motions are the rotation of a methyl group, the flipping of tyrosine rings discussed earlier, and the bending of hydrogen bonds. This class of fluctuations contributes to the mean-square displacement of many atoms in a protein in solution. The

same movements can occur in a crystalline protein (the evidence is discussed below) because protein crystals contain large regions of solvent which surround most of the molecular surface. Protein crystals have few intermolecular contacts and so each molecule is in a nearly aqueous environment which resembles a gel more than it does a crystal of sodium chloride.

The Problem of Lattice Disorder

X-ray diffraction is instantaneous in that the characteristic time for the scattering event is very short, $\sim 10^{-18}$s, but the observed structure is still a double average. Interference between planes of atoms in the crystal makes the calculated electron density an average over all asymmetric units in the crystal. The diffraction pattern is also an averge over the time required to collect the data, on the order of a week for a typical protein. The time average causes the effects of atomic motion to be seen in the spreading of the electron density. The averaging over many molecules in the crystal contributes a problem that interferes with the simple interpretation of individual isotropic B values as atomic motion. If the crystal packing is not perfect, there will be a static lattice disorder that will add to the apparent mean-square displacements. For example, if every protein molecule were in a slightly different position relative to the origin of the unit cell, the crystal-averaged structure would show a spreading of the electron densities due to this static lattice disorder. Lattice disorder can be translational and/or rotational. It may involve the whole molecule, or be confined to discrete regions. Overall translational disorder would contribute equally to the apparent mobility of every atom in the protein. Rotational lattice disorder would contribute most to those atoms furthest away from the axis of rotation.

The total mean-square displacement of an atom in a protein crystal can now be written as:

$$\langle x^2 \rangle_{\text{x-ray}} = \langle x^2 \rangle_v + \langle x^2 \rangle_c + \langle x^2 \rangle_{ld},$$

where the subscripts v, c and ld stand for vibrations, conformational fluctuations, and lattice disorder, respectively. To arrive at values that are independent of crystal imperfection $\langle x^2 \rangle_{ld}$ must be subtracted out. We have devised a method for the subtraction that is based on the Mössbauer effect in ^{57}Fe. The recoilless fraction is determined by the mean-square displacement, $\langle x^2 \rangle_{\text{Mössbauer}}$, of the emitting or absorbing nucleus. At temperatures above ~250 K, conformational transitions are faster than the characteristic time of the ^{57}Fe decay and the mean-square displacement is given by (Frauenfelder et al., 1979):

$$\langle x^2 \rangle_{\text{Mössbauer}} = \langle x^2 \rangle_v + \langle x^2 \rangle_c,$$

Stationary disorder does not affect the recoilless fraction because each nucleus emits or absorbs undependably. By determining the mean-square displacement for the same atom by both x-ray diffraction and Mössbauer effect, the lattice contribution consequently can be obtained by subtraction. Using Parak's values for $\langle x^2 \rangle_{\text{Mössbauer}}$ at various temperatures (Parak and Formanek, 1971; Parak, private communication), and our own values we find a mean value of $\langle x^2 \rangle_{ld}$ of 0.045 ± 0.006 Å^2 corresponding to B units of 3.55 Å^2 out of an overall isotropic B value for Met iron myoglobin of ~11 Å^2. This estimate of the lattice disorder at the iron position was subtracted from the isotropic displacement parameter of every atom at each of the four temperatures to yield $\langle x^2 \rangle_{cv} = \langle x^2 \rangle_v + \langle x^2 \rangle_c$ for all nonhydrogen atoms. Assuming that the lattice disorder at the iron is identical to the disorder everywhere in the molecule is equivalent to assuming that the disorder is purely translational. Indirect evidence implies that this assumption is reasonable. The values of $\langle x^2 \rangle_{cv}$ that result are neither too negative nor all large positive. A number of them are quite small, on the order of atomic vibrations in crystalline solids. Further, there are widely differing values of $\langle x^2 \rangle_{cv}$ for atoms on the surface of the protein, ruling out a dominant rotational disorder. Finally, the correlation of $\langle x^2 \rangle_{cv}$ with structure, discussed below, is so good that there is little possibility of a large lattice disorder swamping out the true displacements.

