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Permeability of lysozyme tetragonal crystals to water

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Abstract. Diffusion of water within cross-linked tetragonal crystals of hen egg-white lysozyme has been measured and simulated on a computer using the X-ray structure of water-filled channels within the crystal lattice. Relative to the self-diffusion coefficient of bulk water molecules, the experimental diffusion coefficient of water within the crystal was found to be 13 times reduced in the (001) crystallographic plane and 5 times reduced in the [001] direction. Comparison of the experimental and computer simulated diffusion coefficients shows that steric limitations for water diffusion are mostly responsible for this reduction of the water diffusion in the crystal, with the self-diffusion coefficient of intracrystalline water reduced by no more than 30–40% as compared to that of bulk water.

Key words: Lysozyme – Protein crystal – Water diffusion – permeability

Introduction

Studies of water transport within monocrystals of globular proteins are of interest from several points of view. First, cross-linked protein crystals have recently been proposed for industrial and clinical applications as extremely stable catalysts (St. Clar and Navia 1992) and as sensitive elements for (bio)chemosensors (Morozov and Morozova 1989, 1992). Properties of intracrystalline water, determining transport of substrates through the crystals, are important in these applications. Second, protein crystals provide a unique model to study physical properties of porous materials, since the structure of water-filled pores in the crystals may be determined to atomic resolution by X-ray diffraction. Pores in protein crystals range in width from 0.3 nm to 5 nm and occupy 25–75% of the crystal volume (Mattews 1968). The porosity of protein crystals, $(1–3)\times10^6$ m^2/kg (Morozov and Morozova 1989), is comparable with that of the best porous materials such as charcoal and silica-gel. Third, analysis of ion and water transport through well defined pores in protein crystals may throw more light on the physical properties of water in immediate proximity to surfaces, which is important to know in order to understand functioning of channels in biological and synthetic membranes. Solvent transport in semipermeable membranes has been intensively studied (Churaev 1990; Dytnerskii 1986; Yaroshchuk and Mesheryakova 1989; Zawodzinski et al. 1991); however, arbitrary assumptions concerning the size and shape of channels makes interpretation of the experimental results ambiguous. In contrast to this, protein crystals offer an opportunity to compare experimental transport characteristics with those calculated from atomic structure of the channels, as we show in this study.

We describe here an experimental method to measure pressure-induced flow of water through the lysozyme crystal along the different crystallographic directions and a theoretical method to calculate water diffusion within the crystal. Comparison of the experimental and theoretical results allows us to estimate the self-diffusion coefficient for intracrystalline water.

Materials and methods

Tetragonal crystals of hen egg-white lysozyme

$P4_32_12$ space group, were grown according to the method of Steinrauf (1959) and cross-linked with glutaraldehyde as described previously (Morozov and Morozova 1983). Membranous samples (0.5×0.5 mm, 7–10 μm thick) were cut with a microtome parallel to specific crystallographic planes. Sample thickness was measured with a Linnik's

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interferometer as described by Morozov and Morozova (1981).

Measurement of water permeability

As shown in Fig. 1, membranous sample-1 was glued with a cyanoacrylic adhesive-2 to one end of a thick-walled glass capillary-7. To make the attachment more secure, the capillary end was subsequently treated with 3-aminopropyltriethoxy silane (as described by Robinson et al. (1981)) and with 5% of glutaraldehyde (1–2 h in 10 m*M* phosphate buffer, pH = 7) before sample attachment. The glueing procedure was performed in a humid box with relative humidity of 95–100%. After polymerization of the adhesive for 20–30 min, capillaries with the samples were stored in the buffer salt solution used to grow the lysozyme crystals (5% NaCl, 50 m*M* Na-acetate buffer, pH = 4.7).

