A Model for Water Motion in Crystals of Lysozyme Based on an Incoherent Quasielastic Neutron-Scattering Study

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ABSTRACT This paper reports an incoherent quasielastic neutron scattering study of the single particle, diffusive motions of water molecules surrounding a globular protein, the hen egg-white lysozyme. For the first time such an analysis has been done on protein crystals. It can thus be directly related and compared with a recent structural study of the same sample. The measurement temperature ranged from 100 to 300 K, but focus was on the room temperature analysis. The very good agreement between the structural and dynamical studies suggested a model for the dynamics of water in triclinic crystals of lysozyme in the time range ~330 ps and at 300 K. Herein, the dynamics of all water molecules is affected by the presence of the protein, and the water molecules can be divided into two populations. The first mainly corresponds to the first hydration shell, in which water molecules reorient themselves fivefold to 10-fold slower than in bulk solvent, and diffuse by jumps from hydration site to hydration site. The long-range diffusion coefficient is five to sixfold less than for bulk solvent. The second group corresponds to water molecules further away from the surface of the protein, in a second incomplete hydration layer, confined between hydrated macromolecules. Within the time scale probed they undergo a translational diffusion with a self-diffusion coefficient reduced ~50-fold compared with bulk solvent. As protein crystals have a highly crowded arrangement close to the packing of macromolecules in cells, our conclusion can be discussed with respect to solvent behavior in intracellular media: as the mobility is highest next to the surface, it suggests that under some crowding conditions, a two-dimensional motion for the transport of metabolites can be dominant.

INTRODUCTION

Water represents ~70% w/w of the cell (Mentre, 1995) and has a major role in the function of the cell. It is not only involved in the various mechanisms of transport within the cell but also plays a role on the proteins themselves: water is implicated for the folding, unfolding, and stabilization of the three-dimensional structure (Cooper, 2000), as well as in the internal dynamics and function of proteins (Ferrand et al., 1993; Careri, 1998; Nagendra et al., 1998). Moreover, it can mediate the interactions of proteins with other components of the cell (Pocker, 2000; Chung et al., 1998). However, proteins also act on water, and the water next to proteins behaves very differently from bulk water (Bone and Pethig, 1985; Halle, 1999). For this reason it is generally termed interfacial water, and a good knowledge of its properties is necessary to understand various biological mechanism related to, e.g., the proton pathways along/within proteins (Royant et al., 2000), and to undertake molecular dynamics, drug design, and protein engineering (Lounnas, 1999; Ehtezazi et al., 2000; Jaenicke, 2000).

The organization of interfacial water has therefore been studied using a multitude of methods, including calorimetry, thermodynamical measurements (for review, see Rupley and Careri, 1991), nuclear magnetic resonance (NMR) analysis (Otting et al., 1991; Denisov and Halle, 1995), x-ray and neutron small-angle scattering (Svergun et al., 1998), high resolution x-ray crystallography (Burling et al., 1996; Podjarny et al., 1997; Teeter et al., 1993; Badger and Caspar, 1991), and high resolution neutron crystallography (Shu et al., 2000; Habash et al., 2000; Niimura et al., 1997; McDowell and Kossiakoff, 1995; Wlodawer et al., 1989; Mason et al., 1984). All these studies showed that water molecules on the protein surface mainly occupy well-defined hydration sites (time and space averaged water molecule position), giving stability to the protein structure and mediating interactions with other components of the cell.

Nonetheless, there are still open questions concerning the quantity of solvent whose structure is affected by the presence of the protein. Most x-ray crystallographic studies have suggested that only the first solvent layer, called first hydration shell, is affected, whereas calorimetric studies suggest that more than one layer is affected (Mentre, 1995; Saenger, 1987). The recent work on the structure of the solvent in triclinic crystals of hen-egg white lysozyme, by neutron diffraction and at room temperature (Bon et al., 1999), threw new light on this question. In this form, crystals of lysozyme contain ~30% (v/v) of solvent, which means one hydration shell, plus a few water molecules in an incomplete second hydration layer. In this study, the solvent was deuterated, which implies that all three atoms of the water molecule were theoretically equally visible. Two-hundred forty-four hydration sites of the ~310 theoretically present in the unit cell were observed, and the water molecules could be split into three populations. The first population (115 hydration sites) corresponds to complete hy-

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dration sites, where the oxygen and two deuterium sites could be localized in the neutron density map. This means that water molecules passing on these sites mainly adopt the same orientation, due to the local environment. The second population (129 hydration sites) corresponds to "partly disordered hydration sites" where one could only observe the oxygen site. In such case, it means that the deuterium sites (and as a consequence the hydrogen bonding network) are either dynamically or statically disordered (a static crystallographic disorder means that the deuterium localization is not the same over crystal unit cells, but this is not due to a dynamic disorder). The third population corresponds to water molecules that could not be visualized in the density maps: in some parts of the solvent, which are in the second incomplete hydration layer and correspond to "holes" between the hydrated molecules of lysozyme, either no density, elongated, or featureless density could be observed. This neutron crystallographic study was then compared with an x-ray study at atomic resolution (Walsh et al., 1998). It was observed that the hydration sites observed from x-ray crystallography, at room temperature, mainly correspond to the more ordered (from the Debye-Waller factor point of view) complete neutron hydration sites plus a few incomplete neutron sites corresponding to the more ordered incomplete neutron hydration sites. Some other less ordered hydration sites could be retrieved in the x-ray structure at 100 K. As x-ray diffraction techniques mainly see the oxygen of the water molecule, even at atomic resolution, it seems more difficult to see a single, smeared-out oxygen than a distribution of three connected and equally visible atoms with neutrons. Information about some of the more disordered hydration sites can therefore sometimes be retrieved using neutron diffraction, and it was concluded from the study of triclinic lysozyme that the solvent structure in the crystal is affected beyond the first hydration layer.

Moreover, due to the large surface to volume ratio of proteins and the high density within the cell, there can on average only be two to three hydration layers between proteins (Mentre, 1995). It is therefore obvious to raise the question of how the solvent can be both structured enough to maintain the protein structure and fluid enough to allow the rapid transport of metabolites and protons within the cell, and several dynamical studies have therefore already been undertaken to answer this question. Among those, NMR studies, mainly on protein solutions (Denisov and Halle, 1995; Otting et al., 1991), gave information about the average residence time and reorientation time on the protein surface. Likewise, incoherent quasielastic neutron scattering experiments probe single particle diffusive motions of water under 10^{-12} to 10^{-10} s and on an atomic scale. They have been undertaken at various temperatures on bulk water (Teixeira et al., 1985), on protein powders rehydrated at various level (Bellissent-Funel et al., 1996; Settles and Doster, 1996), on protein in solution (Perez et al., 1999), on purple membrane (Fitter et al., 1996), and on various media, which were taken as models of the hydrophobic or the hydrophilic interface (Bellissent-Funel et al., 1993, 1996). These studies permitted to show that water at the surface of proteins, at room temperature, have a long-range diffusion coefficient close to the long-range diffusion coefficient of bulk water but with a longer residence time, much like the behavior of supercooled water at 273 K (Bellissent-Funel et al., 1996).

Molecular dynamics simulations also give information about the time scale (faster than 1 ns) and the geometric scale at atomic resolution of the diffusive motions of individual water molecules and is thus an important source of information about the atomic mechanism of water motion. Several studies have therefore been undertaken on protein solution or protein crystals (Alary et al., 1993; Lounnas and Pettitt, 1994; Makarov et al., 1998), sometimes leading, although, to contradictory results.

However, the various investigations of water have not been done on identical samples, which makes it difficult to compare the structural and dynamical results obtained. Here we report an analysis by incoherent quasielastic neutron scattering of the water dynamics in triclinic crystals of hen egg-white lysozyme, which have already been extensively studied structurally by crystallography (Bon et al., 1999). This is the first time that the technique is used on protein crystals, and although some care is needed in the preparation of the sample, the advantage is that the solvent layers around the proteins are reasonably well defined. Moreover, protein crystals are probably the best representation of crowded intracellular solutions available for measurements near atomic resolution (Srere, 1981). As the structural and dynamical results were in very good agreement, this allowed us to propose a model of the dynamics of water in a crowded environment at atomic resolution, which we believe can be of use beyond the specific case of triclinic lysozyme.

