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REVIEW

Structure and dynamics of the water around myoglobin

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Abstract

The interplay between simulations at various levels of hydration and experimental observables has led to a picture of the role of solvent in thermodynamics and dynamics of protein systems. One of the most studied protein–solvent systems is myoglobin, which serves as a paradigm for the development of structure–function relationships in many biophysical studies. We review here some aspects of the solvation of myoglobin and the resulting implications. In particular, recent theoretical and simulation studies unify much of the diverse set of experimental results on water near proteins.

Keywords: diffraction analysis; hydration; myoglobin; protein solutions; solvation; water dynamics

Myoglobin has a long history as a testing ground for ideas about the nature of proteins. The first protein whose structure was obtained at sufficient resolution to determine a complete set of atomic coordinates was myoglobin. This pioneering work revealed the now-classic globin fold of the 8 α-helices packed together into the tertiary structure (Kendrew et al., 1960, 1961). Because of its simple structure relative to other proteins, its accessibility to various spectroscopies, and its physiological importance for oxygen storage, myoglobin has been extensively considered from both theoretical and experimental points of view. Even now, the relation between the structure and its function is not completely understood and much work continues on this protein (see Pauling, 1964; Olson et al., 1988; Rohlfs et al., 1990; Genberg et al., 1991; Springer et al., 1994).

It is apparent that the environment surrounding the protein is critical in determining observable properties. Many experimental measurements depend in a sensitive way on the state of the system, including the solvating environment (or lack thereof). The term solvation refers to the general solution process; hydration refers specifically to the role of water as a solvent. In this review on the solvation of myoglobin, we concentrate mostly on aspects of the structure of water around myoglobin. Of primary interest to us are the cases where there are substantial overlaps in the study of hydration properties by different methods, and where there are also abundant comparisons between theory and experiment.

Diffraction experiments carried out on protein crystals provide time-averaged atomic positions of both the solvent and the protein. In one sense, they give the best spatial information available at this time. There are difficulties in the interpretation of the less-ordered water molecules. Perhaps surprisingly, results derived from X-ray and neutron diffraction methods seem to differ in certain details, especially those concerned with the solvent. Other methods such as NMR and Mössbauer radiation spectroscopy provide information complementary to that of diffraction experiments, and the challenge now is to assemble these different results into a coherent picture.

Computer simulations and simpler theoretical models have recently provided an interesting, and possibly unifying, view of many details concerning how proteins might be hydrated. Some such theoretical methods depend strongly on experimental input, whereas others seek to reveal this picture of proteins from little more than analysis of models of static positions of individual atoms. Each has its strengths and weaknesses. More important for this discussion is the comparison of results from such approaches with direct experimental counterparts. In the cases where the comparisons yield similar results, further interrogation of the simulation or theory can yield hints about mechanistic details; where the results differ, we are pointed to areas needing improvement on the interpretation or theoretical side.

From works that have caught our attention, we attempt to highlight the comparisons between different theories and ex-

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periments on the hydration of myoglobin; we do not claim to produce an exhaustive review. Where such comparisons are warranted, we also occasionally draw on the information accumulated on proteins other than myoglobin. In organizing this material, it seemed important to us to emphasize the diverse results from a range of experiments. We first introduce the reader to a selected set of experiments that are aimed at probing the waters of hydration around proteins in general and myoglobin in particular. This is followed by a review of recent theoretical and simulation-based studies. Some of the theoretical studies help to clarify the picture amassed from multiple experimental methods. Finally, we end with an outlook on this area.

Experiments

A wide variety of experiments have yielded aspects of our current picture of the hydration of myoglobin. Each experiment yields a picture of but a subset of the properties. In the areas of structure and hydration, experiments give a range of complementary but often confusing or contradictory results. This is due fundamentally to the different measurement processes. Evidence on solvation properties exists from sorption thermodynamic measurements, viscosity, microwave absorption, NMR, electron spin resonance (ESR), and Rayleigh scattering of Mössbauer radiation spectroscopy (RSMR) experiments performed on protein powders in various states of hydration (Parak, 1986; Goldanskii & Krupyanskii, 1989; Rupley & Careri, 1991). Such measurements give different perspectives from that of the diffraction experiments.

Thermodynamics

Sorption thermodynamics is a common tool for the study of solvation. The specific heat capacity, C_p, isotherms measured as a function of the ratio of the mass of added water to the mass of the protein or hydration number, h, exhibit discontinuities revealing chemical changes in the nature of hydration. For myoglobin, the point of full hydration, which defines the amount of added water beyond which the measured property does not significantly deviate from bulk, varies by more than a factor of 2, depending on the experimental measure used.

The heat capacity change at both constant pressure (ΔC_p) and constant volume (ΔC_v) can be measured in solution and as a function of hydration. When considering folding, the change between the denatured state and the native state is of most interest ($\Delta_N^D C_p$). This change is usually positive and correlates with the change in solvent-accessible surface area and, thus, with degree of hydration on going from native to denatured form (Murphy & Gill, 1990; Privalov & Makhatadze, 1992). Recently, this interpretation has been challenged and reinterpreted in terms of the expenditure of work against cohesive van der Waals and electrostatic forces between the two states (Hinz & Meyer, 1994). This reinterpretation was prompted by the unexpected sign in the difference between isobaric and isochoric heat capacity of the native versus denatured forms of a variety of proteins.