Before measurement the capillary was filled with buffer solution by dipping it into the solution and boiling for 10–20 sec at room temperature under reduced pressure to replace air inside the capillary with water vapor. On pressure release the buffer filled the capillary. Part of the buffer column was then removed to produce a meniscus-8 in the middle of a 3–4 cm long capillary. The capillary was fixed in a brass jacket thermostated at 25 °C. The end with the glued sample rested on a soft porous support-4 which prevented breakage of the membranous sample under applied pressure. The porous support was soaked in the same buffer solution-6 that filled the capillary. Control experiments revealed no dependence of the water flow rate upon the type of ultrafiltration membrane, used as a porous support.

The rate of permeation of the buffer solution through the crystalline membrane was measured under an optical microscope equipped with a micrometer eyepiece. The shift in position of the meniscus was recorded every 10 min within 2–3 h.

The permeability of the crystal section cut perpendicular to the crystal direction [hkl] was characterized with the specific permeability, $\mathbf{I}_{[hkl]}$, calculated as:

$$\mathbf{I}_{[hkl]} = \mathbf{J}_{[hkl]}\,\mathbf{h}/\Delta\mathbf{P} \qquad (1)$$

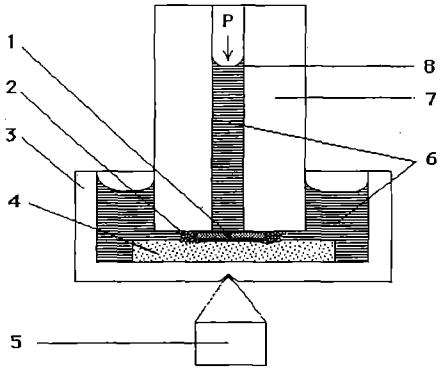

Fig. 1. Schematic of the apparatus used for measurements of water permeability of a cross-section of cross-linked protein crystal. 1 – protein sample, 2 – glue, 3 – cell, 4 – porous material, 5 – rod, 6 – buffer solution, 7 – glass capillary, 8 – liquid meniscus

Here **h** and **ΔP** are thickness of the sample and difference in pressures between the interior and exterior of the capillary, respectively. $\mathbf{J}_{[hkl]}$ is the water flow rate in the [hkl] direction of the crystal:

$$\mathbf{J}_{[hkl]} = \Delta\mathbf{Q}_{[hkl]}/\mathbf{S}\Delta\mathbf{t} \qquad (2)$$

with $\Delta\mathbf{Q}_{[hkl]}$ denoting volume of the solution passing in the [hkl] direction through the area, **S**, of the crystal section in time interval, **Δt**. Here and below we will use [hkl] indexes to denote tensor components along the main crystallographic directions, which coincide with the principal axes of second order tensors in the tetragonal crystal.

Control experiments show that $\mathbf{J}_{[hkl]}$ is proportional to applied pressure up to 5 atm. and under these pressures $\mathbf{I}_{[hkl]}$ did not show any changes within several hours.

All the measurements were made at 25 °C using a complex buffer consisting of $NaCH_3COO$, Na_2HPO_4, $Na_2B_4O_7$, 5 m*M* each, with 20 m*M* or 1 M NaCl. pH was adjusted with NaOH or HCl solutions.

According to the solution-diffusion mechanism of Merten (1966), the diffusion coefficient of water can be calculated from experimentally measured specific permeability according to the thermodynamic relation:

$$\mathbf{D}_{[hkl]} = \mathbf{I}_{[hkl]}\,\mathbf{RT}/\mathbf{C}_w\mathbf{V}_w^2 \qquad (3)$$

where **R** and **T** are the gas constant and absolute temperature, $\mathbf{C}_w$ and $\mathbf{V}_w$ the molar concentration of water within the crystal and molar volume of water, respectively.