MATERIALS AND METHODS

Incoherent quasielastic neutron scattering takes advantage of the fact that cold neutrons have an energy comparable with the energy barriers that hinder molecular reorientation and diffusion and a wavelength comparable with the interatomic distances. It can consequently supply information about the time scale of molecular motions (in the range of 10^{-12} to 10^{-10} s) as well as a model for the geometry of these motions on an atomic scale. Thanks to the large incoherent cross section of hydrogen, the scattered signal of a biological sample is dominated by the incoherent contribution of hydrogen atoms, and this allows particularly to study the single-particle diffusive motions of water molecules by following the single-particle diffusive motions of the protons involved.

Sample preparation

Crystals used were all $\sim$0.5 mm^3 in volume and were obtained by batch technique. The material used was hen egg-white lysozyme from Boehringer (Mannheim, Germany), three times recrystallized. The protein as well as the crystallisation solution were hydrogenated. Equal amounts of a 1.0%

solution of lysozyme and a 2.8% solution of $NaNO_3$ in 50 mM acetate buffer (pH 4.7) were mixed and left at room temperature in large, closed plastic vials for the crystals to grow over a period of several years.

Each sample was made from ~1.5 g of nonoriented protein crystals. To discriminate between the protein and the water dynamics, two types of samples were prepared, without and with deuterium. The "all-hydrogenated" sample, in which all hydrogens on the protein and within the solvent are kept hydrogenated, gives spectra that hold information about both the protein and solvent dynamics. The "solvent-deuterated" sample, in which protons of the solvent (and the labile protons of the protein) were exchanged by deuterium, will to a very large extend only supply information about the protein dynamics.

The "solvent-deuterated" samples were prepared using vapor diffusion exchange. The triclinic lysozyme crystals were placed in a small cup with only a few drops of mother liquor, after which they were equilibrated for 3 weeks in a confined atmosphere with a large excess of deuterated mother liquor.

Special care was taken to eliminate the water on the surface of the crystals, as this water will have a dynamic behavior different from the molecules inside the crystals. This surface water was removed by equilibrating crystals against a concentrated salt solution as suggested by Salunke et al. (1985). $NaNO_3$ was retained as agent as this salt is part of the crystallization solution. A week before the experiment, lysozyme crystals were placed in the sample holder with minimal mother liquor and equilibrated against a 5% NaNO3 solution (deuterated for the "solvent-deuterated" sample). Careful visual inspection of the samples showed that the crystal surfaces were dry but shiny, and crystal diffraction techniques were used to ensure that there was no crystal damage and that the cell parameters did not change.

Data collection

Data collection was done at two different resolutions using the two time-of-flight spectrometers of ILL (Grenoble). On the IN6 spectrometer, the incident wavelength was chosen to be 5.12 Å, giving a Gaussian resolution function with a Q-dependent full width at half maximum (FWHM) between 78 and 114 μeV, and a scattering vector Q ($4\pi \sin(\Theta)/\lambda$, in which 2Θ is the scattering angle and λ the neutron wavelength) comprised between 0.43 and 1.93 $Å^{-1}$. The IN5 spectrometer was used to probe slower motions; the incident wavelength was 9 Å, giving a triangular resolution function with a FWHM of 21 μeV, which is approximatively Q-independent, and a scattering vector Q comprised between 1.14 and 1.44 $Å^{-1}$.

On each spectrometer the experiment started by the recording of a set of spectra on vanadium at 285 K, covering all the experimental resolutions. This measurement took 2 h 30 min. with a vanadium disk of 1.2-mm thickness and 50-mm diameter. Vanadium has a mainly incoherent scattering pattern, and the spectra were used to obtain the resolution function for the spectrometers. Then the recording of the spectrum of an empty cell at room temperature followed, which took 2 h.

The sets of spectra from the "all-hydrogenated" and "solvent-deuterated" samples were recorded for 4 h on IN6 and for 6 h on IN5, starting at room temperature and cooling down to 180 K by steps of 10 or 20 K. In addition a set of spectra was measured at 100 K. The transmission was evaluated to be more than 80%. All data were collected with aluminium flat-plate sample holder disks of 1.5-mm thickness and 50-mm diameter, oriented at 135° relative to the incident beam.

Data reduction

Data reduction was done with the INX software developed at ILL (Rieutord, 1990), which allows different signal corrections: calibration of the different detectors using vanadium spectra; removal of the scattering contribution of the empty cell; transmission corrections (Rieutord, 1990); transformation of the time-of-flight spectra into energy spectra; and grouping of the 89 and 90 spectra of each set recorded on IN6 and IN5, respectively, into 10 and 12 spectra to improve the quality of the statistics.

Before grouping, it was checked that the cristallinity of the sample did not influence the spectra. In a crystalline sample a coherent signal resulting from interferences is diffracted into Bragg peaks. This signal, in the case of a single crystal, is diffracted at discrete Q-values for particular directions. As the present sample is composed of misoriented crystals, this signal is diffracted at discrete Q values for all directions. This diffracted intensity could superimpose on the elastic peak of the spectra recorded, and if this extra intensity is nonnegligeable compared with the incoherent elastic intensity, it would erroneously be considered as incoherent signal. As Bragg peaks appear for discrete values of Q, such occurrence would show up by plotting the elastic peak intensity versus Q. In the present study, the plot indicated a smooth, regular decrease of the elastic peak versus Q, as expected from thermal fluctuations.

No correction for multiple scattering was undertaken, as the transmission was evaluated to be more than 80%. Finally it should be noted that although the "solvent-deuterated" spectra to a good approximation reflect the diffusive motions within the protein, a number of labile protons inside the protein are also exchanged by deuterium. To study the solvent dynamics, it was therefore decided to use directly the "all-hydrogenated" spectra rather than to use difference spectra and to take the protein contribution into account in the analysis. Indeed, the derivation of "all-hydrogenated" minus "solvent-deuterated" spectra thereby subtracting the signal from the protein would have induced considerable errors due the difficulty of estimating the relative scattering weights of the two samples.

Model fitting: the general procedure

The "solvent-deuterated" spectra were first compared with the "all-hydrogenated" spectra to probe the relative contributions of the protein and the solvent to the "all-hydrogenated" spectra. Following this the analysis of the data was done in two stages, and at each stage (because of the E-range investigated) the vibrational contributions were approximated by the global mean square displacement of atoms $\langle u^2 \rangle$ plus a flat background $\beta(Q)$ (Bee, 1988).

In the first stage the quasielastic part of the spectra for the two instruments were examined in more details. At first, a "phenomenological study" was done both on "solvent-deuterated" and "all-hydrogenated spectra". For the various data sets the procedure was the following: each spectrum was fitted individually to a number of functions using the Profit software developed at ILL (Ghosh, 1995) to identify the different kinds of motion encountered, as well as their main characteristics. The group of protons, which appear as static within the spectrometer resolution, gives rise to an elastic contribution with the shape of the instrumental function. In addition, for each group of mobile protons with a closely resembling dynamic behavior, a "mean" Lorentzian will occur in the spectra, centered around the zero energy transfer of neutrons ($\Delta E = 0$). The characteristics of each Lorentzian are the half width at half maximum (HWHM) and the intensity (I_1). Moreover, if the geometry of the motion of these groups of mobile protons is confined, a purely elastic contribution will superimpose on the elastic peak. The elastic and purely elastic contributions cannot a priori be distinguished from the spectra, and the total elastic intensity, I_d, thus reflects the sum of these two contributions.

First the IN6 spectra were studied, as they probe a shorter time scale than the IN5 spectra. On each set of spectra ("solvent-deuterated" and "all-hydrogenated"), one single Lorentzian was observed, characterizing a "rapid motion" called so because the FWHM of the resolution function is comprised between 78 and 114 μeV. On IN6 spectrometer it is possible to identify Lorentzians larger by 10% than the resolution function (due to the high incident flux) and this corresponds to a time scale probed of ~50 ps.