No Mössbauer data are available for oxy cobalt myoglobin, so the lattice disorder had to be estimated empirically. We used two methods: equating the $\langle x^2 \rangle_{cv}$ of the atoms in the internal, rigid center of the heme group with those in Met iron myoglobin at the same temperature, and making about the same number of near zero or negative values of $\langle x^2 \rangle_{cv}$ overall for oxy cobalt myoglobin and the Met iron structure at the same temperature. Both methods suggested a larger lattice disorder for the oxy cobalt structure, between 5 and 6 B units. This result also appears sensible from the physical behavior of the oxy cobalt myoglobin crystals: they were more fragile than the Met iron protein crystals. As a

conservative choice, a value of 5 was used, yielding $\langle x^2 \rangle_{ld} = 0.063$ Å^2 for oxy cobalt myoglobin. This value was subtracted from the individual atomic $\langle x^2 \rangle$ values to give $\langle x^2 \rangle_{cv}$ at 250 K.

Although oxy cobalt myoglobin was studied at only one temperature, the temperature dependence of the $\langle x^2 \rangle_{cv}$ values for the Met iron form was analyzed extensively. This analysis is discussed in detail in our previous paper (Frauenfelder et al., 1979) and only some general conclusions will be mentioned below.

RESULTS

Correlation of $\langle x^2 \rangle_{cv}$ with Structure

The mean-square displacements of the atoms in Met iron myoglobin are presented graphically in Frauenfelder et al.(1979). Fig. 3 shows the data for the atom in each side-chain with the largest displacement. Though the $\langle x^2 \rangle_{cv}$ values for the main chain atoms are, on the average, smaller, they follow the same general trend. Displacements are greatest at the N terminal and, particularly, the C terminal regions of the molecule, with the region around the distal histidine, residue 64, being significantly more rigid. There are regions of high mobility around residues 120 and 45. Myoglobin is composed largely of eight α-helical segments, denoted *A – H*. Residue 120 is a proline which creates the external, non-helical bend between the *G* and *H* helices, and residue 45 is an arginine which is in the middle of the eight residue bend between the *C* and *D* helices. In general, regions where the chain bends between helices are substantially more flexible than regions in the centers of helical segments.

The heme prosthetic group in myoglobin is bound in a pocket lined with hydrophobic residues. Most of its close interatomic contacts are with amino acids in the middle of helix *E* (residues 64–72) and the beginning of helix *G* (residues 99–104). Both the main chain and side-chain atoms in this region show relatively small mobilities. The tightness of this heme binding region is reflected in the mean-square displacements of the atoms in the heme itself. The four pyrrole nitrogens have an average $\langle x^2 \rangle_{cv}$ of 0.044 Å^2 at 300 K, a value typical of the vibrations of atoms in crystalline porphyrine. The iron atom is slightly more flexible, with $\langle x^2 \rangle_{cv} = 0.063$ Å^2 at 300 K, but this value is very small compared with the mean-square displacement of 0.33 Å^2 for the β-carbon of the highly mobile *c*-terminal glutamine-152. Indeed, a glance at Fig. 3 shows dozens of atoms with $\langle x^2 \rangle_{cv} > 0.2$ Å^2, implying root-mean-square displacements of almost half an Ångstrom. In contrast, values of $\langle x^2 \rangle$ found for atoms in small organic crystal structures are rarely outside the range 0.025 Å^2 to 0.126 Å^2 (Stout and Jensen, 1968). The magnitude of the motions involved is even more striking when it is remembered tha these are average fluctuations. The harmonic model used to fit the *B* values to the spread of electron density assumes that the probability of finding an atom a distance x from its equilibrium position is Gaussian. Therefore, a mean-square displacement of 0.3 Å^2 implies that instantaneous displacements of 1.5–2 Å occur with reasonable probability.

