

# Water-Coupled Low-Frequency Modes of Myoglobin and Lysozyme Observed by Inelastic Neutron Scattering

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**ABSTRACT** Conformational changes of proteins often involve the relative motion of rigid structural domains. Normal mode analysis and molecular dynamics simulations of small globular proteins predict delocalized vibrations with frequencies below  $20\text{ cm}^{-1}$ , which may be overdamped in solution due to solvent friction. In search of these modes, we have studied deuterium-exchanged myoglobin and lysozyme using inelastic neutron scattering in the low-frequency range at full and low hydration to modify the degree of damping. At room temperature, the hydrated samples exhibit a more pronounced quasielastic spectrum due to diffusive motions than the dehydrated samples. The analysis of the corresponding lineshapes suggests that water modifies mainly the amplitude, but not the characteristic time of fast protein motions. At low temperatures, in contrast, the dehydrated samples exhibit larger motional amplitudes than the hydrated ones. The excess scattering, culminating at  $16\text{ cm}^{-1}$ , is suggested to reflect water-coupled librations of polar side chains that are depressed in the hydrated system by strong intermolecular hydrogen bonding. Both myoglobin and lysozyme exhibit ultra-low-frequency modes below  $10\text{ cm}^{-1}$  in the dry state, possibly related to the breathing modes predicted by harmonic analysis.

## INTRODUCTION

Conformational changes in many proteins involve the relative movement of nearly rigid structural elements. This applies to the R-T transition of hemoglobin where the  $\alpha/\beta$ -subunits rotate upon oxygen binding as well as to hinge-bending motions of structural domains. Such open/closed structures of lysozyme, hexokinase, and citrate synthase have been probed by X-ray crystallography and normal mode analysis (Faber and Matthews, 1990; Bennett and Steitz, 1980; Wiegand and Remington, 1986; Marques and Sanejouand, 1995) suggesting that the conformational path between the open and closed form of these proteins involves a few delocalized low-frequency modes. By normal mode analysis it was further found that the conformational change in myoglobin upon oxygenation proceeds essentially via six modes with frequencies  $<12\text{ cm}^{-1}$  (Seno and Go, 1990). The frequency of the softest mode was at  $5\text{ cm}^{-1}$ , comparable to results with trypsin inhibitor, lysozyme, and hexokinase. The softest modes exhibit the largest thermal amplitudes,  $u_n$ , according to  $\langle u_n^2 \rangle \propto k_B T / \omega_n^2$ , and should thus be easy to observe. However, the corresponding vibrational periods are of the order of 1 ps, which falls in the range of fast dissipative processes. The common time scale allows the dissipation of mechanical energy within a few cycles, which raises doubts whether such vibrations remain underdamped at room temperature. Furthermore, the vibrational spectrum interferes with a quasielastic component centered at  $\omega = 0$ .

The far-infrared range below  $20\text{ cm}^{-1}$  is still difficult to explore, which applies also to Raman scattering and dielec-

tric spectroscopy. This so-called interaction region reflects collective motions in contrast to vibrations of individual molecular groups (Nielsen, 1993). The first low-frequency Raman experiments performed by Brown et al. (1972) on lyophilized chymotrypsin showed a broad maximum at  $30\text{ cm}^{-1}$ . The maximum was much less pronounced in the denatured form. The authors suggested intramolecular vibrations of elements of secondary structure as a likely explanation. Genzel et al. (1976) performed similar Raman experiments with lysozyme crystals and found a peak at  $25\text{ cm}^{-1}$  that was absent in solution. In contrast to Brown et al. (1972), they attributed this band to intermolecular vibrations of the crystal. Bartunik et al. (1982) observed a broad peak centered at  $25\text{ cm}^{-1}$  in neutron scattering experiments on polycrystalline lysozyme. Similar results were obtained by Raman scattering for a number of proteins (Painter et al., 1982). A small minimum in the far infrared transmission spectrum of dry lysozyme was observed at  $19\text{ cm}^{-1}$  using synchrotron radiation (Moeller et al., 1992). Neutron scattering experiments with dry trypsin inhibitor also revealed a broad maximum in the dynamical structure factor at  $\sim 25\text{ cm}^{-1}$  (Cusack, 1989). A similar peak was found with hydrated myoglobin at low temperature (Cusack and Doster, 1990; Doster et al., 1990), lyophilized hemoglobin, and even red blood cells (Martel et al., 1991). At room temperature, in contrast, this feature was buried by strong quasielastic scattering. With dry collagen, however, several peaks at 17, 27, 40, and  $57\text{ cm}^{-1}$  were identified by neutron spectroscopy at room temperature (Berney et al., 1987). The functional relevance of this frequency range was stressed by the result that the light-induced electron transfer in reaction centers couples to a protein mode in the vicinity of  $15\text{ cm}^{-1}$  (Vos et al., 1993).