The function used for analysis was the following:

$$I(Q,\omega) = \left(I_{d6}(Q)R_6(Q,\omega) \otimes \delta(\omega) + I_{l1}(Q)\frac{1}{\pi}\left(\frac{\mathrm{HWHM}_1(Q)}{\mathrm{HWHM}_1(Q)^2+\omega^2}\right) \otimes R_6(Q,\omega)\right) e^{-\langle u^2\rangle Q^2/3} + \beta(Q) \quad (1)$$

in which the parameter was fixed or known: $R_6(Q, \omega)$ is the IN6 resolution function, obtained from the vanadium spectra. The parameters allowed to vary were: $I_{d6}(Q)$ is the intensity of the elastic plus purely elastic contribution within the time scale accessible on IN6. $I_{l1}(Q)$ and $\mathrm{HWHM}_1(Q)$ is the intensity and HWHM of the Lorentzian 1. $\langle u^2\rangle$ is the global mean square displacement of atoms. $\beta(Q)$ is the flat background in the quasielastic part resulting from inelastic scattering.

From these parameters, the following experimental elastic incoherent structure factor (EISF) was derived, which describes the geometry of atomic confinement:

$$\mathrm{EISF}_{\mathrm{exp},6}(Q) = \frac{I_{d6}(Q)}{I_{d6}(Q) + I_{l1}(Q)} \quad (2)$$

Next, the IN5 spectra were studied, and in this case the FWHM of the resolution function was ~21 μeV, at 9 Å incident wavelength, independent of Q. For this instrument, Lorentzians ~20% larger than the resolution function can be distinguished, and this corresponds to a time scale of ~330 ps. The time scale probed was thus larger than on IN6 spectra, and on each set of spectra ("solvent-deuterated" and "all-hydrogenated"), in addition to the corresponding Lorentzian 1, a second motion, termed "slow motion," resulting in a second Lorentzian (Lorentzian 2) could be retrieved. Based on the relative contribution of the protein and protein + solvent to the quasielastic intensity, and on the "phenomenological study" of the "solvent-deuterated" spectra, the two motions that we could detect on "all-hydrogenated" spectra were attributed to the solvent. Then the previous structural work (Bon et al., 1999) allowed us to make the assumption that the two motions observed could be attributed to two distinct populations of water molecules. The first population would correspond to hydration sites seen completely or incompletely in the structural study, and the second would be for the water molecules not seen at all. Thus, the function used for analysis of all-hydrogenated spectra was the following:

$$I(Q,\omega) = \left(I_{d5}(Q)R_5(Q,\omega) \otimes \delta(\omega) + I_{l1}(Q)\frac{1}{\pi}\left(\frac{\mathrm{HWHM}_1(Q)}{\mathrm{HWHM}_1(Q)^2+\omega^2}\right) \otimes R_5(Q,\omega) + I_{l2}(Q)\frac{1}{\pi}\left(\frac{\mathrm{HWHM}_2(Q)}{\mathrm{HWHM}_2(Q)^2+\omega^2}\right) \otimes R_5(Q,\omega)\right) e^{-\langle u^2\rangle Q^2/3} + \beta(Q) \quad (3)$$

in which the parameters fixed or known were: $R_5(Q, \omega)$ is the IN5 resolution function, obtained from the vanadium spectra. $\mathrm{HWHM}_1(Q)$ is fixed at the values obtained from IN6 spectra. Motion 1 and 2 do not occur on the same time scale, which allows to fix HWHM_1, and let HWHM_2 vary. The parameters allowed to vary were: $I_{l1}(Q)$ and $I_{l2}(Q)$ are the intensities of Lorentzian 1 and 2 in the IN5 spectra. $\mathrm{HWHM}_2(Q)$ is the HWHM of Lorentzian 2. $I_{d5}(Q)$ is the intensity of the elastic plus purely elastic contributions within IN5 resolution. $\langle u^2\rangle$ is the global mean square displacement of atoms. $\beta(Q)$ is the flat background resulting from the inelastic part.

In the following, indices 1 and 2 will refer to the "all-hydrogenated spectra," whereas indices 1,prot and 2,prot will refer to the "solvent-deuterated spectra." The variation of HWHM_1 and HWHM_2 with Q allowed us the assignment of two models corresponding to the two motions. Motion 1 can be regarded as a long-range diffusion within an impermeable sphere and with localized jumps. This model has been described by Volino and Dianoux (1980). Motion 2 has the characteristics of a long-range translation (Bee, 1988). From $\mathrm{HWHM}_1(Q)$, values of the radius of confinement, a, the root mean square distance of jump, and the long-range diffusion coefficient were extracted. From $\mathrm{HWHM}_2(Q)$, the long-range diffusion coefficient was extracted. On IN5 "solvent-deuterated spectra," the main protein contribution (Lorentzian 1,prot) results in a small broadening of the elastic peak, constant throughout the whole Q-range, and it was represented as a diffusional motion on a sphere. The evolution of its HWHM and EISF with Q allowed us to attribute to this population of protons a model of diffusion on a sphere of radius $b = 0, 8 \pm 0, 2$ Å. This may reflect the rotational motion of methyl groups, which have been shown to be the major part of lysozyme internal motions on the picosecond timescale (Shirley and Bryant, 1982). Such a model is a rough approximation of the internal protein dynamics but is in agreement with the fact that "solvent-deuterated" spectra reflect the dynamics of the core of the protein (labile proton are exchanged for deuterium), and that some diffusive motions within the protein could be prevented in the crystal. The protein internal dynamics has been studied more precisely by other authors (Fitter et al., 1996; Settles and Doster, 1996), but the focus here was on the solvent dynamics.

In the second stage the analysis was pushed further using the "all-hydrogenated spectra" obtained at room temperature on IN5 to study both solvent motions simultaneously. At this temperature it is possible to directly compare the results with the previous structural study, and this is crucial for a more global description of the solvent dynamics. Mathematical developments of the models for the two motions attributed to the solvent were used. They allowed for a more precise description of the geometries and time scales of the two motions, and in addition the proportion of protons undergoing each type of motion was derived. The "all-hydrogenated spectra" were well reproduced taking into account the contribution of the two solvent motions. Nonetheless, at this stage the small contribution of the protein protons to the quasielastic intensity was also represented, although very roughly: only the main contribution of the protein to the quasielastic intensity (Lorentzian 1) was taken into account. A radius of 0.8 ± 0.2 had been extracted from the "solvent-deuterated spectra"; it seemed reasonable to fix the radius at 1 Å for this fit. This allowed us to check that the protein contribution to the "all-hydrogenated spectra" was negligeable compared with the solvent contribution. The quality of the fit improved a bit, without varying the parameters describing solvent motions more than the incertainty previously estimated. All calculations were done with the software Agathe developed at ILL (Bee, 1996), and the scattering law used for analysis was the following:

$$S(Q,\omega) = (S_{el}(Q,\omega) + S_{qel}(Q,\omega))e^{-\langle u^2\rangle Q^2/3} + \beta(Q) \quad (4)$$

$$S_{el}(Q,\omega) = (p_1 + (1-p_1)(p_2p_3A_0^{sph}(Q) + (1-p_2)A_0^{prot}(Q)))\delta(\omega) \otimes R_5(Q,\omega). \quad (5)$$

$$S_{qel}(Q,\omega) = (1-p_1)(p_2(p_3L^{sph}(Q,\omega) + (1-p_3)L^{trans}(Q,\omega))) \otimes R_5(Q,\omega) + (1-p_1)(1-p_2)(1-A_0^{prot})L^{prot}(Q,\omega) \otimes R_5(Q,\omega) \quad (6)$$

in which $R_5(Q, \omega)$ is the IN5 resolution function, obtained from the vanadium spectra. $A_0^{prot}(Q) = (j_0(Qb))^2$ is the purely elastic component resulting from the protein motion; $j_0(x) = \sin x/x$ and $b = 1$ Å. $A_0^{sph}(Q) = (3j_1(Qa)/(Qa))^2$ is the purely elastic component resulting from the motion confined within an impermeable sphere. j_1 is the 1-order Bessel function, and a is the radius of confinement of the motion. $L^{prot}(Q, \omega)$ is a normalized Lorentzian. $L^{sph}(Q, \omega)$ is the Lorentzian corresponding to the model of diffusion within an impermeable sphere:

$$L^{sph}(Q, \omega) = 1/\pi \times \sum_{(l;n)\neq(0;0)} (2l + 1)A_n^l(Q) \times \left(\left((x_n^l)^2 D^{sph}/a^2\right)/\left(\left((x_n^l)^2 D^{sph}/a^2\right)^2 + \omega^2\right)\right) \quad (7)$$