Clearly, defining the effect of solvent on protein and vice versa depends on the properties used as a measure. Using sorption thermodynamics, a hydration number, h, of 0.35 has been measured. However, the numbers in other measurements range from 0.39 in microwave absorption to 0.6 in RSMR. This represents equivalently 400–600 nonbulk water molecules that are characterized by a reduced rotational and translational mobility relative to bulk water. Recent theoretical computer simulations yield a number as low as 350 water molecules for "complete" hydration (Steinbach & Brooks, 1993). This number is less than a monolayer of coverage and clearly demonstrates that the concept of complete hydration depends on the property of interest.

Spectroscopy

Recently developed NMR techniques applied to biomolecules in aqueous solutions prove to be informative on both positions and residence times of water inside and around proteins (Kuntz & Kauzmann, 1974; Fung, 1986; Bryant, 1988; Otting et al., 1991). The ^{1}H spin-lattice relaxation has been measured for several different states of protein–water systems. Here we digress from myoglobin and consider another globular protein, lysozyme. Several sample preparations have been used in one particularly interesting study. Systems considered included dehydrating solutions of lysozyme (approaching the dry state), rehydrating lyophilized lysozyme powder via isopiestic equilibration, and titration with water to examine the more dilute regimes (Fullerton et al., 1986). The NMR response was found to change or break in a number of hydration ranges, in particular for the hydration number, h, equal to 0.05, and in the ranges 0.22–0.27 and 1.22–1.62. By comparing the sorption isotherm data with an analysis of the ^{1}H resonance data, one obtains about 19 tightly bound water molecules per lysozyme, 148 weakly bound water water molecules, and approximately 2,000 molecules of disturbed or "multilayer" water (Lioutas et al., 1987).

Complementary to this is the use of multinuclear experiments. NMR experiments have also been preferred on lysozyme powders utilizing both ^{17}O and ^{2}H. The experiments show a discontinuity in the measured signals at a high hydration number of 1.7, which corresponds to around 1,400 waters per molecule (Lioutas et al., 1986). Thus, it is clearly challenging to find a constant interpretation for these experiments with those mentioned in the preceding paragraph. In comparison with lysozyme, myoglobin is a larger protein; based on a simple analysis, one would expect that the corresponding hydration numbers or water molecules of hydration per myoglobin would be approximately 2/3 those found for the lysozyme case.

Another measurement complementary to the NMR experiments involves the use of ESR utilizing noncovalently bound spin probes. These experiments are also found to be sensitive to the protein–water state or the hydration level (Rupley et al., 1980). In some ways, the ESR experiments provide a partial bridge between certain thermodynamic results and NMR measurements, showing breaks at 0.07 and 0.25 h, which had been seen in heat capacity isotherms. The correlation time for the spin probe is also observed to decrease continuously toward the bulk solution value. The final equilibrium plateau for this measure is reached at a level of hydration around 1.8–2.0 h.

In comparison, it is clear that the thermodynamic methods are sensitive to sample changes in a limited range of hydration number. The magnetic spectroscopies, on the other hand, show a richer set of molecular behaviors. It is difficult to assign these various transitions exactly, but to a first approximation, the ones seen both in thermodynamic measurements and in magnetic resonance experiments are consistent with equilibrium behavior. It is consistent to hypothesize that there are related structural

transitions corresponding to these discontinuities. The other changes not seen in the thermodynamic measurements but seen by methods such as ESR and NMR suggest that there exist certain motional behaviors or nonequilibrium dynamics changes as the degree of hydration is changed.

Because certain dynamic probes and thermodynamic properties show corresponding changes for hydration levels less than the "monolayer" solvent coverage of a protein (Yang & Rupley, 1979; Goldanskii & Krupyanskii, 1989), one might hypothesize that the same trend should hold for changes with more than a monolayer of coverage. This hypothesis has been argued in the case of the ESR measurements (Rupley et al., 1980). However, the need for an extrinsic spin probe may require several layers: water in excess of the monolayer might be needed to solvate the probe even though less water may be needed to affect the protein. This approach is further complicated by the use of models in the interpretation of the measurements.

The response to microwave radiation can also be used to study water dynamics in protein crystals and solutions (Pethig, 1979; Parak, 1986; Pethig & Kell, 1987). Relaxation times of water surrounding (and in) myoglobin crystals have been studied in the microwave region over a wide range of temperatures (Singh et al., 1981). In contrast, no dielectric discontinuity was observed near 0 °C, indicating that the 400 water molecules estimated to be present in the crystal were quite perturbed by the presence of the protein.