There is a strong correlation between $\langle x^2 \rangle_{cv}$ and position of an atom within the myoglobin molecule. Myoglobin is ellipsoidal, with overall dimensions ~45×35×25 Å. The minimum radius of the molecule is ~13 Å, and the center of gravity is located near the bottom of the heme pocket. The mean-square displacement of atoms close to the center is on average almost half that of those within 3 Å of the surface of the protein. The displacements increase further as one leaves the surface: long external side-chains have $\langle x^2 \rangle$ which increase sharply with chain length (an example is shown in Fig. 6). Although the refinement method used in determining $\langle x^2 \rangle$ values applies restraints on bonded atoms, the restraints are not the origin of the increase in displacements as one proceeds down the side-chain. We know this because

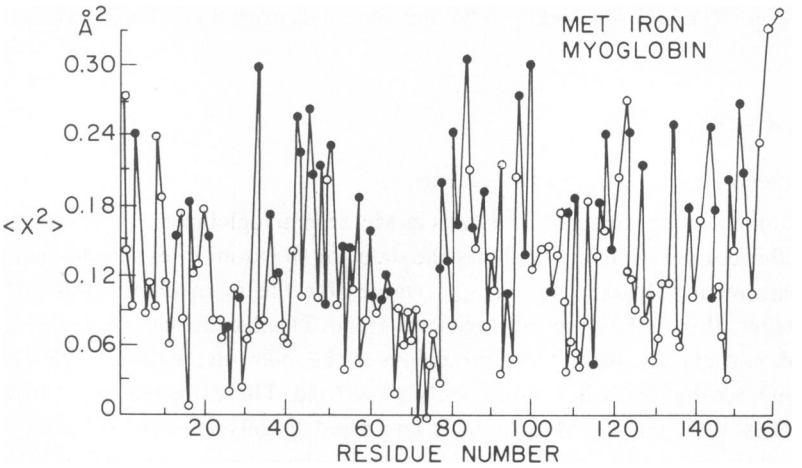

Figure 3 Plot of the mean-square displacement $\langle x^2 \rangle_{av}$ for the side-chain atoms of Met iron myoglobin at 250 K. Only the atom with the largest displacement in each residue is plotted. Solid circles indicate charged side-chains.

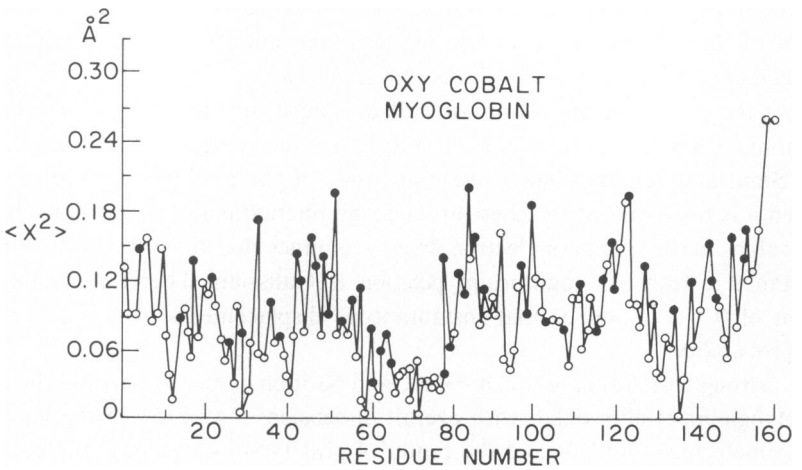

Figure 4 Plot of the mean-square displacement $\langle x^2 \rangle_{av}$ for the side-chain atoms of oxy cobalt myoglobin at 250 K. Only the atom with the largest displacement in each residue is plotted. Solid circles indicate charged side-chains.

long residues that are internal do not display this trend (an example is shown in Artymiuk et al., 1979). Also, we have run cycles of refinement without restraints and the results are the same. Finally, the electron density of the side-chains clearly indicates increases of $\langle x^2 \rangle$ with chain length; often, the terminal atoms barely show density above the noise level.

The plot of mean-square displacements for side-chain atoms shown in Fig. 3 reveals some interesting fine structure. For example, the *A* helix has one side exposed to the surface and one side facing the tightly packed center of the molecule. This asymmetry is reflected in the cyclic side chain displacements from residue 8–17, where side-chains on the exposed surface have greater $\langle x^2 \rangle_{cv}$ than those on the internal surface.

Charged side-chains tend to be more flexible than uncharged ones. The origin of this effect may lie in their greater average length, but the very low $\langle x^2 \rangle_{cv}$ for phenylalanines (e.g., residue 43) and the occasionally low $\langle x^2 \rangle_{cv}$ for a buried charge group (e.g., glutamic acid 105) suggest that the strongest determinant is degree of exposure to solvent. Myoglobin is the prototypical "oil drop" protein; most of the charged side-chains are on the surface. The extreme mobility of these residues can be seen in Fig. 3, where the solid circles are amino acids with charged side-chains.