The dimensionless numbers x_n^l and $A_n^l(Q)$ values were calculated by Volino and Dianoux (1980). Taking into account the a value obtained from previous fits, only the first nine Lorentzians were included. It should be noted that in the calculation on the IN5 spectra, the local jump motion was not taken into account, as it does not affect the IN5 spectra. Moreover, water reorientation was not treated with an explicit model of rotation, as had been done in the bulk water study (Teixeira et al., 1985). Indeed, the reorientation of water molecules on the protein surface is highly anisotropic and dictated by the local hydrogen bond network. In our model, the water reorientation is thus reflected by the proton local jump dynamics. $L^{trans}(Q, \omega)$ is the Lorentzian resulting from the long-range translational motion. Its HWHM follows the law $HWHM(Q) = D^{trans}Q^2$.

Six parameters were allowed to vary. p_1 is the proportion of total protons (protein + solvent) seen as static within spectrometer resolution. p_2 is the proportion of the $(1 - p_1)$ mobile protons having a motion attributed to the solvent. p_3 is the proportion of the $(1 - p_1)p_2$ mobile solvent protons having a diffusive motion confined within a sphere. a is the radius of confinement for the diffusive motion confined within an impermeable sphere. D^{trans} is the diffusion coefficient of the translational motion. D^{sph} is the diffusion coefficient of the motion confined within a sphere. First, all the spectra were fitted individually. Then the values of these parameters were set to the mean value obtained for each Q, and all spectra were fitted as a whole. To quantify the fit uncertainty, spectra were then fitted as a whole starting from distinct values obtained for each Q.

FIRST NUMERICAL RESULTS AND DISCUSSION

To start with, for each Q-value, the "all-hydrogenated" spectrum was compared with the spectrum from the "solvent-deuterated" sample, which reflects motions of the nonexchangeable protons of the protein. On Fig. 1 is shown the spectrum obtained at 300 K, $Q = 1.2$ Å^{-1} on IN5 for the "all-hydrogenated" sample as well as the spectrum obtained under the same conditions for the "solvent-deuterated" sample. The two spectra are normalized to the maximum, and the resolution function of IN5 is represented in full line. This shows that to a first approximation the contribution of the protein to the "all-hydrogenated" quasielastic intensity can be neglected when compared with the contribution from the solvent.

Then followed the first analysis of the elastic peak and the quasielastic broadening. The quasielastic broadening could be observed until 180 K. Below this temperature it seems that all motions are freezed both on "all-hydrogenated" and "solvent-deuterated" spectra. Indeed, the broadening was very small already at 260 K, and Lorentzian parameters could not be fitted below this temperature. Above 260 K, two Lorentzians could be identified in the "all-hydrogenated spectra," and two other Lorentzians could be identified in the "solvent-deuterated spectra." The evolution versus Q of $HWHM_{1,prot}$ and $HWHM_{2,prot}$ was distinct from the evolution of $HWHM_1$ and $HWHM_2$ (Fig. 2). This gave us another confidence that the two motions observed on "all-hydrogenated" spectra correspond to two populations of protons within the solvent. The evolution versus Q of $HWHM_1$ and $HWHM_2$, for temperatures above 260 K is given on Fig. 2. The evolution of $EISF_{exp,6}$ versus Q is

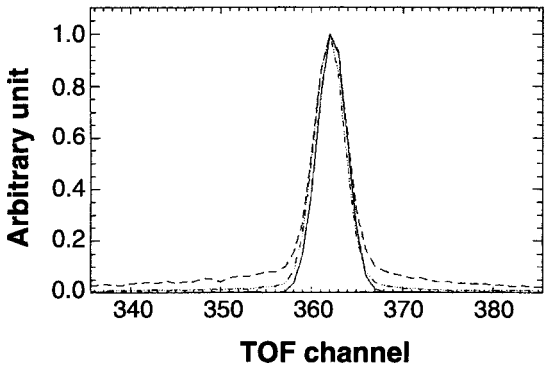

FIGURE 1 Superposition of quasielastic broadening of the IN5 spectra obtained at 300 K, $Q = 1.2$ Å^{-1}, on the "all-hydrogenated" sample (*broken line*) and the "solvent-deuterated" sample (*dashed-dotted line*). The spectrometer resolution function is shown in full line.

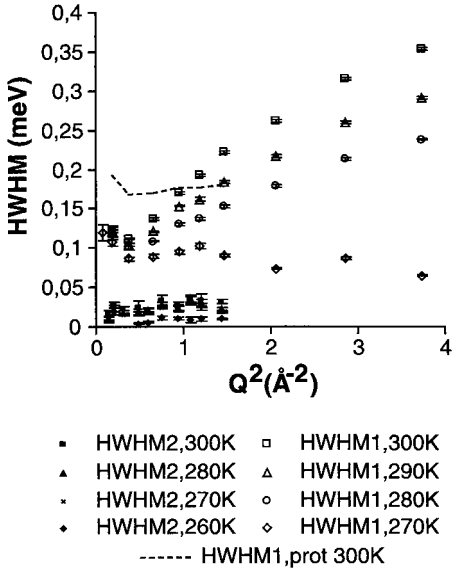

FIGURE 2 Evolution of experimental $HWHM_1$ and $HWHM_2$ versus Q^2, for various temperatures. Error bars are not given, but from 300 to 270 K, they are equal or less than the size of the marks. The evolution of experimental $HWHM_{1,prot}$ versus Q^2, at 300 K, has been added in broken line for comparison.

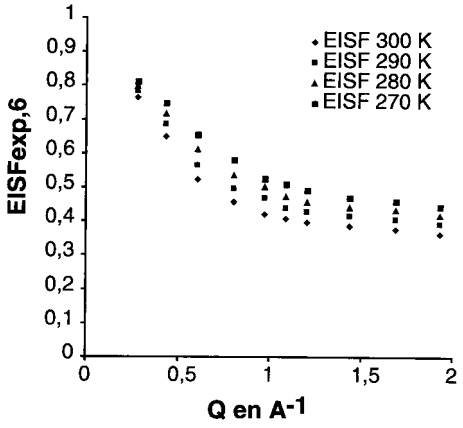

FIGURE 3 Evolution of $EISF_{exp,6}$ versus Q for various temperatures.

given on Fig. 3. The motions corresponding to the two Lorentzians are described below.

Rapid motion

$HWHM_1$ exhibits a plateau for low Q and follows a quasi-Q^2 law for intermediate values. This is characteristic of a diffusive motion confined within an impermeable sphere (Volino and Dianoux, 1980). From this plateau value at low Q, we find that the radius of confinement is of the order of 3 Å. Moreover, it exhibits a plateau for the higher Q-value, which is typical for a local jump diffusion. In this model of jump diffusion, the plateau value $HWHM_{max}$ is related to the mean residence time before jumping, τ_0, by the relation $HWHM_{max} = h/\tau_0$, in which h is Planck's constant.

Table 1 gives the values deduced from the evolution of HWHM1 versus Q, in which 1 is the length of the proton jump. Values obtained for bulk solvent (Teixeira et al., 1985) have been added in brackets for comparison (the bulk solvent τ_0 and $\langle l^2\rangle^{1/2}$ values were actually measured at 293, 285, 278, and 268 K, respectively, whereas the bulk solvent D value is given at 298 and 273 K, respectively).

