Internal molecular motions of bacteriorhodopsin: Hydration-induced flexibility studied by quasielastic incoherent neutron scattering using oriented purple membranes

(protein dynamics/diffusive motions)

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ABSTRACT Quasielastic incoherent neutron scattering from hydrogen atoms, which are distributed nearly homogeneously in biological molecules, allows the investigation of diffusive motions occurring on the picosecond to nanosecond time scale. A quasielastic incoherent neutron scattering study was performed on the integral membrane protein bacteriorhodopsin (BR), which is a light-driven proton pump in Halobacterium salinarium. BR is embedded in lipids, forming patches in the cell membrane of the organism, which are the so-called purple membranes (PMs). Measurements were carried out at room temperature on oriented PM-stacks hydrated at two different levels (low hydration, \( h = 0.03 \) g of D\(_2\)O per g of PM; high hydration, \( h = 0.28 \) g of D\(_2\)O per g of PM) using time-of-flight spectrometers. From the measured spectra, different diffusive components were identified and analyzed with respect to the influence of hydration. This study supports the idea that a decrease in hydration results in an appreciable decrease in internal molecular flexibility of the protein structure. Because it is known from studies on the function of BR that the pump activity is reduced if the hydration level of the protein is insufficient, we conclude that the observed diffusive motions are essential for the function of this protein. A detailed analysis and classification of the different kinds of diffusive motions, predominantly occurring in PMs under physiological conditions, is presented.

The main properties of a molecular machinery like an enzyme or an active transport protein are its specific architecture and internal flexibility. The study of dynamical features on a picosecond time scale reveals information on the internal flexibility, which might be correlated to the different levels of the architecture of a biological macromolecule. Fast stochastic structural fluctuations in a protein are supposed to be essential for conformational changes on a slower time scale (e.g., millisecond), which are necessary for processes like intermolecular recognition, enzymatic reactions, or other biological functions. The incoherent scattering from hydrogen atoms is dominating the total neutron scattering of biological samples (the incoherent cross section of hydrogen nuclei is \( \approx 40 \) times larger than the cross sections of other elements) and is therefore a powerful tool for the investigation of molecular motions within a time range from \( 10^{-12} \) to \( 10^{-10} \) seconds. To a good approximation, the hydrogen atoms are distributed homogeneously in a biological macromolecule. Therefore, incoherent neutron scattering from hydrogen atoms monitors the internal motions. Early work using inelastic incoherent neutron scattering yielded dynamical information on biological systems and demonstrated the potential of this technique in the field of biophysics (1–3). Recent inelastic incoherent neutron scattering studies as well as Mössbauer spectroscopy showed that at low temperatures protein motion can largely be explained by a harmonic behavior. At temperatures between 180 and 230 K, a dynamical transition was found for proteins with sufficient hydration, which is characterized by a dramatic increase of a global average “mean square displacement” (including all kinds of motions in the observed time range) obtained by a global Debye–Waller factor analysis (4–6). Furthermore, the incoherent neutron scattering shows an additional quasielastic contribution with increasing temperature, which is due to the onset of diffusive motions in the macromolecule. Such diffusive motions are probably part of the conformational fluctuations leading to transitions between different conformational substates according to a model introduced by Frauenfelder (7–9). At low temperatures, the potential barriers separating different substates largely exceed thermal energy and only essentially harmonic vibrational motions occur. Proteins are in general “fully functional” only under physiological conditions characterized by a special range of pH, temperature, and a sufficient hydration. At these conditions an appreciable fraction of the internal motions are no longer harmonic, but they become anharmonic and to a large extent even predominantly diffusive. A detailed analysis of the quasielastic incoherent neutron scattering will give information especially on these diffusive motions.

The membrane protein bacteriorhodopsin (BR), which functions as a light-driven proton pump in Halobacterium salinarium, is one of the best characterized membrane proteins. It is the only protein in the plasma membrane of the organism aggregated to highly ordered two-dimensional layers, called purple membranes (PMs). Because many biophysical methods have been applied to this system, it is an important prototypic membrane protein. The structure of BR was mainly investigated by cryo-electron microscopy to moderate resolution (10). This study revealed that BR consists of seven \( \alpha \)-helices spanning the membrane, connected by “loop” regions protruding into the aqueous phase. Neutron diffraction experiments showed that the working cycle of BR is accompanied by structural changes of the order of 10% in the observed intensity up to 7 Å resolution (11). In time-resolved x-ray diffraction experiments, these structural changes were monitored with a time resolution of a few milliseconds (12).