Comparison With Oxy Cobalt Myoglobin

Fig. 4 shows a plot of $\langle x^2 \rangle_{cv}$ for the side-chain atom with the largest displacement in each residue of oxy cobalt myoglobin. Overall, the oxy structure is significantly tighter than the Met form. The lowest $\langle x^2 \rangle$ values are comparable in both cases, and are of the same magnitude as vibrations in small organic structures. The large fluctuations, in contrast, are much lower for the oxygenated protein. We interpret this to mean that the number of available conformational substates is smaller for oxy cobalt myoglobin.

Close comparison of Figs. 3 and 4 indicates that the patterns of displacement in the two crystal structures are strikingly similar. Nearly all of the fine structure in the Met iron plot is reproduced, though with different amplitudes, in the oxy plot. Examples are the rigid region near the distal Histidine-64, the highly mobile regions around residues 120 and 45, and even such details as the sharp drop in mobility for Isoleucine 28, which is in a hydrophobic cluster on the distal histidine side of the heme. The close correspondence for these two independent sets of data is encouraging demonstration of the ability of crystallography to detect molecular motion.

We have searched for a structural explanation for the differences in flexibility between the two structures. The oxy cobalt structure has been described previously (Petsko et al., 1978); briefly, the oxygen is bound to the cobalt in the bent, end-on configuration with a Co-O_1 distance of 1.89 ± 0.08 Å, an O_1-O_2 distance of 1.26 ± 0.08 Å, and a Co-O_1-O_2 angle of 131 ± 12 °. The distal histidine is hydrogen-bonded to one of the oxygens, probably O_1. Both the distal histidine and the $C_{\gamma 1}$ of valine 68 are in van der Waals contact with O_2. The cobalt is out-of-plane by 0.25 Å on the proximal side of the heme. Met iron myoglobin has a water molecule bound to the iron. The metal is 0.40 Å out-of-plane and the Fe-O distance is 2.0 Å. The distal histidine is hydrogen-bonded to the bound water. In Fig. 5 we compare the $\langle x^2 \rangle_{cv}$ for the side chain atoms of Val 68 in the two structures. The displacements are similar except for $C_{\gamma 1}$, which is much less mobile in the oxy protein. The close van der Waals contact between this atom and the O_2 of the bound oxygen molecule may explain the difference. The second oxygen is missing in the Met structure, possibly permitting greater freedom of movement. A similar effect occurs for the $C_{\delta 2}$ of His 64, which is also in van der Waals contact with the second oxygen in oxy cobalt myoglobin. The mean-square displacement of this atom is almost

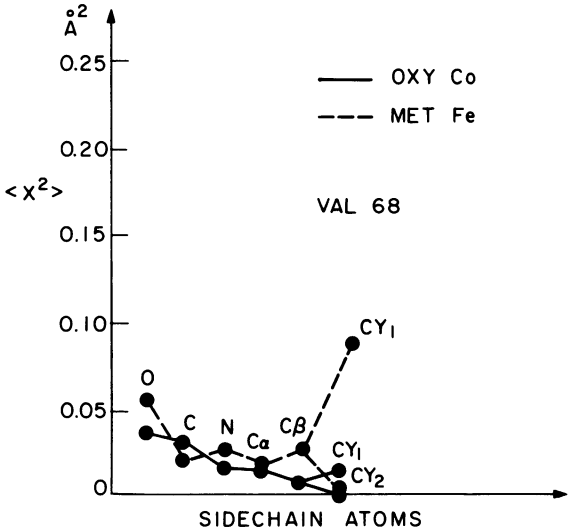

Figure 5 The mean-square displacements $\langle x^2 \rangle_{cv}$ for the atoms in valine 68 in Met iron myoglobin (broken lines) and oxy cobalt myoglobin (solid lines).

twice as large in Met iron myoglobin. The N_ϵ of this residue, which is hydrogen bonded to O_1 in oxy myoglobin and to the water oxygen in Met myoglobin, has the same $\langle x^2 \rangle_{cv}$ in the two structures, within experimental error.