Although these values reflect a rather slow dynamics, they are in good agreement with those obtained from neutron scattering studies of water around various proteins or model hydrated with approximately one layer of solvent: C-phycocyanin (amorphous protein rehydrated): $D^{sph} = 12\ 10^{-6}$ cm^2/s, $\tau_0 = 6.6$ ps, and $a = 4.3$ Å at 298 K; $D^{sph} = 7.6\ 10^{-6}$ cm^2/s, $\tau_0 = 8.2$ ps, and $a = 4$ Å at 273 K (Bellissent-Funel et al., 1996); purple membrane (bacteriorhodopsin trimers in a lipid bilayer matrix) at 300 K: long-range diffusive coefficient along the bilayer: $4.4\ 10^{-6}$ cm^2/s, rotational diffusive rate reduced sixfold compared with bulk (Lechner et al., 1994a,b); and vycor glass: a model to study the behavior of solvent in a hydrophilic confined space, amorphous material rehydrated: $D^{sph} = 9.2\ 10^{-6}$ cm^2/s, $\tau_0 = 15$ ps, and $a = 4$ Å at 298 K, $D^{sph} = 3.8\ 10^{-6}$ cm^2/s, $\tau_0 = 20$ ps, and $a = 3$ Å at 268 K (Bellissent-Funel et al., 1996).

TABLE 1 Parameters describing the rapid motion of solvent, and comparison with the parameters describing the bulk solvent dynamics

	D^{sph} (0.10^{-6} cm^2/s)	τ_0 (ps)	$\langle l^2\rangle^{1/2}$ (Å)
300 K	2.5 (23)	11	1.3 (1.29)
290 K	1.7	13	1.2 (1.25)
280 K	1.3	16	1.1 (1.32)
270 K	0.1 (11)	20	0.3 (1.54)

In contrast, the long-range diffusion coefficient around myoglobin powder, rehydrated with approximately one hydration shell, has been found between 20 and 100 times slower than in bulk (Settles and Doster, 1996). Settles and Doster propose that the differences between their results and results obtained for C-phycocyanin and purple membrane arise from approximations used in the latter studies; one of them is the decoupling approximation that allows the separation of translation from rotation for hydration water. Nonetheless, such an approximation has not been used in our study, as the rotation has been taken into account in the local proton jump diffusion (see model fitting for more details).

The average proton jump length (~1.3 Å) corresponds approximately to the distance between hydrogen atoms in a water molecule or the distance between a hydrogen atom site and one of the lone pair sites. Moreover, the average proton residence time before jump is ~11 ps. It thus seems reasonable to suggest that an important part of the local jump diffusion comes from reorientations of the water molecules. In the study of solvent along the purple membrane, an average jump length of 4.1 Å was observed (Fitter et al., 1996). The difference with the value we obtained should be related with the difference between the model of interpretation used: in Fitter's study $\langle l^2\rangle^{1/2}$ represents the jump of water molecules between hydration sites.

Within the time scale accessible on the IN5 spectrometer, protons are confined to a sphere with a radius of ~3 Å. Consequently (as the average jump length is only around 1.3 Å) the proton jumps do not only correspond to the 180° flipping of the water molecule or the other reorientations invoked above, but the water molecules also perform global jumps from site to site. These jumps can for example be reorientations around a hydrogen atom rather than around the oxygen atom and will in the long run lead to a diffusion. The time taken for this jump from site to site cannot be obtained, but its minimum is given by the time scale of 330 ps for IN5, during which time the water molecule stays within the impermeable sphere of the model.

Slow motion

On Fig. 2 is shown the Q^2-dependence of HWHM2. This slow motion could only be observed on the IN5 spectrom-

eter. The local behavior of this motion was not accessible because of the Q-range probed, and because the statistics obtained on IN5 did not allow us to propose a tighter model. From Fig. 2 an average diffusion coefficient, called D^{trans}, of $\sim 3.5\ 10^{-7}$ cm^2/s for all temperatures above 270 K was extracted.

Temperature dependence

The diffusive motion confined within a sphere seems to be affected by the decrease of temperature down to 270 K, whereas the slow motion is less influenced by the temperature change in this range. For the rapid motion, as the temperature is dropped, the diffusion coefficient decreases and the reorientation time increases smoothly. This reflects that water molecules undergoing the rapid motion are more and more confined and that they reorient themselves more and more slowly. The average jump length is partially reduced, which could be correlated to the shrinkage of the crystal cell parameters and/or a thermal shrinkage of the solvent.

Around 270 K, a more drastic effect is observed on the solvent dynamics. It affects both the rapid and slow diffusive motions. The average jump length of the rapid water molecules becomes very small, which reflects a strongly reduced probability of reorientation. Such a "transition" in the water dynamics, although, does not imply the crystallization of water molecules. Water molecules within the crystal seem to interact strongly with the protein, leading to a behavior of supercooled water. This transition could be related to the fact that between 250 and 270 K, one observes a decrease in the internal flexibility of bacteriorhodopsin, hydrated above 0.18 g/g protein (Fitter et al., 1997). However, in the latter study, part of the water molecules crystallized, which according to the authors explained the loss of flexibility observed.

Although, only around 180 K was observed a "global freezing" of both protein and solvent diffusive motions, where no quasielastic broadening could be seen in neither the "solvent-deuterated" nor the "all-hydrogenated" spectra. This transition has already been observed on myoglobin and bacteriorhodopsin both by neutron spectroscopy (Doster et al., 1989; Ferrand et al., 1993) and with other techniques (Smith et al., 1990). It seems that this transition reflects a partial freezing of the protein, which changes from an anharmonic to a harmonic regime induced by the glass transition in the surrounding solvent. The accurate temperature of transition for lysozyme, 175 K, was determined by Pissis (1992).

Details of solvent dynamics at room temperature

Following the first survey, a more refined analysis was done on the room temperature "all-hydrogenated" spectra as explained previously. Fig. 4 shows the quality of the fit obtained for the highest and lowest momentum transfer spectra. Parameters fitted are shown in Table 2.

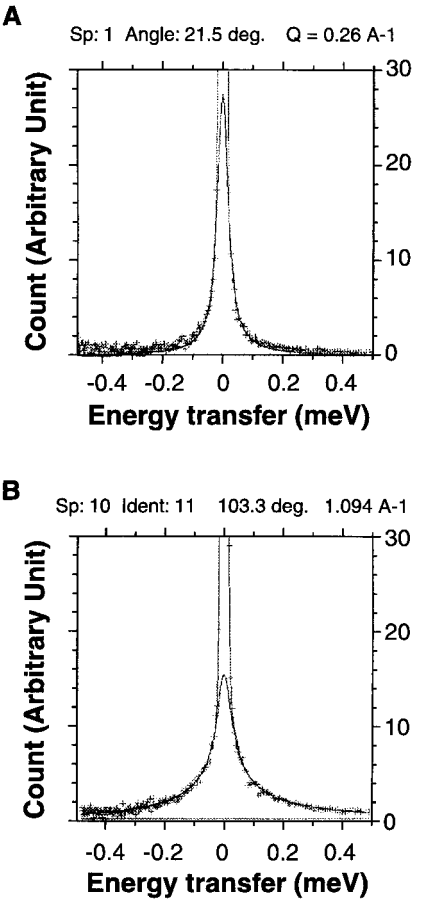

FIGURE 4 Experimental points (*crosses*), fitted quasielastic broadening (*solid line*), and resolution function (*solid line*) on IN5 at room temperature, for the spectra at $Q = 0.26$ Å^{-1} and $Q = 1.094$ Å^{-1}.

Proportion of "immobile" and mobile protons

Of the total population of protons within the sample, 3.5 ± 1.5% are "immobile" within the time scale probed. These protons could either represent water molecules trapped within cavities or at the surface of the protein, having a very slow dynamics, or they could correspond to protons undergoing a very rapid diffusion dynamics, in which case the resulting Lorentzian would be very wide and could be

TABLE 2 Proportion of protons involved in each motion and parameters describing each motion, obtained from advanced analysis of room temperature IN5 spectra

p1 (%)	p2 (%)	p3 (%)	a (Å)	D^{sph} 10^{-6} cm^2/s	D^{trans} 10^{-7} cm^2/s
3.5 ± 1.5	59 ± 5	75 ± 5	3.6 ± 0.4	3.9 ± 0.8	3.6 ± 1.0

hidden in the inelastic background. In total it means that for times shorter than ~330 ps very few protons are immobile within the sample. This agrees with an NMR study by Otting et al. (1991), which shows that even on very ordered crystallographic hydration sites, at 4°C, water molecule have a residence time below 500 ps. It is therefore likely to expect even shorter residence times at room temperature.