The aim of the present work is to analyze various types of diffusive motions occurring in the protein–lipid complex of PM at room temperature and at two different levels of hydration. To separate motions in the solvent (i.e., that of water molecules) from motions in the PM, samples were hydrated with H\(_2\)O and D\(_2\)O, respectively. To a good approximation, the D\(_2\)O-hydrated sample exhibits only scattering from the PM.

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Abbreviations: BR, bacteriorhodopsin; PM, purple membrane; EISF, elastic incoherent structure factor; QISF, quasielastic incoherent structure factor.

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MATERIALS AND MEASUREMENTS

Sample Preparation. Purple membranes were isolated from *H. salinarium*. The PM suspension was deposited on aluminium foils and was dried under controlled conditions for 3 days. Oriented films of PM stacks were obtained with membrane plane parallel to the surface of the aluminium foil. Samples were rehydrated with D$_2$O and equilibrated at two different hydration levels using vapor exchange over pure D$_2$O (high hydration level, “wet” $h = 0.28$ g of D$_2$O per g of PM, corresponding to 340 water molecules/BR molecule) and over saturated KCL solution, respectively (low hydration level, “dry” $h = 0.03$ g of D$_2$O per g of PM, corresponding to 40 water molecules/BR molecule). Finally, the samples were sealed in a circular slab-shaped aluminium container and positioned in the neutron beam.

Measurements. The experiments were carried out at a temperature of 297 K using two different time-of-flight spectrometers. Spectra from samples at both hydration levels were obtained using the spectrometer MIBEMOL (Laboratoire Léon Brillouin, Saclay) with an elastic energy resolution of $\sim 50$ $\mu$eV [full-width at half-maximum (FWHM)] to study motions with correlation times between 0.1 and 30 ps. Further measurements using only wet samples were carried out with the inverted time-of-flight spectrometer IRIS (Rutherford Appleton Laboratory, Didcot), which allows an analysis of motions in a time range from 1 to 100 ps (elastic energy resolution, 16 $\mu$eV (FWHM)). To observe anisotropy effects in the dynamical behavior, measurements were performed with two different sample orientations $a$. The sample orientations were chosen so that for the scattering angle $\phi = 90^\circ$ the elastic $Q$-vector was perpendicular ($\alpha = 45^\circ$) and parallel ($\alpha = 135^\circ$) to the membrane plane, respectively (see inset of Fig. 1). More experimental details concerning these quasielastic incoherent neutron scattering experiments are given elsewhere (13).

THEORY OF DATA ANALYSIS

In a neutron scattering experiment with predominantly incoherent scattering from hydrogen nuclei the double-differential cross section,

$$\frac{\hat{d}^2\sigma}{d\Omega d\omega} = \frac{1}{4\pi} \left| \frac{k_1}{k_0} \right|^2 \cdot b_{inc} \cdot S_{inc}(Q,\omega), \tag{[1]}$$

determines the number of neutrons scattered into a solid angle element $d\Omega$ and an energy transfer element $d\omega$. Here $b_{inc}$ is the incoherent scattering length, while $k_0$ and $k_1$ are the incident and the scattered wave vectors, respectively. Information on the dynamics of individual hydrogen atoms can be obtained from the incoherent scattering function $S_{inc}(Q,\omega)$ using the formalism of self correlation functions developed by Van Hove (14). The self correlation function $G_t(r,t)$ is the Fourier transform in space and time of the incoherent scattering function,

$$S_{inc}(Q,\omega) = \frac{1}{2\pi} \int_{-\infty}^{\infty} e^{-iQ \cdot r} \cdot G_t(r,t) \, dr dt. \tag{[2]}$$

In the classical approximation (see for example refs. 15 and 16), $G_t(r,t)$ describes the average time-dependent probability density distribution of hydrogen atoms.