Computer simulations of water diffusion in the protein crystal

The effective coefficient of water diffusion has been calculated from parameters, characterizing a random walk of a spherical probing body within the crystal channels. Atomic coordinates for tetragonal lysozyme (7LYZ) were taken from Brookhaven Data Bank. Crystal unit cell was filled with atoms as described by Kisluk et al. (1994). Atoms were approximated by balls of radius, $\mathbf{a}_j = \mathbf{a}_{wj} + \mathbf{a}_o$. Van der Waals radii of different atoms, $\mathbf{a}_{wj}$, were chosen according to Lee and Richards (1971), and the additional radius, $\mathbf{a}_o$, accounting for a finite size of the probing diffusive body, was varied in calculations.

The crystal unit cell was represented as a 3-dimensional grid with axis parallel to those of the unit cell. The density of nodal points on the grid was varied from 15 to 30 per 1 nm in different simulations. Channels were defined according to the CHANNEL program (Kisluk et al. 1994). The probe was inserted randomly into the crystal channel and displaced randomly to the nearest nodal point along either of three axes, *x*, *y* or *z*, at each step of the calculation. If a step terminated in a nodal point occupied by an atom, this was considered as a collision with the channel wall. A new random step was initiated in this case from the position preceding the collision. The mean square displacement vector of the probe, $\langle\Delta\mathbf{R}^2\rangle$, was calculated in a series of 20–30 random walks, consisting of $\mathbf{N} = 3\times10^5$ steps each. This number of steps was shown to be sufficient to make m.s. deviations in position of the probing

body independent of its starting coordinates. Values of the effective diffusion tensor (along the principal axes coinciding with the crystallographic ones in the tetragonal crystal) were calculated from the Einstein-Smolukhovskii relation:

$$\mathbf{D}^{\mathrm{eff}}_{[hkl]} = <\Delta \mathbf{R}^2_{[hkl]}> / 2\mathbf{N} \quad (4)$$

Here $\langle \Delta \mathbf{R}^2_{[hkl]} \rangle$ is the mean square of the projection of the displacement vector, on any one of the three main crystallographic directions ([100], [010] and [001]). All calculations were performed on a micro VAX 3600 computer.

Results and discussion

Since crystal permeability is described by a second order tensor, the permeability tensor surface for $P4_32_12$ space group of the tetragonal crystal is characterized by an ellipsoid of revolution (Sirotin and Shaskolskaya 1975) with two principal values: the permeabilities along the main axis, one parallel to the [001] direction, and the other in any direction in the (001) plane. Our measurements of permeabilities of the tetragonal lysozyme crystal along [001], [100] and [010] directions for the low salt buffer solution (pH = 8.0, 20 mM NaCl) have given $\mathbf{I}_{[001]} = (1.2 \pm 0.1) \times 10^{-18}$ m^4/s N and $\mathbf{I}_{[010]} = \mathbf{I}_{[100]} = (0.48 \pm 0.05) \times 10^{-18}$ m^4/s N. Data from 5–8 independent measurements with different samples were averaged. We may, thus, visualize the permeability tensor surface of the tetragonal lysozyme crystal as an ellipsoid of revolution 2.5 times as extended along [001] axes.

Calculations according to Eq. (3) give $(1.8 \pm 0.2) \times 10^{-10}$ m^2/s for the diffusion coefficient of water in the (001) plane and $(4.6 \pm 0.4) \times 10^{-10}$ m^2/s for that in the [001] direction (see notes for Table 1 for other parameters used in the calculations). As compared to the self-diffusion coefficient of water molecules in bulk water ($\mathbf{D}_b = 2.4 \times 10^{-9}$ m^2/s (Zatsepina 1987)) these are reduced by factors of 13 and 5, respectively.

