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WATER IN BIOLOGICAL SYSTEMS: THE NMR PICTURE

Bertil HALLE
Condensed Matter Magnetic Resonance Group, Department of Chemistry
Lund University, P.O. Box 124, S-22100 Lund, Sweden

Abstract This review outlines the consensus picture of biomolecular hydration that is now emerging from results provided mainly by solution NMR relaxation methods, X-ray crystallography, and computer simulation.

1. From Controversy to Consensus

In order to describe complex molecular systems in a simple way, we have invented a high-level language incorporating terms such as "hydration", "bound water", and "hydrophobic effect" that hide not only the underlying complexity but also our ignorance. While they have undoubtedly served a useful purpose, these deceptively familiar terms have often stood in the way of quantitative understanding. In the most general sense, the term "hydration" encompasses all solute-induced perturbations of the structure, energetics, and dynamics of the aqueous solvent. Since different experimental techniques probe the system on different scales of length, energy, and time, they provide us with different pictures of hydration. This has given rise to a plethora of *operational* (method-dependent) definitions of hydration, making it difficult to assess the mutual consistency of the different pictures of hydration.

The most striking feature of the prodigious literature on biomolecular hydration is the lack of consensus. This, of course, tells us that we are dealing with a hard problem. Several extensive and penetrating reviews are available that portray the field in its various stages of development [1-3]. In the past few years there has been a resurgence of interest in biomolecular hydration, fuelled by methodological developments and an increasing awareness of the importance of hydration for biomolecular stability and function. As a result of recent advances in the field, a consensus picture of biomolecular hydration is finally emerging. This picture is largely based on the complementary and mutually consistent results provided by solution NMR relaxation methods, single-crystal X-ray diffraction, and molecular dynamics (MD) computer simulation. The aim of the present Chapter is to summarise the current understanding of biomolecular hydration and to critically review the results on which it is based. Although the emphasis is laid on results obtained by NMR, an attempt is made to integrate these results with those provided by crystallography and computer simulations. Most of the results discussed here refer to globular proteins, but many of the conclusions are valid also for other biopolymers and for nonbiological interfaces. The material is divided in two parts, focusing on internal and external hydration. In each part, both structural and dynamic aspects are considered.

2. Internal Water

2.1. Structure

The crystallographic data base is undoubtedly the richest source of structural information about biomolecular hydration [4]. Most protein crystals contain 30 – 60 vol% water [5], classified as internal, surface, or interstitial water. Crystallographers refer to a water molecule as *internal* if it cannot be connected to external water by a continuous chain of (inferred) water-water H-bonds [6]. In the present review, we use a less restrictive definition of internal water, including not only water molecules completely buried in cavities, but also those partly buried in deep and narrow crevices or pockets and those coordinating protein-bound multivalent metal ions (Fig. 1). Internal waters that are not metal-coordinated will be referred to as buried.

A small (15 kDa) globular protein typically contains half a dozen internal waters, but the variation is large [6]. Buried water molecules heal packing defects and extend the H-bond framework, thus contributing importantly to protein structure and stability, and are sometimes involved in enzyme catalysis [7]. They are conserved among homologous proteins to the same extent as amino acid residues [4] and cannot be removed by drying [8]. Singly buried water molecules observed by crystallography are generally highly ordered with B factors of 10 – 15 $Å^2$ (comparable to nearby protein atoms) and usually make 3 or 4 strong H-bonds with the protein (as inferred from short distances to protein O or N atoms). Water clusters buried in larger cavities are less ordered, as reflected in larger B factors and smaller (NMR-derived) orientational order parameters , e.g., for the 6-water cluster in trypsin [9,10] and the 20-water cluster in the intestinal fatty acid binding protein [11,12].

Although buried water molecules must be considered an integral part of the protein in a structural and energetic sense, they do exchange with the external solvent. This exchange makes it possible to detect buried water molecules in protein solutions by NOE spectroscopy [13] and magnetic relaxation dispersion (MRD) [14]. Since crystal contacts are not expected to perturb the internal hydration of proteins, the main virtue of the solution NMR methods is not in verifying the presence in solution of crystallographically identified internal hydration sites, but rather in providing complementary information. NOE spectroscopy can detect water molecules in nonpolar cavities, where positional disorder [15] or partial occupancy [16] render them invisible to X-ray diffraction. The MRD method, on the other hand, can provide orientational order parameters of internal water molecules which can be converted to librational amplitudes and rotational entropies, information that cannot be obtained even from neutron diffraction (which can "see" water hydrogens) at current resolutions.

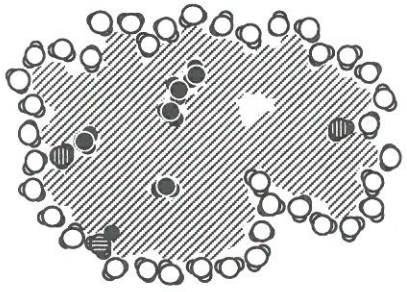

Fig. 1 Schematic cross-section of a protein, showing "internal" water molecules that are completely buried (black), partly buried in surface pockets (vertical stripe), or metal-coordinated (horizontal stripe), as well as external water molecules (white).