The hydration water is located in the interior of the protein, on the surface of protein and lipids, and in the interlayer space, the latter containing most of the water at medium and high hydration levels.

$$G_t(r,t) = N^{-1} \sum_{i=1}^{N} (\delta r_i + \hat{R}(0) - \hat{R}(t)), \tag{[3]}$$

where $\hat{R}(0)$ and $\hat{R}(t)$ are position vectors of atom $i$ for the time $t = 0$ and for time $t$. In general, it is not possible to calculate the trajectories $\hat{R}(t)$ directly from the measured scattering function, so that in practice special analytical models are used to investigate the movements of the individual atoms (see for example ref. 15). Using this kind of analysis, a calculated theoretical scattering function, $S_{theo}(Q,\omega)$, is fitted to a measured scattering function $S_{meas}(Q,\omega)$ as follows:

$$S_{meas}(Q,\omega) \propto e^{-\omega^2\delta^2}[S_{theo}(Q,\omega) \otimes S_{rel}(Q,\omega)]. \tag{[4]}$$

While the quasielastic lines are represented by quasielastic scattering ($S_{theo}(Q,\omega)$, convoluted with the experimental resolution function $S_{rel}(Q,\omega)$), the attenuating effects of vibrational motions on the latter are expressed by the Debye-Waller factor, which contains the mean square displacement $<\delta r^2>$ of vibrational motions as a parameter. To study local quasielastic motions of molecular subunits, the measured scattering function was fitted with jump diffusion models. This kind of motion is obviously restricted to the limited volume available for the displacement of hydrogen atoms and is parameterized by jump distances $d$ as well as by correlation times $\tau$ (for details see refs. 15–17). In the case of biological macromolecules very many different molecular subunits (e.g., sidegroups of the polypeptide chain) may make quite different contributions to the quasielastic scattering and even identical subunits may experience different “motional environments” and thus also differ in their contributions. Therefore we do not expect that a single Lorentzian will provide a description of the quasielastic incoherent neutron scattering spectra that is valid over a large energy range. In fact, we definitely know from our neutron investigations on PM, using several different energy resolutions, that the observed quasielastic scattering cannot be described by only one single Lorentzian (see also Results). On the other hand, it is generally not possible to describe the motions of all hydrogen atoms with analytical models on a molecular level. Thus we were led to attempt a description containing several different quasielastic components. A finite number of models describing jump diffusion can be used to characterize different types of motions and can approximate the dynamical behavior, when the corresponding parameters are understood as averages over distributions. Following this idea, we classified all protons into five different categories, which are considered as potential candidates for special types of motions occurring in the protein-lipid complex of PM.

All protons in molecular elements, which might participate in molecular motions already familiar from other studies, were attributed to one of the following three categories.

(i) Category 1: An important category is represented by protons located in methyl groups. It is known from many studies that the latter tend to perform uniaxial rotational diffusion (e.g., three-site jump diffusion) (18–20). In the case of our sample, methyl groups are located in polypeptide sidegroups and in hydrophobic chains of PM lipids (21, 22). With the exception of amino acid methionine, in which only 3% of all methyl groups are located, the methyl groups possess C-C bonds as rotation axes. A study of methyl groups with C-C bonds as rotation axes has been carried out by Volino et al. (23). They showed that at room temperature rotational motions can very well occur on the picosecond timescale, which appears to be the order of magnitude corresponding to the largest component of our quasielastic spectra.

(ii) Category 2: Furthermore, it is expected that some hydrogen atoms are remaining in hydrogen bonds, although the natural solvent H$_2$O has been replaced by D$_2$O (24). These
hydrogen atoms may participate in a two-site jump diffusion, where acceptor and donor site are separated by a potential barrier (25).

(iii) Category 3: Because the scattering from D₂O molecules is much less intense (by a factor of 11) than that from H₂O molecules, scattering of the deuterons is only a few percent of the total. In previous publications we have already analyzed the dynamical behavior of hydration water on purple membrane, so that we may approximate the motions of D₂O molecules by a properly weighted term developed for H₂O molecules in the above-mentioned investigations (13, 26).

The remaining hydrogen atoms, which have not yet been described by the above three categories, were taken into account by two additional ones.

(iv) Category 4: All protons participating in further diffusive motions corresponding, for example, to reorientations of polypeptide side chains or other molecular subunits, were described by a general two-site jump diffusion. Because the various groups are structurally different from each other, jump distances as well as jump rates have to be taken as values averaged over corresponding distributions.

(v) Category 5: Apart from all above mentioned types of hydrogen atoms located in different parts of the PM, there are also those that move very slowly and cannot be resolved by the energy resolution used in the present experiment. These hydrogen atoms are therefore classified as “fixed.”

The analytical expressions for these five categories were implemented into a theoretical scattering function. Because the spectra measured with the IRIS spectrometer are also suitable to study slower motions (as compared with motions that can be resolved with MIBEMOL), we divided for practical purposes the general two-site jump diffusion component into two parts. One “fast” component can be resolved in spectra measured with both instruments, and another “slow” component is only resolved in spectra measured on the IRIS spectrometer. Considering the number of protons participating in the corresponding types of motion, each category was given a statistical weight F (see Table 1). A correction for multiple scattering using the Sears expansion (27) was applied and has been discussed elsewhere (28).