Mössbauer experiments that probe the heme iron site provide independent experimental evidence of hydration effects on globins. RSMR, performed on metmyoglobin (and on human serum albumin) as a function of degree of hydration from 0.05 to 0.75, does not display spectral additivity (Fullerton et al., 1986; Krupyanskii et al., 1986; Kurinov et al., 1987a, 1987b; Goldanskii & Krupyanskii, 1989). Using a mechanical analogy, this can be restated as implying that the protein dynamics are changed in a continuous, nonlinear fashion with changing hydration. Thus, the solvent increases the protein mobility, thereby lowering the elastic scattering. In turn, the dynamics of water approach the characteristic properties for bulk water when h is greater than 0.6 or 0.7. This effect reflects mutual dynamic influences of water and protein. In small peptides, it is known that water drastically affects the intramolecular energy, or more properly, the free energy surface, greatly reducing a number of barriers and increasing the number of possible conformations (Pettitt & Karplus, 1985). Thus, the reduced barriers and the increase in number of conformations can be viewed as having a profound loosening effect as compared to the dry state (Brooks & Karplus, 1989). The influences of a change in viscosity (drag or friction) versus h complicate the interpretation for RSMR experiments. In several cases, however, this view produces a reasonable picture for such systems.

X-ray diffraction

X-ray crystallography is a powerful tool for visualizing the tightly bound, well-ordered water molecules on the molecular surface. Recent structures of myoglobin usually have more than 100 specific water molecules identified on or near the surface of the protein. In the case of some smaller proteins, such as crambin, which are extremely static in crystals and hence diffract to very high resolution, rings and partial clathrates or cages can be clearly seen around the protein side chains (Teeter, 1984, 1991).

These strongly bound water molecules are usually characterized by an occupancy factor and thermal parameter during the process of refinement of the X-ray structure. With these parameters, very tightly bound water molecules are often distinguished from the more loosely bound ones. There is, however, a rather low upper limit on how mobile the water can be before it becomes impossible to "see" in the diffraction experiments. Typically, if the temperature factor is greater than about 50–100 Å (corresponding to an RMS translational variation of about 0.5 Å), the water molecule cannot be reliably assigned a unique position in the structure. The hydration probability density is thus characterized by many local maxima. The results for different crystal forms and for different scattering beams can be compared (see Fig. 1) (Lounnas & Pettitt, 1994a).

The water molecules are seen to form irregular networks on the surface of the protein, usually with geometries that suggest hydrogen bonding to the polar side chains of the protein. Occasionally, water molecules are seen in cavities, particularly in the ligand-binding pocket of deoxy forms of myoglobin, where hydrogen bonding to the distal histidine allows a stable attachment site for the water molecule. Such polar interactions are usually required for high population of interior water sites.

These water molecules in the ligand-binding pocket of myoglobin have profound functional effects directly connected to the binding of oxygen or other nonphysiological ligands (Quillin et al., 1993). The water must be displaced prior to the binding of ligands, and the free energy lost by the breakage of that bond must be made up by the new ligand in order to have tight binding. Some engineered mutations that have large hydrophobic side chains instead of histidine do not show this bound water molecule and have correspondingly higher ligand association rates and affinities (Quillin et al., 1993). Because dioxygen can substitute for water as a hydrogen bond acceptor and carbon monoxide cannot, discrimination between O_2 and CO is enhanced relative to simple heme compounds. Thus, in myoglobin, as in many other proteins, the role of water extends beyond that of a simple solvent to a role in physiological function.

Clearly, details of the water structure depend on subtle aspects of the protein structure. In comparing two different crystal forms of sperm whale myoglobin (Fig. 1), some water molecules were found to occupy similar positions and some not. Just as surface side chains are found to vary in different packing environments, so the water structure varies, only more so. In many places where the water molecules are conserved, the side chains are in similar conformations (G.N. Phillips, Jr., unpubl.). Similar variability is seen in other protein crystal structures with more than one protein molecule in the asymmetric unit.

When it comes to analysis of the less tightly bound water molecules, diffraction techniques are much less well developed. Protein crystallographers usually ignore data at small scattering angles (d-spacings greater than 5–10 Å) during routine analysis of their structures. The rationale for this omission in the early stages of a structure solution is that, due to nonspecific heavy atom binding, phases for these low-resolution data cannot be determined accurately using multiple isomorphous replacement methods. In the later stages of analysis, these continue to be ignored because it is difficult to calculate accurate phases due to lack of adequate modeling procedures for the solvent contributions to total scattering. This problem manifests itself in poor