Table 1. Comparison of experimental values of water diffusion coefficients in different direction of the tetragonal lysozyme crystals with those obtained in computing simulations accounting for only steric limitations for diffusion

Crystal direction	Diffusion coefficient of water within the crystal, $D_o \times 10^{10}$ m^2 sec^{-1}	
	Theoretical[a]	Experimental[b]
[100] and [010]	2.4 ± 0.4	1.8 ± 0.2
[001]	6.3 ± 1.0	4.6 ± 0.4

Notes to Table 1: [a] Results of computer simulations with radius of probing body, $a_o = 0.193$ nm, and self-diffusion coefficient, $D_b = (2.4 \pm 0.18) \times 10^{-9}$ m^2 s^{-1} (Zatsepina 1987)

[b] Calculated from experimentally found permeabilities according to the solution-diffusion mechanism (Eq. (3)). As the tetragonal crystal contains (0.45 ± 0.03 kg water per kg of dry protein (Morozov et al. 1988) and unit cell volume per 1 mol of lysozyme is 1.787×10^{-2} m^3 (Blake et al. 1965), water concentration in the crystal is $C_w = (2.0 \pm 0.15) \times 10^4$ mol/m^3 (mol. wt. of lysozyme was taken en 14 300). Molecular volume of intracrystalline water was taken in the calculations as in bulk water, $V_w = 1.8 \times 10^{-5}$ m^3/mol. T = 298 K

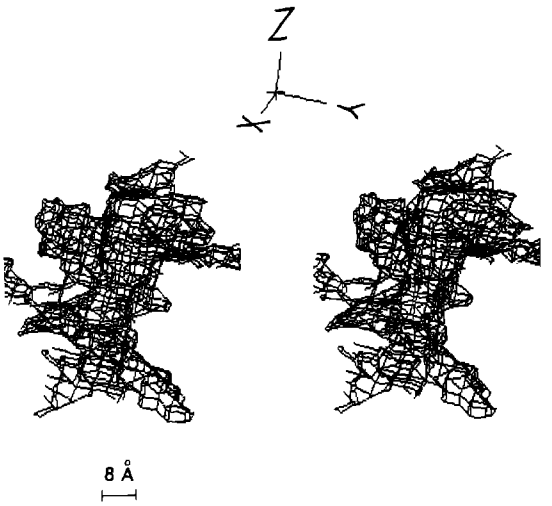

Fig. 2. Stereo diagram of the water-filled channel within a $P4_32_12$ tetragonal lysozyme crystal. Space inside the isoelectronic lines represents the channel body. Isoelectronic lines are drawn for the electronic density intermediate between the density of protein and solvent using the CHANNEL program (Kisluk et al. 1994). Electronic density was calculated using coordinates of 7LYZ taken from the Brookhaven PDB

The marked difference in permeability of the crystal observed along the different directions reveals the structure of cavities in the crystal. As one can see from the stereo pair presented in Fig. 2, the channels look like helical ribbons running along screw axes in the [001] direction. They are characterized with the screw (4_3) symmetry in an arrangement of narrow branches that connect neighboring channels. How can water move within such channels?

Two physically different mechanisms are conventionally used to describe solvent permeability of membranes: viscous flow and diffusion (Brick and Tsapuck 1989; Merten 1966). The main difference between these two mechanisms on a microsopic scale consists in the degree of correlation in the motion of solvent molecules. A diffusive process is characterized by independent uncorrelated motion of individual molecules, whereas in Poiseuille flow motion of individual molecules is highly correlated locally. The solution-diffusion model (Merten 1966) usually describes transport in membranes with small pores (less than 1 nm) adequately, whereas viscous flow models are applied in the case of membranes with much larger pores. Since pore size in our crystals is about 1 nm (see Fig. 2), there is no obvious preference for any of these models and we consider both.

Estimates based on the Poiseuille law give for the effective hydrodynamic radius of channels going along [hkl] direction, $\mathbf{r}_{[hkl]}$, the equation:

$$\mathbf{r}_{[hkl]} = (8\eta \mathbf{I}_{[hkl]} / \pi \mathbf{n})^{1/4} \quad (5)$$

Taking the bulk viscosity of water, $\eta = 1 \times 10^{-3}$ N s m^{-2}, the number of channels per unit of area, $\mathbf{n} = 2\,\mathbf{a}^{-2} = 3.2 \times 10^{16}$ m^{-2} ($\mathbf{a} = 7.91$ nm, is the lattice parameter, 2 is number of channels per unit cell section) and the experimental value of permeability along the [001] direction, $\mathbf{I}_{[001]}$, we obtain the hydrodynamic radius, $\mathbf{r}_{[001]} = 0.56$ nm. Similar calculations with the experimental value