RESULTS

Phenomenological Fit. In a first step we evaluated the qualitative effects that the variation of the hydration level has on the behavior of diffusive motions in the protein–lipid complex, applying “phenomenological fits” to the measured data. Without using special models the total quasielastic scattering of the dry sample was determined by fitting only one Lorentzian, whereas spectra from measurements of the wet sample were fitted by two Lorentzians. In the latter case the values obtained from the fit of the dry sample were used as fixed parameters of one of the Lorentzians. The other Lorentzian represents the additional quasielastic scattering occurring in the sample when the hydration level is raised (i.e., from dry to wet). Note that such a simple fitting procedure is valid only for the purpose of a merely phenomenological comparison of data measured with the same energy resolution. If the latter is varied, the single Lorentzian fit in the case of a complex system such as PM does not yield a unique line width.

This analysis revealed the following general features. (i) The diffusive motions of the dry sample show a significant anisotropy, with a stronger component of diffusive motions parallel (Fig. 1b) than perpendicular to the membrane plane (Fig. 1a). (ii) By increasing the hydration from a lower to a higher level an increase of diffusive motions is observed. This increase is stronger for components occurring perpendicular to the membrane plane, so that at high hydration the anisotropy disappears.

| Table 1. Categories of diffusive motions |
|-----------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Category | Number of H-atoms | Type of motion and corresponding $S_{thor} (\hat{Q}, \omega)$ |
|-----------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| 1 | 834 | Methyl groups (met) (three-site jump diffusion): $S_{met} (\hat{Q}, \omega) = EISF_{met} \delta(\omega) + QISF_{met}L(H_{met}, \omega)$ |
| 2 | 180 | Protons in hydrogen bonds (hb) (two-site jump diffusion): $S_{hb} (\hat{Q}, \omega) = EISF_{hb} \delta(\omega) + QISF_{hb}L(H_{hb})$ |
| 3 | 60 (7)* | Scattering from D₂O molecules (sol) [accounted for by introducing a proton equivalent (11D = 1H) of scattering power]: $S_{sol} (\hat{Q}, \omega) = S_{sol} (\hat{Q}, \omega) \otimes S_{trans} (\hat{Q}, \omega)$ |
| 4 | 1431† | Further diffusive motions [approximated by a general two-site jump diffusion (ts)]: $S_{ts} (\hat{Q}, \omega) = EISF_{ts} \delta(\omega) + QISF_{ts}L(H_{ts})$ |
| 5 | 1431† | Fixed protons (fix): $S_{fix} (\hat{Q}, \omega) = \delta(\omega)$ |

Sum of all H-atoms: 2505 (2452)†

The theoretical scattering functions for different categories of proton motions, their corresponding structure factors [elastic incoherent structure factor (EISF) and quasi-elastic incoherent structure factor (QISF)], and parameters. The underlined parameters (six in the case of MIBEMOL spectra and an additional three for the slow two-site jump diffusion—i.e., nine in the case of IRIS spectra) were used as free fit parameters. $j_0(x) = \sin(x)/x$, spherical Bessel function; $1/\tau$ jump rate; L, Lorentzian with half width at half maximum (H).

*Wet (dry).
†Number of H atoms for the sum of categories 4 and 5.
Diffuse Motions Studied by the Multicomponent Model.