Although the larger size of molecular oxygen and its tight interactions with globin on the distal heme side may account for some of the differences between the mobilities of the two structures, it is hard to invoke these factors for side-chains which extend freely into the solvent. An example is shown in Fig. 6. Lysine-96 makes no bonds to any other residue, and is completely external. The differences in mobilities between this residue in oxy and Met myoglobin increase with distance from the backbone. The large difference for the ϵ nitrogen

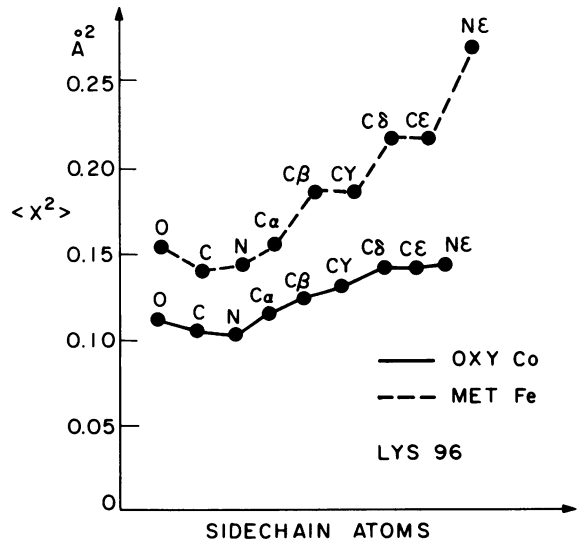

Figure 6 The mean-square displacements $\langle x^2 \rangle_{cv}$ for the atoms in lysine 96 in Met iron myoglobin (broken lines) and oxy cobalt myoglobin (solid lines).

suggests that this atom may be anchored in the oxy structure. Examination of electron density maps shows spherical density 3 Å from the nitrogen in both structures, which may be a bound sulfate ion. The concentration of ammonium sulfate is higher in the oxy mother liquor, which would lead to a higher occupancy for this ion and may contribute to an overall reduction in the lysine mobility. Similar effects may operate for other charged side-chains.

Temperature Dependence of $\langle x^2 \rangle_{cv}$

In the Debye approximation, the mean-square displacement should decrease linearly with decreasing temperature until the Debye temperature is reached. Below the Debye temperature, $\langle x^2 \rangle$ should approach a constant value corresponding to the zero-point motion (Willis and Pryor, 1975). The Debye temperatures of protein crystals are not known, but are expected to be of the order of 100 K. Lonsdale and El Sayed (1965) reported the variation of the overall *B* factor for some organic crystals with temperature; they found linear behavior down to ~100 K.

We have measured the mean-square displacements for all nonhydrogen atoms of myoglobin at 220, 250, 275, and 300 K. If the displacements were entirely due to harmonic vibrations, we should expect $\langle x^2 \rangle \propto T$. In reality, only very few atoms display a temperature-proportional $\langle x^2 \rangle$. Most atoms show different behavior: large displacements are generally temperature independent; most small displacements increase rapidly with temperature (Frauenfelder et al., 1979). These observations are difficult to understand if each atom moves in a simple well but they find a straightforward interpretation in terms of conformational substates and transitions (Gavish, private communication). Two facts emerge from these measurements: (*a*) In order to draw convincing conclusions concerning the motion of individual atoms, values of $\langle x^2 \rangle$ must be determined over as wide a temperature range as possible. Experiments performed at only one temprature are blind to complexities. (*b*) Mean-square displacements must be determined for each individual atom. The average mean-square displacement $\overline{\langle x^2 \rangle}$ of the molecule does not provide enough information about the various regions within a protein.

Recently, Singh et al. (1980) have examined the overall *B* factor in crystals of trypsinogen at various temperatures down to 198K. They observe a solvent dependent, sharp decrease in *B* at temperatures around 225 K. They ascribe this change to a phase transition in the crystal. It is, however, also possible that the change is caused by the freezing of conformational transitions. Singh et al. also found that cooling trypsinogen crystals to 213 K had no effect on the observability of the invisible, flexible chain segments in the molecule. They remark that one explanation for this is "conformational heterogeneity (microstates)." In such extreme cases, where there is no change in $\langle x^2 \rangle$ with temperature, one cannot conclude that the conformational disorder is static. It is still possible that there are many interconverting substates with very small potential barriers between them.