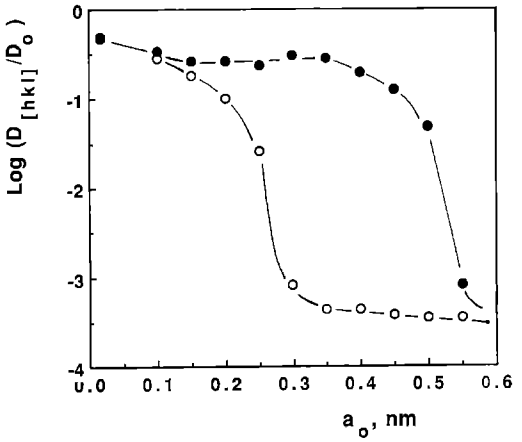

Fig. 3. Results of simulations of the diffusion of a probing body in the lysozyme crystal as a function of radius, $\mathbf{a}_o$, of the probing body. $\mathbf{D}_{[hkl]}$ is the diffusion coefficient along [hkl] direction in the crystal, $\mathbf{D}_o$ is the diffusion coefficient, calculated for a grid free of protein atoms under other identical conditions. (o) denote diffusion in any direction in the (001) plane, (•) for diffusion in the [001] direction

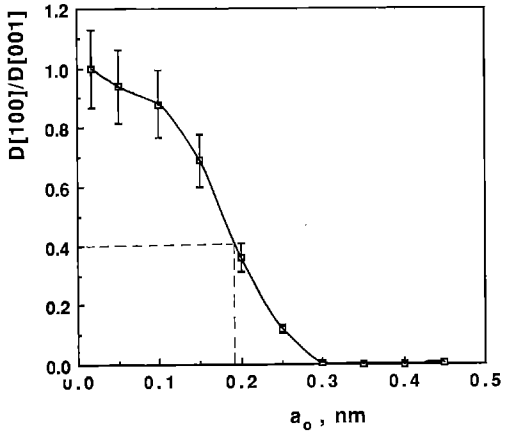

Fig. 4. Ratio of diffusion coefficients along any direction in the (001) plane to that along the [001] direction in the tetragonal lysozyme crystal calculated for a probing body of different radius, $\mathbf{a}_o$. Dashed line shows that experimentally found relation of the diffusion coefficients corresponds to the radius of the probing body of $\mathbf{a}_o = 0.193$ nm

of $\mathbf{I}_{[100]} = \mathbf{I}_{[010]}$, and $\mathbf{n} = 4/\mathbf{ac} = 1.33 \times 10^{17}$ m^{-2} ($\mathbf{c} = 3.79$ nm is the lattice parameter), give for the average radius of pores in the (001) plane, $\mathbf{r}_{[100]} = \mathbf{r}_{[010]} = 0.31$ nm. This simple hydrodynamic model seems able to account surprisingly well for the size of bottle-necks in real channels, since the computer simulations showed, that the spherical probe stops walking along the [001] direction and in the (001) plane of the tetragonal crystal when its radius exceeds 0.55 nm and 0.3 nm, respectively (see Fig. 3). However, this coincidence should be considered with care, since our estimates were based on Poiseuille law which is only applicable for a cylinder channel, it cannot be used to quantitatively calculate flow within the real channels of a complex form. As seen from Fig. 2, even the relatively broad channel along the [001] direction contains bottle necks, across which only 4 water molecules can be placed simultaneously. In the light of these facts, it is reasonable to suggest that motion of water molecules through such pores is more diffusive than viscous.