In a second step these results were analyzed in a more detailed way using the model described in Theory of Data Analysis. A priori, this model is characterized by three parameters (statistical weight \( F \), jump distance \( d \), and jump rate \( 1/r \)) for each category of motion. But, considering that fixed protons only require the weight parameter and that the latter is not independent of the other weights (\( \Sigma F = 1 \)), only 12 parameters are needed for the five proton categories. On the other hand, the phenomenological fit has been possible with only two (dry sample) or four free parameters (wet sample). Therefore a further reduction of the number of model parameters is desirable. This was achieved as follows: The calculated statistical weights (maximum possible fractions of the total number of protons, belonging to the various categories) indicate that the three-site jump diffusion (category 1) and the general two-site jump diffusion (category 4) may be expected to yield the dominating contributions to the scattering function (see Table 1, number of \( H \) atoms). Therefore, the analysis has focused on these two types of motions and fixed values were used for the parameters of category 2 and 3 (see legend of Table 2), which we consider as roughly known from structural information or other previous studies (13, 25, 26). (Note that category 2 represents only 7.2% and category 3 2.4% of the total scattered intensity). Thus the number of free parameters for the total scattering function is reduced to nine (IRIS data) and to six (MIBEMOL data), respectively (see Table 1). But even in this case the separation of the motional components is not completely unambiguous, because the fit parameters remain somewhat correlated. For this reason we were not able to obtain exact parameter values from the fits, but only upper and lower limits for the values of certain parameters. However, the results permit an interpretation of the above mentioned influence of hydration on the dynamical behavior in terms of more detailed models (see Table 2 and Fig. 2). Because of the simplicity of the models applied for the diffusive motions and considering the complexity of the structure, the separation of the measured spectra into only a few different components must be considered as a crude approximation (for more details see ref. 28). Nevertheless it leads to useful results, as follows (see Table 2).

(i) The rotational diffusion of protons in methyl groups (category 1) with correlation times of a few picoseconds is clearly a faster process than local diffusive motions of generally larger molecular subunits, described by the general two-site jump diffusion (category 4).

(ii) At high hydration level (wet), the majority (70–90%) of the protons in methyl groups participate in three-site jump diffusion with sufficiently high jump rates to be observable with the energy resolution of the experiment. The number of protons performing this kind of motion is reduced to \( \approx 50\% \) in the case of low hydration (dry). For both hydration levels, no anisotropy in three-site jump diffusion is observed.

(iii) The values obtained for the jump distances (2.4–4.8\( \AA \)) corresponding to the general two-site jump diffusion are comparable to the size of motional units of polypeptide sidegroups, which indicates that this result is reasonable in the framework of our model.

(iv) In the case of the high hydration level, the general two-site jump motion shows no anisotropy in the diffusive motions. Contrary to this, an anisotropy is observed in the case of the dry sample, which is mainly characterized by a lower number of protons participating in diffusive motions with jump vectors perpendicular to the membrane plane.

(v) The mean square displacement \( \langle \Delta r \rangle^2 \approx 0.05 \pm 0.015 \text{\( \AA \)}^2 \) of vibrational motions obtained from a global Debye–Waller factor shows identical values for both orientations and both hydration levels and exhibits no anisotropy, within the limits of experimental error.

DISCUSSION

In the past the influence of water molecules on the dynamical behavior of protein structures has been studied by various authors with different experimental methods and with molecular dynamics simulation techniques. All these studies on globular, water-soluble proteins have shown that the intramolecular mobility of a wet protein interacting with water molecules is higher than that of the protein in a dry state—i.e., at a low hydration level (29–32). The dynamical properties of proteins are affected by the surrounding solvent through van der Waals interaction and by screening of the electrostatic interaction (33). As a result of this influence, a solvent "damping" of vibrational motions in the protein is observed. This is a consequence of the lowering of part of the potential barriers, leading to more anharmonic motions and "barrier-crossing" and thus to larger amplitudes of motions in wet as
Table 2. Resulting parameter values

<table>
<thead>
<tr>
<th>Category</th>
<th>Type of motion</th>
<th>Orientation</th>
<th>$\tau$, ps</th>
<th>Wet</th>
<th>Dry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-site jump, CH$_3$</td>
<td>$\alpha = 45^\circ$</td>
<td>4–6</td>
<td>25–32</td>
<td>11–14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\alpha = 135^\circ$</td>
<td>4–6</td>
<td>24–31</td>
<td>11–13</td>
</tr>
<tr>
<td>4</td>
<td>2-site jump, “fast”</td>
<td>$\alpha = 45^\circ$</td>
<td>13–19</td>
<td>13–39</td>
<td>7–15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\alpha = 135^\circ$</td>
<td>13–19</td>
<td>15–38</td>
<td>13–20</td>
</tr>
<tr>
<td>4</td>
<td>2-site jump, “slow”</td>
<td>$\alpha = 45^\circ$</td>
<td>80–110</td>
<td>11–26</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\alpha = 135^\circ$</td>
<td>80–110</td>
<td>13–26</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>“Fixed”</td>
<td>$\alpha = 45^\circ$</td>
<td>$\gg 100$</td>
<td>17–28</td>
<td>64–70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\alpha = 135^\circ$</td>
<td>$\gg 100$</td>
<td>18–26</td>
<td>58–64</td>
</tr>
</tbody>
</table>