A multinuclear MRD study of wild-type and mutant forms of BPTI thus showed that the rotational entropies of the 7 investigated internal water molecules span the full range from ice to bulk water, suggesting that the hydration of less polar cavities is entropically driven [17].

Since internal hydration sites result from the specific structure of native proteins, they should be abolished on denaturation. This expectation has been verified by MRD for both thermal [18] and solvent-induced [19] denaturation. Although a fully unfolded protein must be permeated by solvent, this occluded water is expected to resemble the surface water of native proteins in structure and dynamics. Partly collapsed homopolymers [20] and disulfide-intact polypeptides [19], however, have been shown to trap long-lived (> 10 ns) water molecules. The highly compact acid-induced molten globule state, on the other hand, appears to retain most of the native internal hydration [19].

Due to the current nanosecond time scale limitation, regular MD simulations cannot make *bona fide* predictions of cavity hydration. The initial configuration in MD simulations is usually the crystal structure including any buried water molecules. Since buried water molecules do not exchange on subnanosecond time scales, preservation of internal hydration during an MD trajectory does not prove that these internal sites would actually be hydrated at equilibrium with the force field model employed. On the other hand, failure to maintain the crystallographically determined internal hydration [21-23] suggests that the force field is in need of improvement. Given that a buried water molecule is present, however, MD simulations can provide detailed information about positional and orientational disorder [9,24,25] and about flip rates [26]. Furthermore, simulations employing thermodynamic perturbation schemes have been used to calculate the free energy of cavity hydration [27-29]. For superficially buried water molecules with relatively short residence times, MD trajectories can reveal (experimentally inaccessible) details of the exchange mechanism [30].

Water molecules in the primary coordination shell of bound multivalent metal ions are an integral part of many metalloproteins. Even if exposed to external solvent, metal-coordinated waters generally exhibit a high degree of positional order in crystal structures. In protein solutions, the hydration of paramagnetic metal ions has been extensively studied via the paramagnetic relaxation enhancement of the water ^1H resonance. This field has been revitalised by the widespread use of paramagnetic contrast agents in clinical magnetic resonance imaging [31]. Water molecules coordinating diamagnetic metal ions in proteins have been detected and characterised by MRD (^2H and ^{17}O) in only one case so far: the two Ca^{2+} ions in calbindin D_{9k} [32]. The zinc-coordinated hydroxide ion (at pH* 9) in human carbonic anhydrase II has been shown to exchange too slowly (residence time > 2 μs) to contribute to the ^{17}O dispersion [33].

2.2 Residence Time

Diffraction techniques probe structure via *spatial* correlations; other techniques such as magnetic relaxation probe the motion of individual molecules via *dynamical* correlations (time correlation functions). While the MRD and NOE techniques sample the dynamical trajectories of individual water *molecules*, X-ray and neutron diffraction can only see *hydration sites* that, on average, are occupied with high probability by any water molecule. A small thermal *B* factor, therefore, does not necessarily imply slow water dynamics (or vice versa) [2]. The *B* factor is determined by the shape of the potential of mean force near its minimum at the locus of a hydration site. The mean residence time of water molecules

occupying this site, however, is determined by the barriers (or saddle points) in the potential of mean force which, by definition, are sparsely populated and hence invisible to diffraction probes. Reported crystal structures are averaged over periods long compared to the time scale of most molecular motions, but this is usually irrelevant since the configurational space is already fully sampled by spatial averaging over a very large number of unit cells. Time-resolved crystallography can therefore provide dynamical information only if a macroscopically uniform initial state can be prepared and even then can only monitor very slow (> minutes) hydration processes [34,35].

The current conception of globular proteins as dynamically fluctuating conformational ensembles is of relatively recent vintage. In the early days of protein crystallography, it was natural to regard proteins as more or less static entities. Accordingly, buried waters were generally taken to be extremely long-lived, with residence times in the range of seconds or longer [2,36,37]. The first direct proof of buried water exchange came from mass spectrometric studies, showing that buried $H_2{}^{18}O$ in chymotrypsinogen exchanges with bulk $H_2{}^{16}O$ within 20 minutes [38]. The same technique, but with improved temporal resolution, was subsequently used to demonstrate that the buried waters in BPTI, trypsin, lysozyme, and carboxypeptidase exchange within 10 – 15 seconds [39].

During the past decade, buried water molecules have been identified in about a dozen proteins in solution by NOE spectroscopy [13]. The fact that NOESY and ROESY cross-peaks with buried water molecules are invariably observed at the chemical shift of bulk water implies that all NOE-detected buried water molecules have residence times short compared to the inverse of the difference in chemical shift for internal and bulk water. Unfortunately, that shift difference is not known. For the buried waters in BPTI, an upper bound on the residence times was estimated by displacing the bulk water resonance with the aid of a paramagnetic shift reagent [40]. From the observation that the cross-peaks with the buried waters moved to the new bulk solvent shift, it was concluded that the residence times are shorter than 20 ms at 277 K. Since the proton exchange lifetime in bulk H_2O at pH 3.5 and 277 K (the conditions of the NOE study) is merely 1.6 μs [41,42], however, it was not clear if the 20 ms upper bound referred to exchange of entire water molecules or to exchange of water protons. Nevertheless, this upper bound turned out to be consistent with the actual residence time for the most long-lived buried water molecule (W122) in BPTI, subsequently determined to 3 ms at 277 K by ^{17}O and ^{2}H MRD [43]. Although not yet reported in the literature, slowly exchanging water molecules (residence time > ca. 10 ms) should be observable by NOE spectroscopy at the internal water shift.