On the theoretical side, analytical calculations and estimates of the intraprotein hydrogen bond force constants led Chou (1985) to introduce accordionlike excitations of  $\alpha$ -

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helices and  $\beta$ -sheets that have their fundamental frequency between 20 and 30  $\text{cm}^{-1}$ . It is difficult, however, to account for solvent effects in these calculations. Go (1978) proposed that the low-frequency spectrum of a protein could be interpreted in terms of vibrations of an elastic sphere. This implies that the characteristic frequencies vary with the reciprocal radius of the sphere, which is not observed (Painter et al., 1982). Extensive temperature-dependent MD simulations of myoglobin with and without water reproduce the maximum observed in the neutron dynamical structure factor reasonably well (Smith et al., 1990; Steinbach et al., 1991). The maximum, however, is shifted to a lower frequency, 10 to 20  $\text{cm}^{-1}$ . Possible reasons for this discrepancy could be inaccurate force fields related to electrostatic cutoff or intermolecular interactions involving more than three molecules, which are not included in the simulation. In the hydrated powder used in the experiment, neighboring protein molecules are embedded in, and therefore connected by, overlapping hydration shells that form a rigid hydrogen bond network at low temperature. Nevertheless, the mere observation of such a low-frequency band in simulations of a single protein molecule suggests an assignment of the experimental results mainly to intramolecular vibrations. McCammon and Wolynes (1977) have used molecular dynamics to estimate the damping coefficient of the hinge bending mode of lysozyme. They found that the two lobes move in a diffusive manner strongly damped by the solvent. The quasielastic scattering may thus result in part from overdamped soft modes, but a variety of other diffusive motions, such as dihedral transitions, also contribute to the spectrum. Under realistic physiological conditions it seems difficult to discriminate the characteristic spectrum of delocalized modes from the background of other processes.

Below we explore the effect of changing the temperature and degree of hydration to modify the damping coefficient. Molecular dynamics simulations suggest that the rate of dihedral transitions in myoglobin increases upon dehydration because of reduced solvent-damping (Steinbach and Brooks, 1993, 1996). Furthermore, the strong hydrogen bonds formed in the hydrated sample may suppress the collective vibrations at low temperatures. Fewer bonds are expected at low hydration. Finally, by H/D exchange we intend to discriminate between the dynamics of polar and nonpolar residues. The relevant frequency range can be studied conveniently by using cold neutron scattering. In this case, the incoherent scattering by the protein protons is not restrained by selection rules and reflects the proton-weighted vibrational density of states (Smith et al., 1986; Bée, 1988; Cusack and Doster, 1990).

## MATERIALS AND METHODS

The neutron scattering experiments were performed using the time-of-flight spectrometer IN 5 at the Institut Laue Langevin in Grenoble at an incident neutron wavelength  $\lambda_0 = 6 \text{ \AA}$ . For each scattering angle  $2\theta$  the bank of neutron detectors registers the number of neutrons that arrive in the detector acceptance area  $\Omega$  after a flight time  $t_F$ . This quantity is denoted

by  $\partial^2\sigma(2\theta, t_F)/\partial\Omega\partial t_F$ . The time of flight depends on  $\lambda_0$  and the energy gain or loss  $\hbar\omega$  of the neutron scattered by the sample. It denotes the transit time of the scattered neutron for its path from the sample to the detector. Thus the energy exchange ( $1 \text{ meV} = 8 \text{ cm}^{-1}$ ) of the neutron with incident energy  $E_0$  is given by:

$$\hbar\omega(t_F) = \frac{m_n (SD)^2}{2 \left(\frac{SD}{t_F}\right)^2} - E_0 \quad (1)$$

The first term represents the kinetic energy of the scattered neutron expressed by use of the distance  $SD$  between sample and detector and the neutron mass  $m_n$ . The dynamical structure factor  $S(2\theta, \omega)$  was determined from the time-of-flight spectrum according to (Bée, 1988):

$$S(2\theta, \omega) = \frac{k_0}{k} \frac{\partial^2\sigma(2\theta, \omega)}{\partial\Omega\partial\omega} = \frac{\partial^2\sigma(2\theta, t_F)}{\partial\Omega\partial t_F} \times \frac{t_F^4}{SD^3} \frac{\hbar^2/2\pi\lambda_0}{m_n^2} \quad (2)$$

where  $k_0$  and  $k$  denote the initial and final neutron wavenumbers. A configuration of IN5 was used where the instrumental resolution is a Gaussian with  $\hbar\Delta\omega_{1/2} = 30 \text{ \mu eV}$  (half width at half maximum).