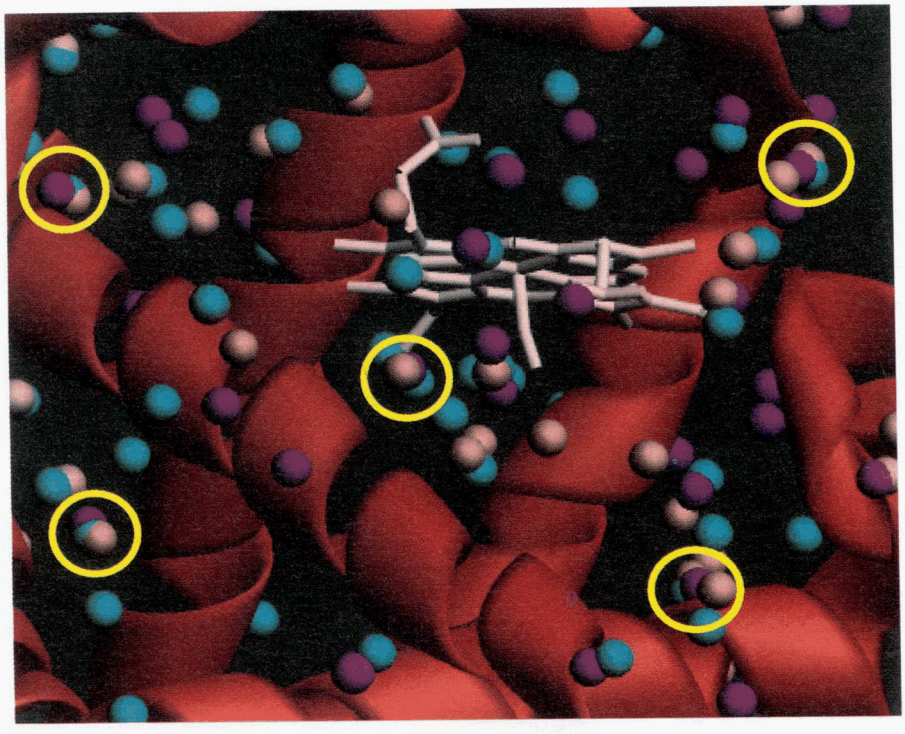

Fig. 1. Comparison of the refined water molecule position from several diffraction experiments on myoglobin crystals. Notice the general lack of correlation between water molecules refined from the different experiments. Molecular dynamics simulations find density maxima at essentially all the refinement sites from these experiments with a range of equilibrium populations (occupancies) (Lounnas & Pettitt, 1994a). Rare places where all three experiments yield close agreement are circled. The experiments are color coded: cyan, P6—Phillips et al. (1990), Brookhaven listing 1MBW; pink, $P2_1$—Kuriyan et al. (1986), Brookhaven listing 1MBC; purple, $P2_1$—Cheng and Schoenborn (1991), (Neutron) Brookhaven listing 2MB5; red, ribbon protein—heme structure from P6 form.

agreement between observed and calculated diffraction intensities (high R-factors). The effect of omitting these low-frequency terms is that the molecular envelope often is not well resolved, and some areas of the electron density are weaker than they would be otherwise. However, because the total scattering power represented by these data is small relative to the entire data set, the effect on refinement and on the final coordinates of the well-ordered protein and the few well-ordered solvent atoms is negligible.

One approach to correcting for the solvent contributions in the calculation of diffraction structure factors has been to modify the atomic scattering factors to reflect this apparent decrease in contrast between protein atoms and the surrounding medium (Langridge et al., 1960; Fraser et al., 1978; Driessen et al., 1989). Following these ideas, it is common to introduce one variable, g, and one fixed parameter, U, that enable diffuse solvent to be modeled by Babinet's principle (Driessen et al., 1989; G. Sheldrick, computer program SHELX-93, unpubl.) The real part of the scattering factor for each non-hydrogen atom is modified as follows:

$$f(new) = f(old) - g \cdot \exp\{-8\pi^2 U[\sin(\theta/\lambda)]^2\}.$$

The large value of U ensures that only the low θ, f, and hence F_c^2 values are affected. In its effect on all calculated F_c^2 values except $F(000)$ (which is calculated ignoring g), subtracting the term in g in this way from the occupied regions of the structure is equivalent to adding a corresponding disordered scattering term in the (empty) solvent regions. For proteins, g usually refines to a value between 2 and 4; for small molecules without significant diffuse solvent regions, it should refine to zero. This approach has been extended to include different g's and U's for different atom types (G.N. Phillips, Jr. & K. Foreman, unpubl.) with slightly improved results, due mainly to the difference between carbon and the more polar oxygen and nitrogen atoms (see also Lounnas et al., 1994).

Another approach is to do the correction in real space, mapping out the parts of the unit cell where the solvent should be. This is done by assuming the solvent has constant density everywhere the protein is not (Phillips, 1980). This approach is commonly used in the analysis of crystal structures with the program XPLOR (Brünger et al., 1987). Although a useful approximation, the solvent density near a protein is far from constant, showing pronounced but inhomogeneous solvent shells (Lounnas et al., 1992).

Either of the methods just described can give calculated structure factors with phases that are useful and, hence, low-resolution data can be included in the analyses. However, both methods presume much about the nature of the solvent scattering (flat density, constant scattering) and neither method allows well for the study of the details of the solvent distribution.

A step in the right direction seems to be to use a grid-based method to refine the electron density corresponding to solvent positions (Schoenborn, 1988; Badger & Caspar, 1991). These studies allow a model-independent determination of the fluctuations in density due to solvent structure, but there are questions about the statistical validity of the approaches. A new method,

producing improved results, has recently been developed to allow peaks on top of the bulk solvent density in a new refinement scheme (Jiang & Brünger, 1994). Comparison with the results from other experiments remains difficult, especially in the case of magnetic spectroscopies. Because the space and time averages are averaged and therefore mixed or confused in crystallography, diffraction techniques will never give a complete picture of solvent behavior.