While NOE spectroscopy does not in general provide an upper bound on the residence time (since the shift difference might be arbitrarily small), the relative sign of the cross-peak intensities in NOESY and ROESY spectra furnish a lower bound. If the effect on cross-relaxation of fast local motions (water librations and 180° flips as well as protein motions) is neglected, a negative intensity ratio implies a correlation time τ_C longer than $\sqrt{5}/(4\pi\nu_0)$, or 300 ps at a ^{1}H resonance frequency of 600 MHz [44]. Provided that the biomolecular tumbling time τ_R is much longer, τ_C can be identified with the mean water residence time τ_W. (It is sometimes incorrectly stated that a negative intensity ratio implies that $\tau_W >> \tau_R$.) Near the zero-crossing, the value of the intensity ratio in principle allows the residence time to be determined [45]. Such detailed analysis is rarely attempted, however, since it is both model-dependent and sensitive to spectral artefacts. A more reliable procedure for determining τ_C is to record the zero-crossing of the NOESY intensity as a function of magnetic field strength (proportional to ν_0) [49].

Most of the available information about internal water residence times has come from MRD studies. Oxygen-17 MRD data generally provide lower and upper residence time bounds that are more restrictive (by orders of magnitude) than NOE-derived bounds and, in favourable cases, can actually determine the residence time with an accuracy of 10 – 20% [14]. In MRD, the upper bound is set by the intrinsic spin relaxation time T_I of an internal water molecule (ranging from a few μs for ^{17}O to a few ms for ^{1}H), while the biomolecular tumbling time (typically 5 – 10 ns) provides the lower bound.

The actual periods of time that different water molecules spend in a given hydration site are of course variable. For relatively long-lived (internal) sites, water exchange can reasonably be assumed to obey first-order kinetics. Although the residence times are then Poisson distributed, the spectral density function $j(\omega)$ probed by MRD is determined by the *mean* residence time τ_W, which may be defined as the integral of the survival probability $Q(\tau) = \exp(-\tau/\tau_W)$. (Throughout the literature, and in the following, τ_W is referred to simply as the residence time.) Since $j(\omega)$ is the Fourier transform of the product of $Q(\tau)$ and the exponential rotational time correlation function (for isotropic protein tumbling), it follows that the observed correlation time is $\tau_C = (1/\tau_W + 1/\tau_R)^{-1}$. Consequently, if the residence time is not much longer than τ_R, but still sufficiently long to give a measurable dispersion step, it can be obtained directly from the shift of the dispersion frequency. This approach has been used to determine residence times in the range 1 – 10 ns [18,45-47]. Longer residence times, in the μs – ms range, can be determined from the violation of the fast-exchange condition $\tau_W << T_I$ [43] since the dispersion amplitude parameter $N_I S_I^2$ then is multiplied by the factor $(1 + \tau_W / T_I)^{-1/2}$ [14].

Accurate residence times (not just bounds) for biomolecular hydration sites have so far been determined in only four cases. For the singly buried water molecule (W122) in BPTI, a difference-MRD study involving wild-type BPTI and the G36S mutant yielded $\tau_W = 170 \pm 20$ μs at 300 K [43]. This water molecule is completely buried in a small cavity near a disulfide bond, it is highly ordered ($B = 11$ Å^2, $S_I > 0.9$), and it makes 4 strong H-bonds to the polypeptide backbone. The other three buried water molecules (W111 – W113) in BPTI occupy a pore-like cavity, with W113 at the bottom and W111 at the mouth. These water molecules are not as long-lived as W122. For at least two of them (W112 and W113), τ_W is in the range 15 ns – 1 μs [48]. While W111 may also be in this range, the available MRD and NOE data are actually consistent with a subnanosecond residence time for this least deeply buried water molecule.

The second and third τ_W determinations refer to water molecules in surface pockets of ribonuclease A and T1. According to the crystal structure, RNase A contains no completely buried water molecules, but six water molecules occupy deep surface pockets. The ^{17}O relaxation dispersion is due to at least three long-lived water molecules, with $\tau_W = 8 \pm 2$ ns at 300 K [18], which most probably are among the six pocket waters. In RNase T1, a difference-MRD experiment yielded $\tau_W = 7 \pm 3$ ns (and $S_I = 0.7$) at 300 K for an extensively H-bonded water molecule residing in a surface pocket and displaced by a sidechain in the T93Q mutant [47]. The shorter residence times for these internal waters (as compared to those in BPTI) is consistent with their more exposed locations.