The rest of the protons are mobile, and ~59 ± 5% of these undergo one or the other of the two motions attributed to the solvent. This proportion of protons corresponds well to the number of protons in the solvent (~50%) plus a few protons from disordered protein side chains with solvent behavior, as suggested by a previous study of parvalbumin (Zanotti et al., 1999). Indeed, a few side chains with multiple conformations were observed in the structure of lysozyme (Bon et al., 1999), making up the difference. Within the solvent, no bulk solvent dynamics has been observed. This is not surprising, as only with degrees of hydration $h >$ 0.6 to 0.7 (w/w), properties of interfacial water become close to bulk solvent properties (Goldanskii et al., 1995). Triclinic hen egg-white lysozyme crystals correspond to a degree of hydration of ~0.38.

Population of protons undergoing a diffusive motion confined within a sphere

Out of the protons assigned to the solvent (including a few side chains) and within the time scale accessible on the instrument, 75% undergo a long-range diffusive motion confined to an impermeable sphere of radius 3.6 ± 0.4 Å with a diffusion coefficient reduced five to sixfold compared with bulk solvent. The local behavior of these protons has been shown to be a jump diffusion with of mean jump length of ~1.3 Å. As discussed above this length approximatively corresponds to the distance between proton sites or proton sites and lone pairs on the same water molecule. Moreover, the average residence time before jump is ~11 ps, i.e., ~10-fold more than the average reorientation time of bulk water molecules (1.2 ps at room temperature (Teixeira et al., 1985)). The average proton residence time can thus been attributed to the average time between reorientations of the water molecules.

Within the time scale accessible on IN5 spectrometer, protons are confined in a sphere of radius 3.6 ± 0.4 Å, and the water molecules therefore not only reorient themselves, but they also in the long run jump from hydration site to hydration site. The long-range diffusion coefficient is ~3.9 10^{-6} cm^2/s, which is approximately fivefold less than for bulk solvent, and it is worth noting that the long-range diffusion coefficient at a bilayer interface determined by NMR and IQENS studies are respectively of the order of 6 10^{-6} cm^2/s and 4.4 10^{-6} cm^2/s at 298 K (Hodges et al., 1997; Fitter et al., 1996), thus within a factor of two of the above value.

All together these water molecules can therefore be imagined as sitting in local, reasonable well-defined hydration sites, where they can either reorient or jump to connected locations. These sites can be expected to occur mostly near the protein surface, and it therefore seems reasonable to identify this population of rapid protons with the water molecules visible by diffraction techniques. Indeed, counting the complete and incomplete hydration sites identified in the neutron diffraction study near the protein surface (Bon et al., 1999), gives a total of 80% of the solvent, corresponding well to the value of 75% derived above.

The dynamic behavior found is in agreement with NMR studies (Halle, 1999), which showed that at 273 K the mean reorientation time of water molecules near the protein surface is ~23 ps. Another NMR study (Steinhoff et al., 1993) showed a mean reorientation time of 68 ± 10 ps within 5 Å from the protein surface, at 298 K, which seems slightly overestimated compared with the present study. Moreover, other NMR studies of the solvent dynamics around haemoglobin in solution (Polnaszek and Bryant, 1984; Steinhoff et al., 1993) and in myoglobin crystals (Kotitschke et al., 1990) showed a long-range diffusion coefficient reduced five and twofold, respectively, compared with bulk solvent.

Population of protons undergoing a simple translation

The other protons having a solvent-like behavior, ~25%, have been shown to undergo a long range translation with a diffusion coefficient reduced ~50-fold compared with bulk solvent. Unfortunately, however, the spectrometer configuration chosen on the IN5 spectrometer, which allowed to probe a large time scale and thus to observe this slow motion, did not permit to probe simultaneously the local behavior of this population of protons. However, this type of motion, pure translation, is compatible with the fact that averaged over time and space, these water molecules would not appear even partially organized in the neutron scattering density. This population therefore seems to correspond to the 20% of the water molecules furthest away from the protein, which have not been localized by diffraction methods.

A model for the solvent dynamics at room temperature

Fig. 5 summarizes the model for the behavior of water molecules derived from the structural and dynamical studies on triclinic crystals of hen-egg white lysozyme, at room temperature, and in the time range around 330 ps. In this model, no part of the solvent behaves as bulk solvent.

In addition, nearly no water molecules are immobile. On hydration sites near the protein surface (in black on Fig. 5), it seems that on average every 11 ps the water molecule reorients itself, i.e., ~10 times slower than in bulk solvent. Near the ordered parts of the protein surface, reorientational

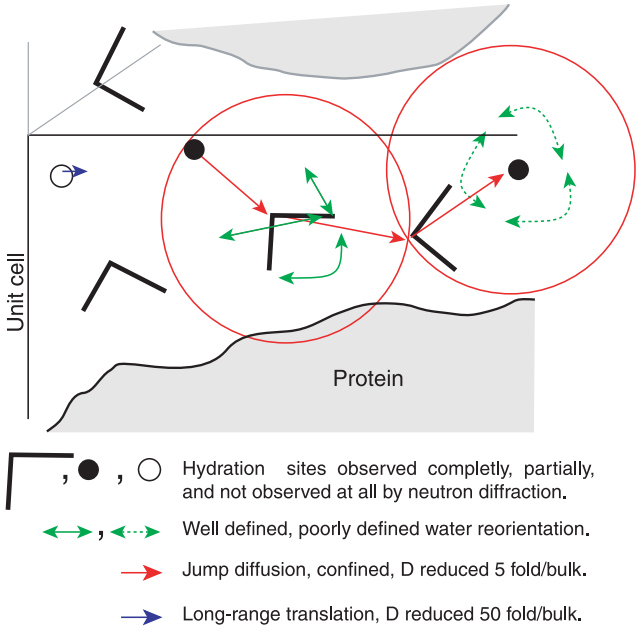

FIGURE 5 Model of the dynamics of water molecules in triclinic crystals of hen-egg white lysozyme, at room temperature, and in the time range ~330 ps. The dynamics is drawn for each of the three types of hydration sites: hydration sites seen as complete, incomplete by neutron crystallography, and hydration sites not seen at all by neutron crystallography are respectively drawn as black angles, full black circles, and open circles. In green, The plain and dashed double arrows represent respectively well and poorly defined reorientation of water molecules. In red, The circles represent the radius of confinement of each water molecule, within 330 ps; the arrows represents the jump between hydration sites, with a long range diffusion reduced approximately fivefold/bulk solvent. In blue, The arrow represents long-range translation with a long range diffusion reduced approximately 50-fold/bulk solvent.

motions seem very well defined, while being less and less so when moving away from the protein or near quite mobile parts of the protein. Within the 330 ps observed, the water molecules do not only reorient themselves, but they also jump from hydration sites to neighboring hydration sites, and overall they explore a space confined within a sphere of radius 3.6 ± 0.4 Å. The long-range diffusion coefficient of these water molecules is reduced approximately five to sixfold compared with bulk solvent.

Water molecules further from the protein surface seem to undergo a long-range translation. They are confined within the pockets between hydrated proteins, which could explain why their long-range diffusion coefficient is reduced approximately 50-fold compared with bulk solvent. These water molecules might act as an additional reservoir of molecules.