Neutron diffraction

Neutron diffraction is a complementary diffraction technique that is often useful for the same systems amenable to X-ray diffraction analysis. This powerful technique can yield both spatial and temporal information that can be used to analyze the hydration of protein crystals (Wlodawer, 1982; Kossiakoff, 1983) or solutions. The spatial information, although similar in many respects to that from X-ray experiments, differs by the nature of the physical processes involved in the neutron scattering process. Neutrons scatter according to the nuclear spin state and isotope of the atomic species in the sample rather than probing the electron distribution, as in X-ray diffraction studies. Thus, nuclei are located instead of electrons. Given this advantage, isotopic substitution is a valuable tool, and the use of H_2O and D_2O as solvents in neutron scattering of protein samples allows the determination of the water probability density. The different neutron scattering abilities of deuterium and hydrogen can enhance the solvent features by a factor of 2 relative to those observed with X-rays. Results on myoglobin show two distinct solvation shells, and 87 water molecules of hydration were refined (Cheng & Schoenborn, 1990). Interestingly, hydration sites often differ from the water molecules of hydration from the X-ray results, pointing out the limits of these approaches and/or unexpected differences in H_2O and D_2O solvation (Fig. 1).

Theory and simulation

Surface area/shape

Molecular dynamics simulations show the persistence of water structure beyond the first solvation shell (Lounnas et al., 1992). This result is in qualitative agreement with the diffraction results discussed above. The probability distribution of water at values above bulk density forms tubelike networks along the surface of the protein and is strongly correlated with the topography of the protein.

Several proteins have been shown to have correlations between local protein surface contours and hydration sites as seen in high-resolution crystal structures (Thanki et al., 1988; Kuhn et al., 1992). Applying concepts from fractal geometry (Hausdorff, 1919; Mandelbrot, 1983) to the surface of proteins ("surfractals"), it has been seen that surface groove accessibilities along so-called along reentrant curves correlate with most water molecules in refined X-ray structures (Kuhn et al., 1992; Peitgen et al., 1992). However, problems or biases in the reported water structure affect such an analysis and are difficult to quantify within such an approach. In the computer simulation of the water density, there are known limitations due to sampling and the underlying forcefield accuracy; however, a direct relationship between the protein surface in motion and the solvation shells is seen. Grooves and other surface topographies are seen only on average or as transients. This interplay results in a mutual smearing of the protein and water probability density near the interface. This penetration effect is only seen in the simulations for levels of occupancy below 30% of bulk density (Lounnas et al., 1992) and is thus practically inaccessible by either NMR or crystallography. High-probability solvation shells with such interpenetration and the view of the water as penetrating all regions of a protein at some very low probability yield a picture consistent with both diffraction placement of waters in the highest probability regions and titrations that require access to inner protons by solvent for exchange.

Distributions from simulations

The early work of Scanlon and Eisenberg (1975) clearly delineated the difference between various density maxima in the solvent region and the water molecules of hydration based on X-ray crystallographic refinements (Scanlon & Eisenberg, 1975). Recently, this idea has been continued directly in an analysis of a computer simulation of myoglobin in water (Lounnas & Pettitt, 1994a). This work made use of the difference between individual solvent molecules and hydration sites or local maxima in the average solvent density near the protein to extend the analysis of the proximal solvent to dynamic properties and the underlying distributions. The refined distribution was then interpreted as implying certain dynamic behavior, by taking the B-factor refinement model as a literal model of the microscopic dynamics.

In the simulations, it was found that the shape of a hydration density maximum is related to the local dynamics of the protein's polar or charged groups to which the water molecules are attached by hydrogen bonds. Local protein motions for short times are often reasonably represented by harmonic or quasiharmonic oscillations of atoms around their mean positions (Willis & Pryor, 1975; Brooks et al., 1985; Kuriyan et al., 1986), even though the actual dynamics are known to be intrinsically anisotropic and somewhat anharmonic. This approximation restrains their effective dynamics and the resulting distributions to a multivariate harmonic result, which then represents the effective dynamics of the small-scale thermal motions. B-factors or isotropic mean-square displacements are quantities straightforward to evaluate from a simulation trajectory and have been compared with results from crystallography (Lounnas & Pettitt, 1994a). Hydration sites in simulations can also be approximated by an isotropic Gaussian probability distribution, $P_g(r)$,

$$P_g(r) = \frac{1}{W(2\pi\sigma^2)^{1/2}} \exp\left(\frac{-r^2}{2\sigma^2}\right),$$

centered at a site location (with respect to the protein) that is characterized by a width σ^2 and an occupancy W (Lounnas & Pettitt, 1994a). Generally, the largest features of the distribution are well reflected in such a reconstruction. Modeling of other features requires a more flexible approach (Ichiye, 1985).

Deviations from perfect isotropy in the thermal distribution of the atoms about the average is characterized by unequal variances; anharmonicity is reflected in higher-order moments. The third and fourth moments of the positional distribution are indicative of the degree of anharmonicity of the atomic motions

(Kendall & Stuart, 1977). The asymmetry, or third moment, is reflected in the coefficients of skewness in the x, y, or z directions. The fourth moment is measured by the coefficients of excess kurtosis. Using these other measures to represent the probability distribution can further improve the fit of the functions to the trajectory (Ichiye, 1985; Lounnas & Pettitt, 1994b).

The probability density distribution of the solvent sites is occasionally treated as ellipsoidal. The directions and amplitudes of the fluctuations along the principal axes can be obtained by diagonalization of the symmetric matrix formed with the set of variances and covariances obtained from the complete B-factor refinement. These distributions have an implied harmonic dynamic character.