The fourth τ_W determination is for a chain of 5 water molecules residing at the floor of the narrowed central part of the minor groove in the *B*-DNA duplex of the dodecamer d(CGCGAATTCGCG). Difference-MRD experiments, where these 5 water molecules were selectively displaced by a netropsin molecule, yielded $\tau_W = 0.9 \pm 0.1$ ns at 277 K [45] and 10 ± 1 ns at 253 K [46]. For this DNA duplex, τ_W has also been determined by NOE

spectroscopy at 283 and 288 K [45,49]. Although the minor-groove region where these 5 waters are located admits only a single file of water molecules, these are H-bonded to external water molecules and the groove is ca. 4 Å wide.

In three of these four cases, τ_W has been determined at several temperatures over a 50 K range or more (Fig. 2), allowing the (apparent) activation enthalpy to be determined: 90 ± 5 kJ mol^{-1} for W122 in BPTI , 43 ± 10 kJ mol^{-1} for RNase A, and 52 ± 3 kJ mol^{-1} for DNA. These values are larger than the activation enthalpy of bulk water motion, ca. 20 kJ mol^{-1}. Since the water residence times in the four systems span 6 orders of magnitude (at 300 K), the exchange mechanism may be quite different. While the mechanism has not yet been established, plausible models may be constructed on the basis of the structure around the hydration sites and the activation enthalpy. From the crystal structure of BPTI, it is clear that W122 cannot exchange without substantial disruption of the native protein conformation. Since the exchange mechanism therefore is likely to involve many coupled degrees of freedom within the protein, the Arrhenius single-smooth-barrier picture may be less relevant than a Gaussian random energy model, which then should have an rms ruggedness of ca. 10 kJ mol^{-1} to explain the observed temperature dependence of τ_W [43]. In this view, the residence time is determined primarily by *intra-protein* (rather than water-protein) interactions and W122 should be described as *trapped* rather than bound. Although protein conformational fluctuations may well play a role also in the case of RNase A and T1, a model where water exchanges from a rigid surface pocket cannot be ruled out here. The residence time is then determined mainly by the *geometry* of the hydration site. If the pocket is deep and narrow, the water molecule must pass through a "transition state" where it has sacrificed its protein H-bonds before it can engage in new H-bonds with external waters (and another water molecule can take its place). The residence times are 3 orders of magnitude longer than the time required for a similar displacement in bulk water not because the water molecules interact strongly with the protein (they may have fewer H-bonds than in bulk water) but because of the *absence* of favourable interactions with the protein and with other water molecules in the transition state. This, in turn, is a consequence of the *geometry* of the pocket; if it is sufficiently deep and narrow, the pocket must be completely vacated before a new water molecule can enter. In the minor groove of the *B*-DNA duplex, a similar situation might be envisaged. The finding that τ_W is essentially invariant with respect to nucleotide base sequence (which affects the groove width), however, suggest a coupling to large-scale DNA motion [46]. As these examples demonstrate, the residence times of internal water molecules can provide valuable information about the conformational dynamics of biomolecules.

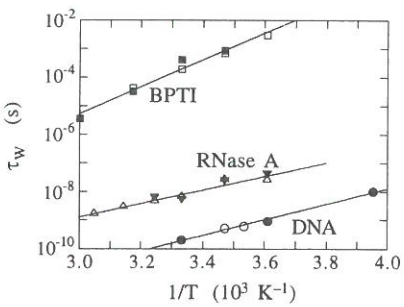

Fig. 2 Temperature dependence of the residence times of the singly buried water molecule W122 in BPTI (■ ^{17}O data, □ ^{2}H data) [43], of 3 – 6 water molecules trapped in pockets on the surface of RNase A (▼ pH* 2, Δ pH* 4) [18], and of 5 water molecules in the minor groove of the *B*-DNA duplex d(CGCGAATTCGCG) (● MRD data, O NOE data) [46,49].

For about a dozen other proteins investigated by ^{17}O MRD, internal water molecules have been detected with residence times in the range 0.01 – 1 μs [10,12,17-19,33,48]. Unless difference-MRD experiments are performed, only the product $N_I S_I^2$ of the number of water molecules with τ_W in this range and their mean-square orientational order parameter is obtained [14]. Overall, a good correlation is found between $N_I S_I^2$ and the number of crystallographically identified internal waters (as defined above), indicating that the majority of internal waters have residence times in the range 0.01 – 1 μs (at 300 K) and order parameters in the range 0.5 – 1. A minority of internal waters escape detection by ^{17}O MRD because they have residence times much longer than 1 μs or order parameters significantly smaller than 0.5. The former are deeply buried in rigid parts of the protein (W122 in BPTI) or coordinated to metal ions (Zn-OH^- in carbonic anhydrase), while the latter occur in large clusters (trypsin and FABP) or in nonpolar cavities (myoglobin and lysozyme). Internal waters with residence times as long as milliseconds can be detected by ^1H MRD and NOE spectroscopy, but then the labile-hydrogen contribution to the ^1H dispersion must be controlled (e.g., by difference-MRD). Experimental residence times should never be quoted without specifying the temperature. For W122 in BPTI, τ_W varies by 3 orders of magnitude over a 50 K interval (Fig. 2). Also, when quoting residence time ranges, it should be made clear whether the range is a property of the investigated *systems* (as the 6 orders of magnitude τ_W range at 300 K in Fig. 2) or merely reflects *methodological* limitations (as the 8 orders of magnitude τ_W range at 277 K derived from NOE studies of BPTI).