The parameter values for categories 1, 4, and 5 resulting from the fitting procedure using the multicomponent model. Excepting the jump distance of the three-site jump diffusion of methyl groups $d = \sqrt{3}\tau = 1.78 \AA$ (category 1), all parameters given were determined in the fit. To save space, the statistical weight factors $F$ (fractions) are given in %. For categories 2 and 3, we used the fixed values of $F_{\text{hb}} = 7.2\%$, $d_{\text{hb}} = 0.85 \AA$, $\tau_{\text{hb}} = 6.6$ ps, and $F_{\text{aa}} = 2.4\%$ (see text and Table 1). $\tau = \tau_{\text{aa}}$ for the three-site jump model; $\tau = \tau_{\text{hb}}$ for the two-site jump model (fast); and $\tau = \tau_{\text{hb}}$ for the two-site jump model (slow). Spectrometers: I, IRIS; and M, MIBEMOL.

The obtained values of jump distances, statistical weights, and jump rates, which are characterizing the diffusive motions in the PM (see Table 2), indicate that the applied models are adequate for the present purpose. In the case of the general two-site jump diffusion, the jump distances as well as the jump rates are in good agreement with the assumption of polypeptide side group reorientations. In contrast to the motion of methyl protons, we observed for this category an anisotropy in the dynamical behavior at low hydration. This dynamical anisotropy may correspond to the loop regions, which protrude into the interbilayer space between two adjacent PMs. If the PM is fully hydrated this space is filled with a layer of hydration water (interbilayer spacing 10 Å), which may increase the mobility of the loop regions of BR. In the case of low hydration, many water molecules are removed from the hydration layer (interbilayer spacing 3 Å), and the mobility, especially in the direction perpendicular to the membrane plane, is reduced. Furthermore the lipids or structural subunits of the lipids are candidates for anisotropic dynamical behavior (35). However, with the experimental data available at present and at this level of analysis, we are not yet able to localize the origin of the anisotropy in more detail.

For the methyl protons a fraction of 70–90% of the total (wet sample) was found to participate in a three-site jump diffusion fast enough to be seen with the given resolution. The dynamical behavior of this category appears to be isotropic, which is not surprising, because methyl groups are distributed nearly uniformly in the PM and do not show a particular orientation with respect to the membrane plane. NMR analysis of methyl group rotational motion in lysozyme revealed this kind of motion as the major part of internal motion occurring on the picosecond time scale (36). On the other hand, a different behavior was found in a combined computer simulation and inelastic neutron scattering analysis of alanine dipeptide (37). In the latter work the authors found that the side-chain methyl groups possess significant intramolecular intrinsic torsional barriers and, therefore, do not perform fast rotational jumps. Our analysis shows that a fast three-site jump diffusion of a major part of the methyl protons in the PM is consistent with our experiments. But with the available data, we are not able to determine the precise percentage of methyl protons participating in this fast rotational motion.

Although up to now we carried out experiments with PM samples only in the dark-adapted groundstate ($BR_{00}$) without entering the photocycle, the question of correlation between the analyzed dynamical behavior and the proton pump mechanism is still of fundamental interest. The influence of the hydration level on proton pumping and on the photocycle, studied by time-resolved absorption spectroscopy, has revealed two important effects: in contrast to the behavior in wet samples, at low hydration levels BR shows a dramatic decrease of the pump activity (38) and the decay of the M-Intermediate
Fig. 2. Hydration dependence of the incoherent structure factors as obtained from the multicomponent fit of the spectra for both sample orientations: $\alpha = 45^\circ$ (a) and $\alpha = 135^\circ$ (b). Three different structure factors are shown for fits of spectra measured at both hydration levels (dry, solid lines; wet, broken lines). The spectra were corrected for multiple scattering. The incoherent structure factors of the total elastic scattering (EISF), of the quasielastic scattering for the three-site jump diffusion (QISF$_{3}$), and of the quasielastic scattering for the general two-site jump diffusion (QISF$_{2}$) are shown. The sum of these structure factors, including QISF$_{0}$ (not shown in this figure), is equal to unity.

is slowed down (39). The formation and decay of the M-intermediate is an important step in the proton pump mechanism and in addition correlated to a conformational change of the protein structure (11). Because the existence of the observed fast diffusive reorientations might be essential for such slower conformational changes, it appears plausible that “reduced” diffusive motions in dry samples, leading to reduced internal flexibility, may be responsible for insufficient protein activity.

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