The occupancy weights and temperature parameters characterizing each hydration site in a simulation have been used to reconstruct the complete network of hydration according to both isotropic harmonic and anisotropic harmonic models (Lounnas & Pettitt, 1994a, 1994b). An analysis of the simulation in this manner leads to the localization of more than 500 hydration sites (significant maxima) distributed into multiple solvation shells located between 2.6 and 6.8 Å from the average atomic surface of the protein. On considering the local solvent density maxima or hydration sites, it is apparent that the positions of individual water molecules and the existence of hydration sites are distinct concepts in accord with the analysis of Scanlon and Eisenberg (1975).

The overall and local properties of the simulated solvation shells around myoglobin can be cross-compared with both neutron (Cheng & Schoenborn et al., 1990) and X-ray diffraction (Phillips et al., 1990) data on myoglobin. Differences between X-ray and neutron data concerning the locations of the protein-bound water may have many origins, but much can be reconciled by the existence of multiple hydration sites around exposed groups in the simulations. This presents multiple possible initial places for water molecules in the refinement that can be mutually exclusive given the steric constraints associated with individual water molecules (Lounnas & Pettitt, 1994a).

Another interesting interface between theory and experimentation is in the interpretation of solvation and fluctuations in proteins as a function of temperature, particularly at temperatures around 200 K. Neutron experiments show a change in mean square displacements of atoms as extracted from B-factors near 200 K. This has been associated with a glasslike transition in the system. A wide range of experiments has been used to probe this behavior (Parak et al., 1982; Bauminger et al., 1983; Parak & Knapp, 1984; Parak, 1986; Doster et al., 1989). Certain experiments have been interpreted as indicating that this low-temperature glass behavior of protein solutions is induced by the solvent (Doster et al., 1986; Ansari et al., 1987). Although this effect has been seen in other proteins and may be a general phenomenon for proteins, myoglobin has been central to the study of this effect. Computer simulations of myoglobin with a partial layer of surface water (350 molecules) have shown that the number of transitions of dihedral torsions from one potential well to another follows the same pattern as the mean square fluctuations with respect to temperature (Loncharich & Brooks, 1990). This has been interpreted in terms of a model with 2 relaxation times. One is a relatively fast process, corresponding to vibrational relaxation, that happens at all temperatures. The other, longer-time process would then be associated with jumps between torsional substates and would occur only above the glass transition temperature. By looking at the skewness and kurtosis of the atomic distributions, this latter motion was found to be significantly anharmonic (Loncharich & Brooks, 1990). However, more recent simulation analyses (Steinbach & Brooks, 1993) indicate that a change in both mean square displacement and dihedral transitions occurs at the glass transition temperature, whether or not solvent is present, which disagrees with the proposal that the transition is solvation related.

A view of the glass transition phenomenon from a different perspective suggests that side-chain motions are the key aspects (Doster et al., 1989; Furois-Corbin et al., 1993; Kneller & Smith, 1994). In this model, variations in neutron scattering data with respect to temperature are well fit by rigid body motions with diffusive side chains. Solvation then becomes only an influence on the diffusive motion, which would alter quantitative agreement but not the qualitative behavior.

Diffusion and dynamics implied by distribution functions

The solvent mobility in the liquid state is measured by the Stokes–Einstein diffusion coefficient, D, which is obtained from simulations by the slope of the mean square displacement versus time,

$$\lim_{t\to\infty} \frac{d}{dt} \langle |\vec{r}(t) - \vec{r}(0)|^2 \rangle = 6D,$$

where $\vec{r}(t)$ describes the position vector of a solvent molecule at time t. The brackets $\langle \ldots \rangle$ indicate that the quantity $|\vec{r}(t) - \vec{r}(0)|^2$ is averaged over both the solvent molecules and the time dimension origins.

There are difficulties concerning the application of this formula in characterizing the solvent mobility around a large biological macromolecule. This equation is usually used only for homogeneous and isotropic systems where averages are a reflection of the properties of each single particle of the system averaged over a long period of time. However, in an anisotropic situation as found at the protein–solvent interface, different solvation shells and places within each present distinct diffusional characteristics; thus, the diffusion "constant" manifests a spatial dependence.

The mobility in a volume of space around a specific moiety or region of the protein–solvent system can be characterized using an extension of the traditional diffusion relation given above (McCammon et al., 1987; Lounnas & Pettitt, 1994b). The main idea is that diffusive behavior can be attained in a shorter time than the residence time of water molecules around the particular group of interest. In bulk, the value of the diffusion constant is roughly 0.3 $Å^2\ ps^{-1}$ at 300 K (Berendsen et al., 1988; Ji et al., 1991). The diffusion water molecules within 6-Å windows from various protein groups have been determined from the slope of the mean square displacement calculated between 1 and 3 ps (Brooks & Karplus, 1989). This is a different measure than simple residence times and may prove helpful in understanding resonance experiments. Simulations have also been used to compute the diffusional mobility of water, $D(\vec{r})$, at each particular point $\vec{r}$ on a grid, as done for the analysis of the dynamics implied by the spatial distribution functions discussed above. A finite difference expression of the equation can then be em-

ployed, for a restricted range of motion about a particular point in space. The time difference values should be taken after the initial quadratic rise but before significant net mass transport.