3. Surface Water

3.1 Structure

For a long time it has been common to think of hydration in terms of complex formation and binding and to assign definite hydration numbers to small molecules and ions and hydration layers of "bound water" to proteins. This viewpoint is appropriate for gas-phase hydration, but is problematic for hydration in solution. Calorimetric measurements [3] and computer simulations [50] suggest that water molecules in the first hydration layer of proteins on average have about 2 kJ mol^{-1} lower free energy than in bulk water. For a small (15 kDa) globular protein making contact with 400 water molecules, this amounts to 800 kJ mol^{-1}, a factor 10 – 20 more than the typical stability margin with respect to denaturation under physiological conditions. While hydration is obviously important for protein stability, it is misleading to think of the protein-water interaction as a "binding energy". Consider the electrostatic free energy of hydration of a simple monovalent ion. It can be shown that the 135 water molecules in the region between 3 and 10 Å from the ion (excluding the first hydration shell, where the continuum description may fail) contribute on average 1.2 kJ (mol H_2O)$^{-1}$ to the hydration free energy. Although these water molecules together contribute 160 kJ (mol ion)$^{-1}$, they are virtually indistinguishable from bulk water in terms of structure and dynamics [51] and therefore cannot reasonably be considered as "bound". Depending on their sensitivity to small perturbations of water structure, different methods will obviously "see" different amounts of hydration water. Rather than determining some operationally defined amount of "hydration water", it is more useful to characterise the protein-induced solvent perturbation in terms of order parameters, decay lengths, and correlation times that are intrinsic properties of the *system* and independent of the *method* used to measure them.

The crystallographic determination of hydration sites on the exposed surface of a protein relies on refinement protocols that are to some extent subjective. Typically, the number of reported external hydration sites is comparable to the number of amino acid residues in the protein. The *B* factors are larger than for internal waters (typically 20 – 50 Å^2) and are strongly correlated with occupancies. For less well-defined hydration sites, it may not even be meaningful to distinguish these two aspects of the spatial distribution function. Accordingly, full occupancy is often enforced during refinement. The accuracy of external hydration sites reported in the literature is highly variable and sometimes poor. In a comparison of four independent determinations (at 2 Å resolution) of the same crystal form of interleukin 1β (151 residues), only 29 hydration sites coincided to within 1 Å among the four structures and many of these were internal sites [52]. Several strategies have been developed for improving the definition of solvent structure, e.g., analysis of neutron H_2O–D_2O difference maps [53] and inclusion of low-order diffraction data coupled with explicit modelling of interstitial solvent [54-56].

Even accurately determined surface hydration sites in ultrahigh resolution crystal structures [57,58] are not necessarily relevant to protein hydration in solution. For typical small and medium-sized proteins, 30 – 40% of the solvent-accessible surface is buried at crystal contacts [59] and water-mediated protein-protein interactions may be crucial for crystal stability [60]. Electrolytes and organic cosolvents from the mother liquor presumably permeate the crystal to some extent and may then perturb, or even be mistaken for, hydration sites [4,60]. The importance of crystal-specific hydration features can be assessed by comparing the hydration structures of the same protein in different crystal forms or at non-equivalent positions in the asymmetric unit. In such comparisons, it is usually found that less than half of the reported hydration sites (mainly the ones with small *B* factors) are conserved [4]. For BPTI, only 12 external hydration sites were found to be conserved among three crystal forms [61].

Water at protein surfaces as seen in crystals is reported to have ca. 20% higher density than bulk water [62]. This is partly a correlation effect; water oxygens tend to be separated from protein surface atoms by 3 – 4 Å, thereby giving rise to a peak in the water-protein pair distribution function [50]. As expected, the surface waters with the smallest *B* factors are most strongly H-bonded to the protein [4]. One third of the surface waters seen in crystals make two or more such H-bonds [62]. Water in contact with extended nonpolar surface regions tends to be disordered [63,64], rather than adopting the clathrate-like hydration structures typical of small nonpolar solutes.

Information about the orientational order of water at protein surfaces can in principle be derived from static quadrupole splittings of the water ^{2}H and ^{17}O resonances from macroscopically anisotropic systems [65]. A solid-state NMR study of a BPTI single crystal showed, however, that the orientation and temperature dependence of the water ^{2}H splitting can be accounted for by a few highly ordered water molecules, either internal or trapped at protein-protein contacts [66]. Most crystallographically identified water molecules probably have small order parameters (0.01 – 0.1), as found for water in contact with the surfaces of clays and amphiphilic liquid crystals [65,67].

The surface hydration of proteins and nucleic acids plays an important role for the energetics and specificity of their interactions with ligands and other biopolymers [68,69]. A favourable entropic contribution to the free energy of complex formation is often invoked on the assumption that the displaced hydration water is more ordered than bulk water. While this may sometimes be true, the generality of this notion is challenged by the

demonstration by MRD that even multiply H-bonded internal waters can have entropies comparable to that of bulk water [17]. In this connection, it should be noted that while order parameters are related to the thermodynamics of complex formation, water residence times are not [70].