The following four samples were examined: D<sub>2</sub>O-hydrated horse myoglobin (Sigma Chemical Co., St. Louis, MO) at  $h = 0.35 \text{ g/g}$  (Mb-D<sub>2</sub>O) representing  $\sim 350$  water molecules per protein; a deuterium-exchanged sample Mb-dry(D) which was dried to below  $h = 0.05 \text{ g/g}$ , representing  $< 50$  water molecules per protein; and a normally protonated myoglobin sample Mb-dry(H) which was also dried to below  $h = 0.05 \text{ g/g}$ . We also studied a dehydrated, D-exchanged lysozyme sample Lys-dry(D). By adjusting the amount of sample ( $\sim 400 \text{ mg}$ ) a common transmission of 92% was achieved. The samples were measured for 12 h at 320 and 150 K and corrected for detector efficiency using a vanadium standard. The spectrum of the sample can (aluminum) was subtracted from the data. The incoherent contribution of the protein protons dominates the neutron scattering spectra, because of their large cross-section in relation to other nuclei (Bée, 1988).

## RESULTS

Fig. 1 shows selected scattering functions of deuterated myoglobin at full (*closed symbols*) and low (*open symbols*) hydration, evaluated according to Eqs. 1 and 2. The full line represents the elastic component of the spectrum whose shape is given by the resolution function of the instrument. The elastic intensity decreases with increase in temperature or mobility, which is compensated for by quasielastic and inelastic scattering. The spectra start to deviate from the elastic line at  $\approx 0.05 \text{ meV}$  or  $0.4 \text{ cm}^{-1}$ . At 320 K both spectra decrease with increasing energy transfer, suggesting a maximum at  $\omega = 0$ , characteristic of diffusive displacements. A second broad feature centered at  $\sim 3 \text{ meV}$  exhibits a maximum at finite frequencies, implying vibrational motions.

At 320 K the lyophilized Mb-dry(D) sample shows much less quasielastic scattering below 1 meV than the D<sub>2</sub>O-hydrated one. The difference in quasielastic intensity of Mb-D<sub>2</sub>O versus Mb-dry(D), is assigned to water-induced protein motions because of the small contribution of D<sub>2</sub>O ( $< 5\%$ ) to the spectrum as compared to the nonexchangeable protein protons. At 150 K little remains of the quasielastic component and the spectra of hydrated and lyophilized myoglobin nearly superimpose in this range.

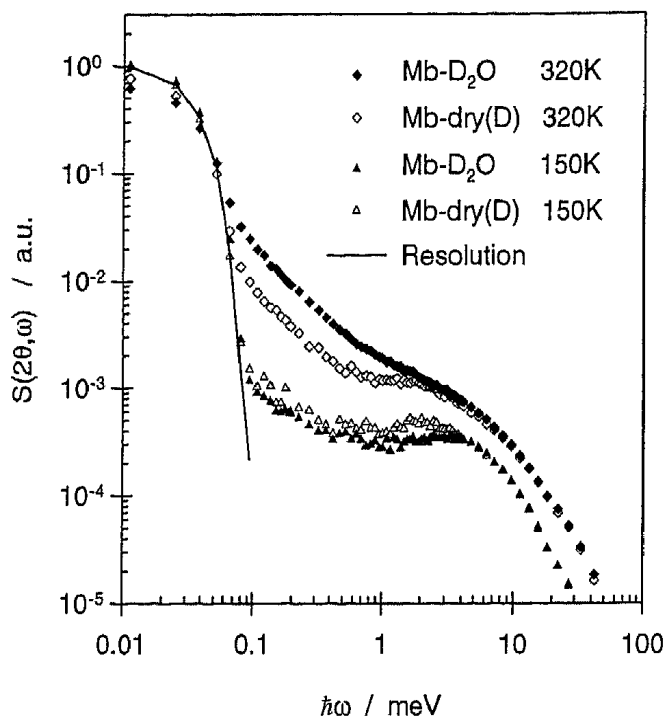


FIGURE 1 Dynamical structure factors (mostly incoherent) of  $D_2O$ -hydrated (Mb- $D_2O$ ) and dry deuterium-exchanged myoglobin [Mb-dry(D)] at 320 and 150 K. The selected spectra refer to a scattering angle  $2\theta = 100^\circ$ , and were normalized by the total cross section of the respective samples. The solid line represents the resolution function of the instrument.