The simulation results for myoglobin showed that the water molecules on the "surface" of the protein comprise regions of both higher and lower mobility than bulk water. Regions of significant but transient penetration show a high mobility as well (Lounnas et al., 1992). This observation is consistent with the "nearly dry" core model of a protein and provides a mechanism for exchange titration experiments. Near the surface of the protein, both hydrophobic and ionized groups showed mobility reductions. Other protein interior regions gave a mixed picture (Lounnas & Pettitt, 1994b), suggesting a complicated correlation between protein and solvent motions.

Residence times and probability distributions

Both the reorientational relaxation and the residence times of the transient water molecules associated with hydration sites can be compared with relevant experimental data, at least in the time domains from NMR and ESR studies. Such an effort reveals the mechanism underlying the observed correlations of occupancy weights with *B*-factors from crystallographic studies. With a computer simulation, residence times may be determined at each hydration site by averaging the time periods during which a single distinct solvent molecule was within a small distance on the order of 1 Å from the site. For myoglobin, they have been found to range from subpicosecond to over 20 ps, with an average around 1 or 2 ps (Lounnas & Pettitt, 1994b).

Furthermore, the orientational correlation function of water molecules can be related to observations from NMR, Raman, IR, and neutron spectra (Impey et al., 1982; Madden & Impey, 1988). These techniques provide a way to quantify the water reorientational motions at the interface with proteins and other solutes. If the rate processes lead to single-exponent relaxation times, they are often interpreted as the actual residence times of water molecules for various solvation shells around proteins (Koenig, 1980; Eisenstadt, 1985). The time correlation functions for water molecules in each hydration site, $C_s(t)$, may be computed from a stored trajectory as a discrete sum over saved coordinates from a simulation,

$$C_s(t) = \frac{1}{\vec{\mu}^2} \sum_{t'=0}^{T} \sum_{\Delta t=0}^{T} [\vec{\mu}(t') \cdot \vec{\mu}(t' + \Delta t)] \qquad t' + \Delta t < T,$$

where T is the length of the production or equilibrated part of the simulation trajectory, and $\vec{\mu}$ is the dipole vector for a water molecule. Assuming a rate law, the reorientation relaxation times are then determined as single (or multi) exponential relaxation time(s), τ, best fitting the correlation functions.

The data from a computer simulation study on myoglobin (Lounnas & Pettitt, 1994b) showed that the overall majority of sites have relatively short average residence times, between 1 and 5 ps. NMR results at temperatures near the freezing point (Otting et al., 1991) yielded times 2 orders of magnitude longer. Similarly, residence times between 1 and 4 ps have also been observed in a simulation of bovine pancreatic trypsin inhibitor (Levitt & Sharon, 1988). The simulations are consistent, and it may be necessary to redo such calculations at a temperature corresponding to the experimental conditions to resolve this question.

In the simulations, some examples were found to correspond to sites with long residence times. Residence times up to 30 ps have been seen, and these are of a magnitude similar to those found for monoatomic monovalent ions (Berendsen et al., 1988; Madden & Impey, 1988). One could speculate on the mechanism for the distribution of residence times; however, it has been observed that the average residence time of water molecules at a particular protein site is not correlated in a simple way with the occupancy.

The occupancy weight and temperature factor of the hydration sites do show a strong correlation with the number of hydrogen bonds shared with the protein (Lounnas & Pettitt, 1994b). There is a correlation of occupancy of a hydration site and the reorientation (relaxation) times of the water molecules in and near that site. Note that this is in contrast to the lack of correlation mentioned above between occupancy and the average residence time. The reorientation times of the water dipole axes, computed as described above, were 2.3, 3.1, 3.9, 6.5, and 12.1 ps for water in the sites characterized by occupancy weights in the [40, 50%], [50, 60%], [60, 57%], [70, 80%], and [80, 100%] intervals, respectively (Lounnas & Pettitt, 1994b). A single exponential (fit) decay time for the orientational correlation was found to be correlated with the occupancy factor. By inference, this is then correlated with the degree of interaction with the protein. In the simulation studied, surface hydrogen bonded hydration sites are often found populated by very strongly oriented water molecules, as reflected by the very slow decay of the dipole correlation function. Different behavior has been seen in cases of hydration sites lacking obvious hydrogen bonding sites. The lack of the usual hydrogen bonding "shoulder" in the orientational correlation functions for this type of site has been interpreted as evidence of rotationally free water molecules.

Structural connections between theory and experimentation

To explicitly quantify the influence of the protein surface on the protein–solvent pair correlation functions mentioned above and to bring together simulated and experimental results, a quantity called $g_\perp(r)$ has been defined (Lounnas et al., 1994). This quantity measures the probability distribution of the solvent molecules as a function of the distance, r, from the closest protein atom, i.

The function of $g_\perp(r)$ is a conditional pair correlation function and describes the solvent structure at a given distance, r, perpendicular to the protein surface. The protein surface is defined by the protein atoms that are in direct contact with the solvent instantaneously. Notice that the traditional pair correlation functions between protein and solvent describe, in some sense, the local solvent distribution around surface atoms of the solute, whereas the perpendicular distribution function is more a characteristic of the whole protein. The perpendicular correlation function method allows the quantitative characterization of aspects of the protein–solvent interface not easily identified by the radial pair distribution function. Thus, it is straightforward to consider distributions as a function of atom types or other convenient categorization such as either the hydrophilic or hydrophobic nature of the selected species, etc. Just as atomic

force field parameters have a variety of atom types for the same atomic species (e.g., carbonyl carbons, tetrahedral carbons, etc.), one can also distinguish between atomic groups as a function of their belonging to the backbone or side chains.