The results of computer simulations of diffusion of a probe within the crystal channels are presented in Figs. 3 and 4. As can be seen from Fig. 3, diffusion in the (001) plane abruptly drops when the radius of the probing body reaches 0.3 nm. For a larger value of the radius, the three-dimensional net of crystal channels disintegrates into a system of separate tubes running along the [001] direction. As the radius increases still further up to 0.55 nm, these channels disintegrate into arrays of isolated cavities with an average size of about 2 nm available for random walking of the probing body.

The ratio of diffusion coefficients along the [100] and [001] directions, $\mathbf{D}_{[100]}/\mathbf{D}_{[001]}$, is strongly dependent upon the radius of the probe, $\mathbf{a}_o$, as shown in Fig. 4. To compare the results of the simulations with experimental data, we need to properly choose the radius of the water molecule. Though its X-ray radius is 0.139 nm, it has been experimentally found, that water molecules undergoing diffusion move like spheres with an effective radius of 0.193 nm, because not single water molecules but clusters are moving elements (Zatsepina 1987). Since in our case we are dealing with diffusion, we should take this last value of the water radius as $\mathbf{a}_o$ in our calculations. We obtain then excellent agreement between the calculated anisotropy of diffusion coefficients, $\mathbf{D}_{[100]}/\mathbf{D}_{[001]} = 0.40 \pm 0.06$, and that of the experimentally determined value, $\mathbf{I}_{[100]}/\mathbf{I}_{[001]} = \mathbf{D}_{[100]}/\mathbf{D}_{[001]} = 0.40 \pm 0.08$. This gives strong support for the validity of our calculations.

Now we can calculate to what extent the geometry of crystal channels restricts diffusion of water through the protein crystal. Taking $\mathbf{a}_o = 0.193$ nm we have calculated and compared diffusion coefficients of water over the grid with and without protein atoms included. We find that the presence of protein molecules which are impenetrable to water reduces the water diffusion coefficient by a factor of $\alpha_1 = 9.8$ in the (001) plane of the crystal and by $\alpha_2 = 3.8$ in the [001] direction.

Provided that the self-diffusion coefficient of water molecules within the crystal channels is the same as in bulk water, $\mathbf{D}_b = 2.4 \times 10^{-9}$ m^2 s^{-1} (Zatsepina 1987), we can calculate the diffusion coefficients of water in the main directions of the crystal by dividing this value by α_1 and α_2. The results of such calculations are presented in Table 1 together with the diffusion coefficient determined from experimentally measured crystal permeabilities according to Eq. (3). The experimentally determined values are 35–37% smaller than those expected from the theoretical calculations which account only for steric limitations to diffusion. Therefore, some other factors must also influence diffusion of water in real crystals.

In addition to the steric limitations due to protein molecules taken into account in our simulations, ther are additional obstacles for diffusion due to the presence of glutaraldehyde cross-links within the channels of the real crystal. The volume occupied by the glutaraldehyde cross-links may be estimated as 10% of the whole volume of the crystal channels, since there is a 10% decrease in the total amount of water (Gevorkian and Morozov 1983; Imoto

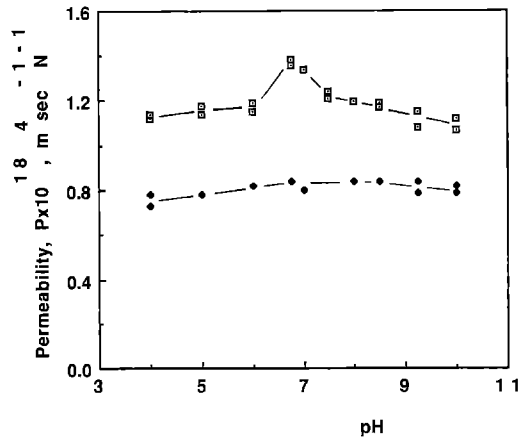

Fig. 5. pH dependence of the specific permeability of glutaraldehyde cross-linked lysozyme crystal along the [001] direction at low (20 mM NaCl, ◇) and high (1 M NaCl, ◆) salt concentrations added to buffer solution. Results of experiments with 2 different samples are presented