The suggested model of the water arrangement around proteins underpins the importance of hydration for the biological function of the protein. It consists of a tight, but mobile and rapidly exchanging hydrogen bonding network, which can interact with the protein surface residues, both maintaining the protein structure and allowing for the protein dynamics. Two points should be underlined. First, no part of the solvent behaves as bulk solvent. This observation is consistent with previous neutron scattering studies on cells (Trantham et al., 1984; Cameron et al., 1997), which showed that no part of the intracellular solvent behaves as bulk solvent, and this should be taken into account when interpreting various experiments performed on biological materials. The second and most striking part of the model is that the solvent near the protein surface undergoes a faster dynamics than the few water molecules in the partial second hydration layer. This could be compared with a recent observation in molecular dynamics simulations (Makarov et al., 1998). In this simulation, it has been shown that the water molecules around macromolecules (proteins as well as DNA) have a higher diffusion rate parallel to the solute surface than perpendicular to it. The reason for this is attributed to a reduction of the dimensionality of space available to the solvent at the interface. It suggests that the water dynamics is in some way organized by layer. In case of two complete hydration layers, the water diffusion would be faster parallel to the protein with a higher diffusion rate in the second hydration layer. On the contrary, for higher protein packing, where a second hydration layer is not complete (as in our crystals), the diffusion rate of water molecules would be kept on the protein surface, whereas the diffusional dynamics in the partial second layer would be highly reduced. Although there is still some controversy, we can mention some studies for which long-range proton migration on lipid layers, purple membranes, and protein monolayers (Gabriel and Teissie, 1996; Lechner et al., 1994b) has been shown to be faster along the protein or lipid surface than perpendicular to it. In the case of protein films, the lateral transfer appears to be strongly controlled by the packing, and it was suggested (Gabriel and Teissie, 1996) that subtle reorganizations of the protein-protein contacts could be biological switches between interfacial and delocalized proton pathways. Although the solvent in triclinic crystals of hen-egg white lysozyme mainly corresponds to a first hydration shell surrounded by pockets of glassy water, the present study suggests that in intracellular media (depending on the local packing) solvent diffusion could be faster near the macromolecules than further away, leading to a "solvent stream" along the surface. Several groups have tried to explain the rapid transport of some metabolites in intracellular media, e.g., by substrate channeling or reduced diffusion dimensionality (Ovadi and Srere, 1992; Mentre, 1995). The present work seems to indicate that the substrate diffusion in these media could be guided by a "solvent stream" along the protein surface, leading to a two-dimensional rather than a three-dimensional diffusion. Of course it should not be forgotten, that the "fast" motion of the water molecules near the surface is slower than in bulk water. The present observation, that the water moves fastest next to the surface, could be a factor in explaining the high rate of some enzymatic reactions within the cell.

CONCLUSION

In this study we have used neutron scattering in a combined structural and dynamical approach for the analysis of the solvent around hen-egg white lysozyme in triclinic crystals. In addition, we used identical samples in all the measurements, as only in this way we could propose a model for the dynamics of water around lysozyme on an atomic scale. Moreover, working on protein crystals gave us the advantage of a well-defined solvent system surrounding the proteins, which is probably at present the best available for producing experimentally tractable models for the many protein dynamic activities occurring within highly crowded media. The study allowed us to underline that the processes may take place without bulk solvent and the model suggests that the solvent in intracellular media might undergo a two-dimensional diffusion along the proteins, which could explain the rapid transport of some metabolites to the proteins within the cell. From a technical point this study proved that such incoherent quasielastic neutron scattering studies using protein crystals are feasible, and it will now be very interesting to study the dynamics of water in other protein crystals using other solvent contents and compositions.

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REFERENCES

Alary, F., J. Durup, and Y.-H. Sanejouand. 1993. Molecular dynamics study of the hydration structure of an antigen-antibody complex. *J. Phys. Chem.* 97:13864–13876.

Badger, J., and D. L. Caspar. 1991. Water structure in cubic insulin crystals. *Proc. Natl. Acad. Sci. U.S.A.* 88:622–626.

Bee, M. 1988. Quasi-Elastic Neutron Scattering. Adam Hilger, Philadelphia, PA.

Bee, M. 1996. Application for General Analysis of Time of Flight and High Resolution Experiment. ILL Report 96BE11T. http://www.ill.fr/tof/agathe_page.html. last access: June 15, 2002; last modified: Apr 18, 2002.

Bellissent-Funel, M. C., K. F. Bradley, S. H. Chen, J. Lal, and J. Teixeira. 1993. Slow dynamics of water molecules in confined space. *Physica A.* 201:277–285.

Bellissent-Funel, M. C., J. M. Zanotti, and S. H. Chen. 1996. Slow dynamics of water molecules on the surface of a globular protein. *Faraday Discuss.* 103:281–294.

Bon, C., M. S. Lehmann, and C. Wilkinson. 1999. Quasi-Laue neutron-diffraction study of the water arrangement in crystals of triclinic hen egg-white lysozyme. *Acta Crystallogr. D Biol. Crystallogr.* 55:978–987.

Bone, S., and R. Pethig. 1985. Dielectric studies of protein hydration and hydration-induced flexibility. *J. Mol. Biol.* 181:323–326.

Burling, F. T., W. I. Weis, K. M. Flaherty, and A. T. Brunger. 1996. Direct observation of protein solvation and discrete disorder with experimental crystallographic phases. *Science.* 271:72–77.

Cameron, I. L., K. M. Kanal, C. R. Keener, and G. D. Fullerton. 1997. A mechanistic view of the non-ideal osmotic and motional behaviour of intracellular water. *Cell Biol. Int.* 21:99–113.

Careri, G. 1998. Cooperative charge fluctuations by migrating protons in globular proteins. *Prog. Biophys. Mol. Biol.* 70:223–249.

Chung, E., D. Henriques, D. Renzoni, M. Zvelebil, J. M. Bradshaw, G. Waksman, C. V. Robinson, and J. E. Ladbury. 1998. Mass spectrometric and thermodynamic studies reveal the role of water molecules in complexes formed between SH2 domains and tyrosyl phosphopeptides. *Structure.* 6:1141–1151.

Cooper, A. 2000. Heat capacity of hydrogen-bonded networks: an alternative view of protein folding thermodynamics. *Biophys. Chem.* 85: 25–39.

Denisov, V. P., and B. Halle. 1995. Hydrogen exchange and protein hydration: the deuteron spin relaxation dispersions of bovine pancreatic trypsin inhibitor and ubiquitin. *J. Mol. Biol.* 245:698–709.

Doster, W., S. Cusack, and W. Petry. 1989. Dynamical transition of myoglobin revealed by inelastic neutron scattering. *Nature.* 337: 754–756.

Ehtezazi, T., T. Govender, and S. Stolnik. 2000. Hydrogen bonding and electrostatic interaction contributions to the interaction of a cationic drug with polyaspartic acid. *Pharm. Res.* 17:871–878.

Ferrand, M., A. J. Dianoux, W. Petry, and G. Zaccai. 1993. Thermal motions and function of bacteriorhodopsin in purple membranes: effects of temperature and hydration studied by neutron scattering. *Proc. Natl. Acad. Sci. U.S.A.* 90:9668–9672.

Fitter, J., R. E. Lechner, G. Buldt, and N. A. Dencher. 1996. Internal molecular motions of bacteriorhodopsin: hydration-induced flexibility studied by quasielastic incoherent neutron scattering using oriented purple membranes. *Proc. Natl. Acad. Sci. U.S.A.* 93:7600–7605.

Fitter, J., R. E. Lechner, and N. A. Dencher. 1997. Picosecond molecular motions in bacteriorhodopsin from neutron scattering. *Biophys. J.* 73: 2126–2137.

Gabriel, B., and J. Teissie. 1996. Proton long-range migration along protein monolayers and its consequences on membrane coupling. *Proc. Natl. Acad. Sci. U.S.A.* 93:14521–14525.

Ghosh, R. 1995. Profit data fitting programme. http://www.ill.fr/tof/TOFHR_software/PROFIT:html. last access: June 15, 2002; last modified: Apr 18, 2002.

Goldanskii, V. I., and Y. F. Krupanskii. 1995. Protein Dynamics in Protein-Solvent Interactions. R. B. Gregory, editor. Marcel Dekker/New York, Basel, Hong Kong.

Habash, J., J. Raftery, R. Nuttall, H. J. Price, C. Wilkinson, A. J. Kalb, and J. R. Helliwell. 2000. Direct determination of the positions of the deuterium atoms of the bound water in -concanavalin A by neutron Laue crystallography. *Acta Crystallogr. D Biol. Crystallogr.* 56:541–550.