3.2 Rotational Motion

NMR has provided more definite information about the dynamics of surface water than about its structure. In particular, the ^2H and ^{17}O relaxation rates probe the rate of water rotation via the (second-rank) rotational correlation time τ_S. Since rotation of surface water is invariably too fast to give rise to a relaxation dispersion, one can only determine the product $N_S \rho_S$ of the number of dynamically perturbed water molecules and the relative dynamic retardation $\rho_S = \tau_S / \tau_{bulk} - 1$ averaged over these waters [14]. A separation of the *range* of the perturbation (N_S) from the *extent* of perturbation (ρ_S) is possible only if the amount of water outside the surface can be varied in a controlled way. This is difficult for proteins; water in incompletely hydrated protein powders [3,71] is not necessarily representative of water at the surface of native proteins in solution. Quantitative information about the range of the surface-induced dynamic perturbation has been obtained in other systems, however. In certain microemulsions, the radius of the water droplets is controlled by the overall water/surfactant ratio. By measuring the water ^{17}O relaxation rate as a function of droplet radius it was found [72] that only waters in direct contact with the surface are significantly perturbed: $\tau_S / \tau_{bulk} = 3.7$ for $N_S = 15$ water molecules, corresponding to the primary hydration of the sulfosuccinate headgroup and Na^+ counterion (Fig. 3). Similar results have been obtained from water ^2H relaxation studies of water outside sodium hectorite clay surfaces ($\tau_S / \tau_{bulk} = 5.4$ for one water layer) [73] and of water outside various phosphatidylcholine lipid bilayer membranes ($\tau_S / \tau_{bulk} = 2.7 - 4.7$ for $N_S = 15$ waters per lipid) [74].

Although *direct* experimental evidence is lacking, there are strong reasons for believing that the dynamic perturbation is similarly short-ranged at protein surfaces. This extrapolation is supported by MD simulations, showing that $\tau_S \approx \tau_{bulk}$ beyond the first hydration layer [75,76]. If the quantity $N_S \rho_S$ derived from ^{17}O MRD profiles is attributed to water molecules in contact with the protein surface and N_S is estimated by dividing the

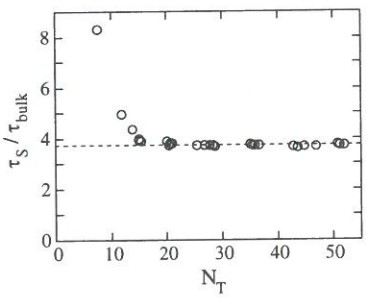

Figure 3: Retardation of water rotation (293 K) at the interface of an aqueous microemulsion droplet, derived from ^{17}O relaxation data [72]. N_T is the total water/surfactant ratio and $N_S \leq 15$.

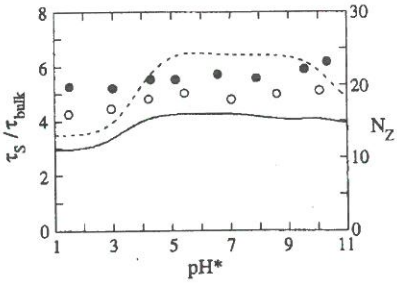

Figure 4: Retardation of water rotation (300 K) at the surface of BPTI (•) and ubiquitin (○), derived from water ^{17}O MRD data [77]. The curves show the total number of charged groups in BPTI (solid) and ubiquitin (dashed).

solvent accessible surface area by 15 Å^2, one obtains for the 7 small proteins BPTI, ubiquitin, calbindin D_{9k}, lysozyme, α-lactalbumin, ribonuclease A, and apomyoglobin τ_S/τ_{bulk} values (at 300 K) in the range 4.9 – 6.4, with an average of 5.5 [18,19,77,78]. For several dodecamer *B*-DNA duplexes, ^{17}O MRD yields $\tau_S/\tau_{bulk} = 6.0$ (at 253 K and with $N_S = 300$) [46]. It is noteworthy that highly charged DNA does not differ significantly from the proteins. The relative contribution to $N_S\rho_S$ from the ionic side chains at the protein surface has been investigated by pH dependent ^{17}O MRD studies of BPTI and ubiquitin (Fig. 4) [77]. The 15% reduction of $N_S\rho_S$ on protonation of the 12 carboxylate groups in ubiquitin (46% reduction in number of charged residues) shows that the ionic form of these groups retards water rotation more strongly but also indicates that the measured global retardation ($N_S\rho_S$) is not dominated by ionic residues. In ubiquitin, protonation of one carboxylate group reduces $N_S\rho_S$ by 21, close to the value 22 found for poly(acrylic acid) [20].