DISCUSSION

Limitations of the Crystallographic Approach

Protein crystallography can provide information about the spatial fluctuations that occur in protein molecules in the crystalline state. It is pertinent to ask whether the movements seen by x-ray diffraction are relevant to the dynamic properties of proteins in solution. The strong correlation of the crystallographic $\langle x^2 \rangle$ values with tertiary structural features suggests the answer is yes. Added evidence comes from consideration of hydrogen exchange. Norvell et al. (1975) report that 64% of the main chain amides, 80% of the ammonium ions, 100% of the

hydroxyls, and 77% of the side chain amides in crystalline carbon monoxy iron myoglobin were able to completely exchange their hydrogens for deuterium in the crystal. An additonal 11% of main chain amide hydrogens were partially exchanged. These values are very close to those found by chemical studies in solution. A D_2O molecule is ~3 Å in diameter and could not penetrate the tightly packed protein structure (Fig. 2) to exchange the internal hydrogens unless the protein were flexible. For such exchange to occur in the crystal, the molecule must be able to undergo the same sorts of fluctuations in the solid state as in solution. The displacements we observe have a reasonable probability of being as large as a few Å. Richards (1979) has recently proposed a mechanism for the exchange process that involves the propagation of 2–4 Å "cavities" or "mobile defects" in the packing of atoms in the interior of the protein. The fluctuations observed in our study are consistent with the kinds of movement needed for this mechanism.

Of course, the crystal packing must influence the motion of some atoms, at least those in the regions of contact between neighboring molecules in the lattice. Fig. 7 shows a "heat picture" of myoglobin; the view is the same as in Fig. 2. Each atom is now shaded according to how far it moves. The darker the atom, the smaller the mean-square displacement. The edge of the heme is dark, indicating its rigidity. There is texture, but most of the surface is more mobile than the heme. A striking exception is the region at the bottom of the picture (residues 55–60), which is very rigid. Although external and largely charged, these amino acids are relatively immobile (Figs. 3 and 4). This region is involved in a strong intermolecular contact in the crystal. Presumably, the mean-square displacements are abnormally low. Fortunately, such regions are rare owing to the irregular shape of protein molecules and the consequent small number of lattice contacts. It is, nevertheless, important to realize that the effects of crystallization may extend some distance within the molecules. Recent electron paramagnetic resonance (EPR) measurements on hemoglobin suggest that the molecular motion involving the heme is less in crystals than in solution (Hampton and Brill, 1979). The displacements given by protein crystal structure analyses should probably be considered lower limits to the values in solution.

Finally, we note that crystallographic data can give only spatial information about protein motions. Spectroscopic and kinetic measurements are complementary because they provide temporal information.

The Lattice Disorder in Oxy Cobalt Myoglobin

Our results have assumed that the lattice disorder in oxy cobalt myoglobin is 40% greater than for Met iron myoglobin. While a reduction of this number will not invalidate most of our conclusions, it will alter the degree of tightening of the oxygenated structure compared with the Met form. Greater rigidity of the oxy structure than the deoxy structure was predicted by Austin et al. (1975) from studies of the temperature dependence of the kinetics of dioxygen rebinding after photodissociation. In this case, we do not know if Met myoglobin is a reasonable model for the dynamic properties of the deoxy protein. (Oxy cobalt myoglobin, on the other hand, appears to be a very good model for oxy iron myoglobin. The structure is essentially identical with that determined for the oxy iron protein by Phillips [1978]).

One conclusion we draw from this study is that comparisons of mean-square displacements for two different structures are best made in the light of detailed information about the magnitudes of the lattice disorder, but that reasonable estimates are possible when such information is not available. Currently, Mössbauer spectroscopy provides the only way of obtaining such data, but it should be possible to use spin labeling, Rayleigh scattering, and, possibly, solid state nuclear magnetic resonance, for this purpose.

Outlook

Maximum information about the spatial aspects of the dynamics of crystalline proteins involves knowledge of the principal directions of motion as well as their equivalent isotropic magnitudes. Such data can be obtained from crystallographic analyses if higher resolution data are available. We are currently trying to measure such data for Met myoglobin at 250 K. Another problem that remains is to determine the effectiveness of the harmonic model for protein motions. Since proteins are so tightly packed with atoms, large movements must be cooperative and involve atomic collisions. It is likely that such atomic motion will be highly anharmonic. Our present exponential form of the "temperature factor" cannot account for the distribution of electron density caused by anharmonic motion. Development of a suitably general model is awaited with interest.