Halle, B. 1999. Water in biologial systems: the NMR picture. *In* Hydration Processes in Biology. M. C. Bellissent-Funel, editor. NATO Sciences series. Series A: Life Sciences. 305:233–249.

Hodges, M. W., D. S. Cafiso, C. F. Polnaszek, C. C. Lester, and R. G. Bryant. 1997. Water translational motion at the bilayer interface: an NMR relaxation dispersion measurement. *Biophys. J.* 73:2575–2579.

Jaenicke, R. 2000. Stability and stabilization of globular proteins in solution. *J. Biotechnol.* 79:193–203.

Kotitschke, K., R. Kimmich, E. Rommel, and F. Parak. 1990. NMR study of diffusion in protein hydration shells. *Prog. Colloid Polym. Sci.* 83:211–215.

Lechner, R. E., N. A. Dencher, J. Fitter, G. Büldt, and A. V. Belushkin. 1994a. Proton diffusion on purple membrane studied by neutron scattering. *Biophys. Chem.* 49:91–99.

Lechner, R. E., N. A. Dencher, J. Fitter, and T. Dippel. 1994b. Two dimensional proton diffusion on purple membrane. *Solid State Ionics.* 70/71:296–304.

Lounnas, V. 1999. Molecular dynamics simulation. *In* Hydration Processes in Biology. M. C. Bellissent-Funel, editor. NATO Sciences series. Series A: Life Sciences. 305:261–290.

Lounnas, V., and B. M. Pettitt. 1994. Distribution function implied dynamics versus residence times and correlations: solvation shells of myoglobin. *Proteins.* 18:148–160.

Makarov, V. A., M. Feig, B. K. Andrews, and B. M. Pettitt. 1998. Diffusion of solvent around biomolecular solutes: a molecular dynamics simulation study. *Biophys. J.* 75:150–158.

Mason, S. A., G. A. Bentley, and G. J. McIntyre. 1984. Deuterium exchange in lysozyme at 1.4-A resolution. *Basic Life Sci.* 27:323–334.

McDowell, R. S., and A. A. Kossiakoff. 1995. A comparison of neutron diffraction and molecular dynamics structures: hydroxyl group and water molecule orientations in trypsin. *J. Mol. Biol.* 250:553–570.

Mentre, P. 1995. L'eau dans la Cellule. Masson, Paris, France.

Nagendra, H. G., N. Sukumar, and M. Vijayan. 1998. Role of water in plasticity, stability, and action of proteins: the crystal structures of lysozyme at very low levels of hydration. *Proteins.* 32:229–240.

Niimura, N., Y. Minezaki, T. Nonaka, J. C. Castagna, F. Cipriani, P. Hoghoj, M. S. Lehmann, and C. Wilkinson. 1997. Neutron Laue diffractometry with an imaging plate provides an effective data collection regime for neutron protein crystallography. *Nat. Struct. Biol.* 4:909–914.

Otting, G., E. Liepinsh, and K. Wuthrich. 1991. Protein hydration in aqueous solution. *Science.* 254:974–980.

Ovadi, J., and P. A. Srere. 1992. Channel your energies. *Trends Biochem. Sci.* 17:445–447.

Perez, J., J. M. Zanotti, and D. Durand. 1999. Evolution of the internal dynamics of two globular proteins from dry powder to solution. *Biophys. J.* 77:454–469.

Pissis, P. 1992. Proton Transfer in Hydrogen Bonded Systems. T. Bountis, editor. *NATO Adv. Study Inst. Ser. B Physics.* 291:207–216.

Pocker, Y. 2000. Water in enzyme reactions: biophysical aspects of hydration-dehydration processes. *Cell Mol. Life Sci.* 57:1008–1017.

Podjarny, A. D., E. I. Howard, A. Urzhumtsev, and J. R. Grigera. 1997. A multicopy modeling of the water distribution in macromolecular crystals. *Proteins.* 28:303–312.

Polnaszek, C. F., and R. G. Bryant. 1984. Nitroxide radical induced solvent proton relaxation: measurement of localized translational diffusion. *J. Chem. Phys.* 8:4038–4045.

Rieutord, F. 1990. INX. Program for TOF data reduction. *ILL Report 90R117T.*

Royant, A., K. Edman, T. Ursby, E. Pebay-Peyroula, E. M. Landau, and R. Neutze. 2000. Helix deformation is coupled to vectorial proton transport in the photocycle of bacteriorhodopsin. *Nature.* 406:645–648.

Rupley, J. A., and G. Careri. 1991. Protein hydration and function. *Adv. Protein Chem.* 41:37–172.

Saenger, W. 1987. Structure and dynamics of water surrounding biomolecules. *Annu. Rev. Biophys. Biophys. Chem.* 16:93–114.

Salunke, D. M., B. Veerapandian, R. Kodandapani, and M. Vijayan. 1985. Water-mediated transformations in protein crystals. *Acta Cryst. B.* 41: 431–436.

Settles, M., and W. Doster. 1996. Anomalous diffusion of adsorbed water: a neutron scattering study of hydrated myoglobin. *Faraday Discuss.* 103:269–279.

Shirley, W. M., and R. G. Bryant. 1982. Dynamical deductions from nuclear magnetic resonance relaxation measurements at the water-protein interface. *J. Am. Chem. Soc.* 104:2910–2918.

Shu, F., V. Ramakrishnan, and B. P. Schoenborn. 2000. Enhanced visibility of hydrogen atoms by neutron crystallography on fully deuterated myoglobin. *Proc. Natl. Acad. Sci. U.S.A.* 97:3872–3877.

Smith, J., K. Kuczera, and M. Karplus. 1990. Dynamics of myoglobin: comparison of simulation results with neutron scattering spectra. *Proc. Natl. Acad. Sci. U.S.A.* 87:1601–1605.

Srere, P. A. 1981. Protein crystals as models for mitochondrial proteins. *Trends Biochem. Sci.* 6:4–6.

Steinhoff, H. J., B. Kramm, G. Hess, C. Owerdieck, and A. Redhardt. 1993. Rotational and translational water diffusion in the hemoglobin hydration shell: dielectric and proton nuclear relaxation measurements. *Biophys. J.* 65:1486–1495.

Svergun, D. I., S. Richard, M. H. Koch, Z. Sayers, S. Kuprin, and G. Zaccai. 1998. Protein hydration in solution: experimental observation by x-ray and neutron scattering. *Proc. Natl. Acad. Sci. U.S.A.* 95: 2267–2272.

Teeter, M. M., S. M. Roe, and N. H. Heo. 1993. Atomic resolution (0.83 A) crystal structure of the hydrophobic protein crambin at 130 K. *J. Mol. Biol.* 230:292–311.

Teixeira, J., M. C. Bellissent-Funel, S. H. Chen, and A. J. Dianoux. 1985. Experimental determination of the nature of diffusive motions of water molecules at low temperatures. *Phys. Rev. A.* 31:1913–1916.

Trantham, E. C., H. E. Rorschach, J. S. Clegg, C. F. Hazlewood, R. M. Nicklow, and N. Wakabayashi. 1984. Diffusive properties of water in Artemia cysts as determined from quasi-elastic neutron scattering spectra. *Biophys. J.* 45:927–938.

Volino, F., and A. J. Dianoux. 1980. Neutron incoherent scattering law for diffusion in a potential of spherical symmetry: general formalism and application to diffusion within a sphere. *Mol. Physics.* 41:271–279.

Walsh, M. A., T. R. Schneider, L. C. Sieker, Z. Dauter, V. S. Lamzin, and K. S. Wilson. 1998. Refinement of triclinic hen egg-white lysozyme at atomic resolution. *Acta Crystallogr. D Biol. Crystallogr.* 54:522–546.

Wlodawer, A., H. Savage, and G. Dodson. 1989. Structure of insulin: results of joint neutron and x-ray refinement. *Acta Crystallogr. B.* 45:99–107.

Zanotti, J. M., M. C. Bellissent-Funel, and J. Parello. 1999. Hydration-coupled dynamics in proteins studied by neutron scattering and NMR: the case of the typical EF-hand calcium-binding parvalbumin. *Biophys. J.* 76:2390–2411.