The quantity $N_S\rho_S$ has been determined from the water ^{17}O relaxation rate in aqueous solutions of a variety of small molecules and ions [79-84]. This substantial data base reveals an *approximate* group additivity, as expected for a short-ranged dynamic perturbation. The contribution to $N_S\rho_S$ is about 4 for CH_2 and CH_3 groups and near zero for most polar groups, e.g., OH, COOH, CONH, and NH_2. Ionic groups have variable effects on $N_S\rho_S$, close to zero for NH_3^+, but 10 – 20 for COO^-, and for simple cations the effect increases with surface charge density, e.g., near zero for KCl and KBr, 5 for NaCl, 7 for LiCl, and 19 for $MgCl_2$. The approximate group additivity extends to polymers and aggregates: $N_S\rho_S \approx 8$ for the $-CH_2CH_2O-$ residue in poly(ethylene oxide) [85] and in alkyl oligo(ethylene oxide) micelles [86], and for the $-CH_2CH(COOH)-$ residue in polyacrylic acid [20]. In $RCOO^-M^+$ micelles, the exposed headgroups and counterions produce roughly the same $N_S\rho_S$ as the corresponding acetate or propionate salts [87]. Furthermore, $N_S\rho_S$ increases with decreasing temperature. For alcohols [84] and ethylene oxide fragments [85,86], the activation enthalpy is 10 – 20 kJ mol^{-1}. This effect, which is also seen for proteins [18,67], implies that τ_S has a higher activation enthalpy than τ_{bulk}.

On average, water at the surface of small globular proteins rotates about a factor 2 slower than expected from small-molecule data. From the water ^{17}O relaxation rate in aqueous solutions of mixtures of amino acids (in their low-pH form) at relative concentrations matching the amino acid content of α-lactalbumin and ribonuclease A [19], one obtains (after correction for the free NH_3^+ and COOH groups and with N_S estimated as for the proteins) $\tau_S/\tau_{bulk} = 2.3$, a factor 2 less than for proteins at low pH (Fig. 4). This difference cannot be explained by the protein having more polar and fewer nonpolar residues at the surface, since polar residues have a smaller effect on $N_S\rho_S$. Since evolution has adapted proteins to an aqueous environment, the geometry of protein surfaces should be correlated to some extent with the properties of water. One such correlation is a higher than random incidence of well-defined hydration sites where a water molecule can make two or more H-bonds to the protein. Such hydration sites will be rare for small molecules, but they account for one third of all crystallographically identified hydration sites on protein surfaces [4,62]. If such surface waters account for 10% of N_S, the difference in $N_S\rho_S$ between proteins and amino acid residues can be explained if $\tau_S/\tau_{bulk} \approx 20$ (i.e., $\tau_S \approx 40$ ps at 300 K) at these sites. This explanation is supported by recent low-temperature (243 and 263 K) ^{2}H and ^{17}O MRD measurements on emulsified BPTI and lysozyme solutions, yielding $\tau_S/\tau_{bulk} \approx 2$ for the surface waters that do not contribute to the dispersion [88].

The few MD simulation studies that have analysed water rotation at protein surfaces have reported surprisingly small dynamic retardations. For ubiquitin [76] and metmyoglobin [75], $\tau_S/\tau_{bulk} = 1.35$ (at 300 K) was obtained as an average over 550 water molecules at the protein surface. This is considerably less than the experimental value, suggesting that there is room for improvement of current force field models. Some MD studies of protein hydration have even found that surface waters rotate *faster* on average than bulk water. This anomalous result appears to be an artefact due to premature truncation of electrostatic interactions [89,90]. MD simulations of small molecules seem to agree better with experiment, e.g., $\tau_S/\tau_{bulk} = 1.6$ for the polar and 2.8 for the nonpolar parts of an alanine dipeptide [91].

3.3 Translational Motion

The residence times of water molecules in hydration sites at exposed protein surfaces are not *directly* accessible by current experimental techniques. For such sites, the rotational and translational diffusion of water should both be rate-limited by H-bond rearrangements and it can therefore be argued that the residence time (the time taken to diffuse ca. 3 Å) should be close to the *first-rank* rotational correlation time (the time taken to rotate through one radian), i.e., $\tau_W \approx 3\ \tau_S$ (where τ_S is the *second-rank* rotational correlation time). This is very nearly the case in bulk water, where $\tau_W = R^2/(6\,D) = 6.5$ ps [92] and $\tau_S = 1.7$ ps (at 298 K) [93], and this is also predicted by MD simulations for water hydrating simple ions [94] and proteins [76]. From ^{17}O MRD results on small globular proteins, we thus predict a residence time $\tau_W = 3 \times 5.5 \times 1.7 \approx 30$ ps (at 300 K) averaged over all surface sites.

Several experimental techniques, e.g., intermolecular spin relaxation and quasi-elastic neutron scattering, provide information related to the translational self-diffusion coefficient D_S of water molecules at protein surfaces. The interpretation of such experiments tends to be model-dependent, however (see below). Furthermore, neutron scattering has mainly been applied to hydrated protein powders [95-97], where water dynamics may differ greatly from that at protein surfaces in aqueous solution.