One question raised by this work is whether the spatial fluctuations in the myoglobin structure have any functional significance. As one speculative example, inspired by a question of J.D. Cowan, we note that the fluctuations even on the outside of myoglobin are smaller in the liganded than the unliganded state. This observation suggests that the cooperativity in hemoglobin may be caused not only by one specific link but by a change in the dynamic properties of the interfaces between subunits. Such a mechanism may be a realization of the Hopfield "distributed energy" model (Hopfield, 1973).

Proteins are not open, rigid structures. They are close-packed, fluctuating molecules whose dynamic properties can be studied by x-ray diffraction as well as by other techniques. The magnitudes of the atomic displacements observed in this study of liganded myoglobin are quite large and correlate well with features of tertiary structure. In view of this, it would seem reasonable to believe that the dynamic properties of proteins may be intimately involved in their biological functions.

We thank Takashi Yonetani for gifts of myoglobin single crystals and Lyle Jensen for information in advance of publication. We are grateful to Fritz Parak for providing the Mössbauer data and to Demetrius Tsernoglou for many useful discussions. Our special thanks to Richard Feldmann for creating the computer graphics pictures of myoglobin. The assistance of Dr. William A. Gilbert in the preparation of Figs. 3–7 is also gratefully acknowledged, as is the work of David Rose , who participated in the collection of the oxy cobalt myoglobin data.

This work was supported by National Science Foundation grant PCM 74-01366 and National Institutes of Health grants GM 18051 and GM 27123. Gregory A. Petsko is an Alfred P. Sloan Foundation Fellow and holds a U. S. Public Health Service Research Career Development Award.

Received for publication 29 December 1979 and in revised form 10 March 1980.

REFERENCES

Alberding, N., R. H. Austin, S. S. Chan, L. Eisenstein, H. Frauenfelder, I. C. Gunsalus, and T. M. Nordlund. 1976. Dynamics of carbon monoxide binding to protoheme. *J. Chem. Phys.* **65**:4701–4711.

Artymiuk, P. J., C. C. F. Blake, D. E .P. Grace, S. J. Oatley, D. C. Phillips, and M.J.E. Sternberg. 1979. Crystallographic studies of the dynamic properties of lysozyme. *Nature (Lond.).* **280**:563–568.

Austin, R. H., K. W. Beeson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus. 1975. Dynamics of ligand binding to myoglobin. *Biochemistry.* **14**:5355–5373.

Campbell, I. D., C. M. Dobson, and R. J. P. Williams. 1975. Nuclear magnetic resonance studies on the structure of lysozyme in solution. *Proc. Roy. Soc. Lond. A Math. Phys. Sci.* **345**:41–59.

Eftink, M. R., and C. A. Ghiron. 1975. Dynamics of a protein matrix revealed by fluorescence quenching. *Proc. Natl. Acad. Sci. U.S.A.* **72**: 3290–3294.

Englander, S.W., N.W. Downer, and H. Teitelbaum. 1972. Hydrogen exchange. *Ann. Rev. Biochem.* **41**:903–924.

Feldmann, R. 1976. The design of computing systems for molecular modeling. *Ann. Rev. Biophys. Bioeng.* **5**:477–510.

Frauenfelder, H. and G. A. Petsko. 1978. Myoglobin structural dynamics. *Abst. Int. Cong. Biophys.*, Kyoto, Japan.

Frauenfelder, H., G. A. Petsko, and D. Tsernoglou. 1979. Temperature dependent x-ray diffraction as a probe of protein structural dynamics. *Nature (Lond.)*.. **280**: 558–563.

Hampton, D. A., and A. S. Brill. 1979. Crystalline state disorder and hyperfine component line widths in ferric hemoglobin chains. *Biophys. J.* **25**:301–311.

Hopfield, J. J. 1973. Relation between structure, cooperativity and spectra in a model of hemoglobin action. *J. Mol. Biol.* **77**:207–222.

Karplus, M., and J. A. McCammon. 1979. Protein structural fluctuations during a period of 100 ps. *Nature (Lond.)*. **277**:578.