It is awkward to complete correlations at each time instant; it is more computationally convenient to define a perpendicular distribution function where solvent positions are preaveraged in time, $g_{\perp}^{A}(r)$ (Lounnas, 1993). Although such a function is slightly different from $g_{\perp}(r)$, it also can be used as a conditional pair distribution function between the surface of the protein and the water molecules of hydration. So, $g_{\perp}^{A}(r)$ may be thought of as correlations between the average positions of both protein and solvent atoms measured with respect to a protein reference frame rather than the traditional space-averaged instantaneous pair correlation. The advantage is that the function $g_{\perp}^{A}(r)$ is much easier to compute than $g_{\perp}(r)$ and is, in the end, still relevant to reconstructions of the density distribution useful for X-ray crystallographic refinements (Lounnas et al., 1994). The three-dimensional density distribution, $\pi^{1}(\vec{r})$, which is obtained from the X-ray experiment, is mathematically related to $g_{\perp}^{A}(r)$.

In a previous study, $g_{\perp}^{A}(r)$ has been decomposed into protein atom-dependent functions for each of carbon, nitrogen, and oxygen. Generally one can perform such a decomposition for any number of atom types, i, as:

$$g_{\perp}^{A}(r) = \frac{\sum_{i} n_i(r) \cdot g_{\perp i}^{A}(r)}{\sum n_i(r)},$$

where n_i is the number of atom i sites that are closest to solvent for a given distance r. Clearly, to calculate $g_{\perp}^{A}(r)$, one must first determine the atomic perpendicular pair functions.

One can then introduce a model fit to reconstruct the average solvent density distribution for the protein–solvent interface (Lounnas et al., 1994). In a particular application of this idea, the model function was expressed in terms of four fitted parameters for each type of atomic species. The modeled distribution function $g_{\perp}^{m}(r)$ provides a basis for modeling the water distribution in crystallographic refinement procedures (Lounnas et al., 1994). This has been shown to yield improved R-factors for the lowest-order reflections, allowing better visualization of solvent structure and distribution. In weighing the utility of this approach, it is important to notice that the number of parameters to be fit or refined is only about a dozen, which in a traditional refinement would be taken up with the first three water molecules placed. The estimates from simulation of the distributions provide a very reasonable starting point, and it was found that all model parameters need not be readjusted. Such a model may find application in addressing the general problem of X-ray crystallographic refinement of globular proteins, where the lack of information on the solvent distribution in the crystal lattice results in some problems concerning the protein structure itself and confusion concerning the role of hydration water in biological systems.

An implication of this approach is the ability to implement a better structural description of the solvent, which will help in protein X-ray crystallography refinement procedures and, possibly, reveal more about the proteins themselves. More work will be needed, however, to firmly connect the time scales inferred from experimentation with simulations, but the different fields of study seem to be converging.

Outlook

In this review, we have attempted to show the diversity of results concerning water surrounding myoglobin. In addition, we find that some unifying principles have emerged from recent theoretical studies. However, no complete picture that includes all the experiments exists as yet. For instance, the view of hydration from heat capacity measurements remains difficult to reconcile completely with theoretical calculations, due to the fact that heat capacities are notoriously difficult to converge in simulations because of their fluctuation-based (second moment) nature.

Yet, we find that the difficulties with interpreting different diffraction experiment results from X-rays, and neutrons, as well as the differences in $P2_1$ and P6 crystal forms can be understood. Here, the unifying concept is that of "hydration sites" as an entity distinct from individual water molecules of hydration. By considering the solvent surrounding myoglobin to be fundamentally disordered as in a liquid (at normal temperatures) or glass (at low temperatures), the natural or convenient conceptual picture becomes that of a probability distribution rather than well-ordered molecules. Using such a distribution approach, it becomes apparent that refining molecules into local density maxima around the protein can be misleading, even when the reduction in crystallographic R-factor is statistically significant. As significant maxima are shown to be spaced, on average, 2 Å apart in the simulations and water molecules are around 2.8 Å in diameter, it is impossible to satisfy two proximal minima at the same time, i.e., a water molecule in each. Although such maxima may have significant populations, their proximity can be seen as a consequence of equilibrium; either one molecule is exchanging between the two sites in question or two (or more) molecules are exchanging into the sites from the bulk surrounding layers of water.

The results of a comparison of the three diffraction experiments on myoglobin show a remarkable agreement for the protein structure. The poor agreement of positions of water molecules is quite disappointing at first glance (see Fig. 1). However, careful comparison of the refined water molecules from the various diffraction experiments shows that, essentially, each of the locations corresponds to a significant local density maximum in the simulation (Lounnas & Pettitt, 1994a).

Many significant problems remain. Although simulations have helped in the interpretation of time-domain data, some issues are not resolved. In particular, we have not reconciled breaks in the NMR response with the breaks seen in heat capacity versus degree of hydration. Other problems are clearly evident as well. We hope that by showing where unifying principles do and do not exist, we can provoke others in the field to consider these problems.

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