The *macroscopic* water diffusion coefficient in a protein solution can be measured *directly* by field gradient NMR or tracer techniques. Since diffusion is then probed on large length scales (μm and upwards), the measured diffusion coefficient is reduced below the bulk water value not only because a fraction of the water molecules are dynamically retarded at the protein surface but also because the protein molecules obstruct the diffusion paths of water molecules. Such experiments are usually interpreted in terms of an amount of "bound water" with $D_S = 0$ [98,99]. Using more realistic models [100], one finds that it is impossible to separate the range and extent of the dynamic perturbation (N_S and D_S) [86]. The finding that the water self-diffusion coefficient in a myoglobin single crystal (with 40% water) is only a factor 2 lower than in bulk water clearly rules out a large reduction of D_S [101]. In oriented amphiphilic liquid crystals, D_S (like τ_S) can be measured more directly; D_{bulk}/D_S is typically 2 – 3 for simple surfactants [102] and 4 – 6 for phospholipids ($N_S \approx 20$) [74,103]. (The larger effect in the latter case may be attributed to a molecular obstruction effect, where water molecules are forced to diffuse around the large and less mobile phosphocholine headgroups.)

Information about the translational motion of water at protein surfaces can be derived from the rate of intermolecular spin relaxation due to the dipole-dipole coupling between a water proton and a protein-bound electron spin (in a covalently attached nitroxide spin label) or an intrinsic protein proton. Relaxation is induced by fluctuations in the length and

orientation of the spin–spin vector due mainly to translation diffusion of water molecules in the neighbourhood of the protein-bound spin. The theoretical modelling of this process is notoriously model-dependent. The spin label approach has been applied to serum albumin [104] and hemoglobin [105], yielding $D_{\mathrm{bulk}}/D_S \approx 5$. The theory used to extract this result involves, as a cutoff for the (assumed uniform) water proton–nitroxide spin pair distribution function, a distance of closest approach. This parameter tends to absorb the effects of various approximations in the model and therefore cannot be simply related to molecular dimensions [104]. The close agreement with the MRD result $\tau_S/\tau_{\mathrm{bulk}} = 5.5$ is striking, but probably somewhat fortuitous. Since the dynamic perturbation is local (see above), it can be argued that the spin label experiment measures the dynamic perturbation induced by the *nitroxide group* rather than that induced by the *protein*.

This problem is avoided in NOE method, where water diffusion can in principle be probed at a variety of surface sites via the cross-relaxation between water and non-labile protein protons that are more than 5 Å distant from any rapidly exchanging proton [13]. On the other hand, since the NOE experiment is usually a *single-frequency* measurement, it is even more model-dependent than the spin label experiment (when this is performed by ^{1}H MRD [104]). Although the dipolar coupling strength falls off with the H – H separation R as $1/R^6$, the number of water protons at a given separation increases as R^2 and so does the time scale of angular modulation of the dipole coupling. The contributions to the cross-relaxation rate from different spatial regions are thus weighted as $1/R^2$ and, when summed up, render the cross-relaxation rate (at low frequencies) dependent on $1/b$ (with b the distance of closest approach) rather than on $1/b^6$ (as for an internal water molecule) [106]. Since the dynamic perturbation is local, the diffusion coefficient varies within the spatial region probed by the cross-relaxation rate. The NOE method usually provides only a single number, the ratio of the NOESY and ROESY cross-peak intensities, from which to estimate a residence time. This is clearly a highly model-dependent exercise. Even the definition of τ_W is problematic for surface sites (see below). It is sometimes claimed that the zero-crossing of the NOESY cross-peak intensity can be used as an indicator of whether τ_W is shorter or longer than 300 ps (at 600 MHz ^{1}H frequency), but this is only true for internal water molecules (without internal motion). Despite these problems, the absence of strong water-protein cross-peaks from surface sites [44] is consistent with the modest dynamic retardation found by MRD and by direct diffusion measurements. When cross-peaks with surface waters are seen, they are an order of magnitude weaker than those from internal waters [107]. Due to the weak intensity, they are susceptible to a variety of instrumental imperfections and some of the reported cross-peaks may even be artefacts [108].

In the past few years, about a dozen MD simulation studies have addressed the problem of water residence times at protein surfaces. While supporting the picture of a short-ranged dynamic perturbation of modest extent, the simulation results do not agree quantitatively with experiment (or even among themselves). Simulated residence times are typically in the range 2 – 10 ps when averaged over the first hydration layer [23,63,75,76,109-111], considerably less than the 30 ps average estimated from MRD data. The simulation results depend sensitively on how τ_W is evaluated; order of magnitude variations may result from changes in the sampling interval and the spatial cutoff bounding the hydration site. Systematic studies of these aspects (and the dependence on force field) are needed. Furthermore, there is qualitative disagreement among the simulations about the relative importance of charged, polar, and nonpolar residues in slowing down water dynamics. Conformational context and surface topography may be as important as chemical properties

for the structure and dynamics of protein hydration [64,112].

The principal mission for MD simulations should not be to come up with numbers that match experiment, but to provide insight into mechanistic details that can only be inferred indirectly from experiment. MD studies focusing on the *mechanism* of water exchange from surface sites with different geometry and H-bonding potential might be revealing [30]. Very large τ_W variations can be expected in going from wide and shallow to narrow and deep surface pockets. The mobility of bulk water is enhanced by a high (local) density. MD simulations attribute this counterintuitive trend to the effect of a fifth water molecule in the first coordination shell, "catalysing" the rearrangement of the H-bond network [113]. Such considerations are relevant also for water at protein surfaces and may explain why fewer H-bonds can lead to slower dynamics.

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