Konnert, J. H. 1976. A restrained-parameter structure-factor least-squares refinement procedure for large asymmetric units. *Acta Cryst. A* **32**:614– 617.

Lonsdale, K., and K. El Sayed. 1965. Variation of the Debye parameter *B* with temperature. *Acta Cryst.* **19**:487–488.

McCammon, J. A., B. R. Gelin, and M. Karplus. 1977. Dynamics of folded proteins. *Nature (Lond.)*. **267**:585–590.

Norvell, J. C., A. C. Nunes, and B. P. Schoenborn. 1975. Neutron diffraction analysis of myoglobin:structure of the carbon monoxide derivative. *Science (Wash. D. C.)*. **190**:568–570.

Parak, F., and H. Formanek, 1971. Untersuchung des Schwingungsanteils und des Kristallgitterfehleranteils des Temperatur-faktors in Myoglobin durch Vergleich von Mössbauerabsorptionsmessungen mit Röntgenstrukturdaten. *Acta Cryst. A* **27**:573–578.

Petsko, G. A. 1975. Protein crystallography at sub-zero temperatures: cryo-protective mother liquors for protein crystals. *J. Mol. Biol.* **96**: 381–392.

Petsko, G. A., D. Rose, M. Ikeda-Saito, and T. Yonetani. 1978. The structure of oxy cobalt myoglobin at 1.5 Å resolution. *In* Frontiers of Biological Energetics, Vol. 2. P. L. Dutton et al., editors. Academic Press,Inc., New York. 1011–1016.

Phillips, S. E. V. 1978. Structure of oxymyoglobin. *Nature (Lond.)*. **273**:247–248.

Richards, F. M. 1979. Packing defects, cavities, volume fluctuations, and access to the interior of proteins. Including some general comments on surface area and protein structure. *Carlsberg Res. Commun.* **44**:47–63.

Singh, T. P., W. Bode, and R. Huber. 1980. Low temperature protein crystallography. Effect on flexibility, temperature factor, mosaic spread, extinction and diffuse scattering in two examples: bovine trypsinogen and F_c fragment. *Acta. Cryst. A.* **36**:in press.

Sternberg, M. J. E., D. E. P. Grace, and D. C. Phillips. 1979. Dynamic information from protein crystallography:an analysis of temperature factors from refinement of the hen egg-white lysozyme structure. *J. Mol. Biol.* **130**: 231-253.

Stout, G. H., and L. H. Jensen. 1968. X-ray structure determination:a practical guide. Macmillan Company, New York. 426–429.

Willis, B. T .M., and A. W. Pryor. 1975. Thermal vibrations in crystallography. Cambridge University Press, Cambridge. 134–140.

Wittebort, R. J., T. M. Rothgeb, A. Szabo, and F. R. N. Gurd. 1979. Aliphatic groups of sperm whale myoglobin: ^{13}C NMR study. *Proc. Natl. Acad. Soc. U.S.A.* **76**:1059–1063.

Woodward, C. K., and B. D. Hilton. 1979. Hydrogen exchange kinetics and internal motions in proteins and nucleic acids. *Ann. Rev. Biophys. Bioeng.* **8**:99–127.

Wüthrich, K. 1976. NMR In Biological Research: Peptides and Proteins. Elsevier North-Holland, Amsterdam. 112–117.

Wyckoff, H. W., M. Doscher, D. Tsernoglou, T. Inagami, L. N. Johnson, K.D. Hardman, N. M. Allewell, D. M. Kelley, and F. M. Richards. 1967. Design of a diffractometer and flow cell system for x-ray analysis of crystalline proteins with applications to the crystal chemistry of ribonuclease-S. *J. Mol. Biol.* **27**:563–578.

DISCUSSION

Session Chairman: Frederic Richards *Scribe*: Lucia Garcia-Iniguez

RICHARDS: We have a written extended comment relevant to this whole fluctuations session from Erik Tüchsen of the Carlsberg Laboratory.

TÜCHSEN: This is a contribution on the dynamic aspect of the atoms in the crystals. We have compared hydrogen exchange rates in crystalline and dissolved hen lysozyme and bovine zinc insulin (4). Crystals were insolubilized by intramolecular crosslinks, introduced by means of glutaraldehyde (1) to permit comparison of dissolved and crystalline protein under identical conditions.