et al.1972; Morozov et al.1988) and only 0.04% decrease of the unit cell volume occurs upon cross-linking. We do not know how the cross-links are distributed over the channel volume. However, since the experimentally measured ratios of the diffusion coefficients in different directions agree well with the theoretically found one, we may suppose that the cross-links are evenly distributed in the channel. If this is the case, the channel cross-section will be decreased by about 6.6%. The largest effect on water diffusion will be due to the decrease in cross section at the narrowest part of the channel. As mentioned above, the radius of these bottle necks is about 0.3 nm. A decrease of 3.3% will correspond to an increase of the radius of the probing body by 0.01 nm. As seen from Fig. 3, such an increase of the radius of water molecules ($\mathbf{a}_o$=0.193 nm) will result in a 17% drop of its diffusion coefficients along [100] and [010] directions. For the [001] direction the effects will be considerably smaller. Hence, glutaraldehyde cross-linking cannot completely account for the observed discrepancy between the experimental and theoretical values of the water diffusion coefficients.

Another factor capable of reducing the mobility of the intracrystalline water molecules is their binding to immobile surface groups of protein molecules and to mobile ions. The last contribution cannot be large, since a considerable change in ionic composition of the intracrystalline solution, produced by a large change in pH and addition of 1 M NaCl, as shown in Fig. 5, change the crystal permeability for water by no more than 30%.

The factors considered above readily explain the observed difference of approximately 40% between the theoretical and experimental values of water self-diffusion coefficients. Therefore, our assumption, that the self-diffusion coefficient of intracrystalline water is the same as in the bulk water is true, and we have to conclude, that the average viscosity of the intracrystalline water and its self-diffusion coefficients change by no more than 20–40% as compared to bulk water.

This conclusion is in a good agreement with the data of the NMR field-gradient technique which revealed an approximately 30% decrease of the water self-diffusion coefficient in the hydration shell of bovine serum albumin (Kotitschke et al. 1990), whereas the overall diffusion coefficient of water through the myoglobin crystal and concentrated albumin solution was 2-fold decreased, which is comparable to sterically-induced reduction of diffusion in our lysozyme crystal. However, other known NMR techniques to measure the self-diffusion of water near a protein surface, namely measurements of proton relaxation in the vicinity of paramagnetic labels covalently attached to a protein surface, give more considerable reduction for water diffusion near the protein surface. Thus, Polnaszek and Bryant (1984 a, b), Steinhoff et al. (1993) found about a 5-fold decrease of the water self-diffusion coefficient within the 0.5–1 nm layer around a protein molecule using this method. Such a discrepancy between the data of different methods may result from different sensitivity of these methods to mobility of water molecules along the protein surface and off the surface. Whereas diffusion in protein crystals, measured in our experiments and in the NMR field-gradient method, is mainly determined by walking along the protein surface, translational diffusion of water off the paramagnetic label and off the protein surface mainly contribute to measurements by the second NMR method. Since water molecules need to break their bonds with the protein surface when leaving the hydration shell it seems reasonable to expect that water molecules jumps more frequently along the surface than off. Diffusion of bound water molecules along the surface may be even faster than in the bulk phase as was experimentally demonstrated in NMR studies of cotton fibers (Anisimov and Dautova 1993). At the same time, diffusion off the surface may strongly depend upon surface quality. As shown in the computer simulations of Knapp and Muegge (1994) diffusion coefficient of water off the surface of a BPTI molecule is a factor of 4 slower than in bulk water for 75% of the hydration water, and a factor of 15 faster of the other 25%.

In conclusion, despite the well known fact that some fraction of hydrated water molecules is strongly bound to the protein surface (Rupley and Carreri 1991), according to our data the average mobility of water in protein crystal channels is not considerably reduced as compared to that of bulk water. This explains why crystalline proteins are easily accessible to solute molecules, provided the size of the latter is less than those of the bottle-necks of the crystal channels.

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