The inelastic spectrum of the hydrated system consists of a broad band, with the maximum centered at 3 meV. This feature has been observed for several proteins, as discussed in the Introduction. At 150 K, in contrast to high temperatures, it is the dry sample which exhibits stronger inelastic scattering than the hydrated one. This is shown in detail in Fig. 2 for myoglobin [Mb-dry(D)] and lysozyme [Lys-dry(D)]. The extra intensity gives rise to a maximum in the structure factor at 2 meV ( $16 \text{ cm}^{-1}$ ) and shoulders at 1 and 3 meV. The spectra merge again above 4 meV, suggesting

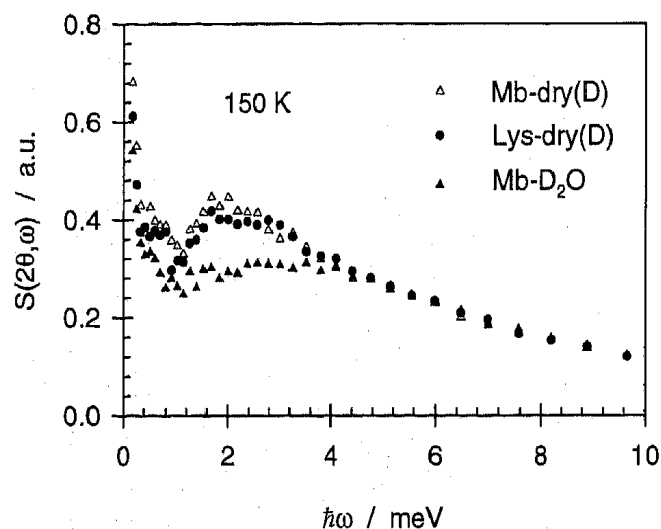


FIGURE 2 Inelastic low frequency excitations of Mb- $D_2O$ , Mb-dry(D), and Lys-dry(D) at 150 K. A deficit of modes in the hydrated protein (Mb- $D_2O$ ) leads to a lower intensity between 0.5 and 4 meV.

a background of water-independent modes. In previous work it was found that the hydrated samples of myoglobin and lysozyme exhibit virtually identical spectral lineshapes (Settles, 1996). The small differences above  $40 \text{ cm}^{-1}$  observed previously (Doster et al., 1993) could be related to the translational band of  $D_2O$ , which becomes noticeable at water concentrations above 0.4 g/g.

To discriminate in mobility between polar and nonpolar residues we compare the spectra of protonated and deuterium-exchanged protein. Myoglobin, known as the prototype of the oil drop model of protein stability, consists of a polar surface and a highly nonpolar core. The isotopic H/D exchange thus highlights the difference in mobility between the surface and internal residues. The comparison has to be made at low water content, because  $H_2O$  would give a strong contribution to the spectrum. Fig. 3 displays the ratio of the structure factors Mb-dry(H)/Mb-dry(D) versus energy exchange  $\hbar\omega$  at 320 K and 150 K. This ratio appears to be constant and compatible with the calculated ratio of cross-sections (solid line) over the entire frequency range within experimental uncertainty. The broad peak in the vicinity of 70 meV represents the hindered rotation band of residual structural water,  $H_2O$ , in Mb-dry(H). The comparison of the band intensities with 0.4 g/g  $H_2O$ -hydrated myoglobin (not shown) leads to an estimate of  $40 (\pm 10)$  residual water molecules in Mb-dry(H). Comparing Mb-dry(H) and Mb-dry(D),  $\sim 300$  of the almost 1300 protein protons were exchanged by deuterons, the latter giving a negligible contribution to the spectrum. The removal of polar protons apparently did not affect the spectral lineshape to a significant extent. It follows that both types of hydrogens report similar or even identical motions at 150 and 320 K. This implies either negligible site heterogeneity or delocalized, collective displacements.

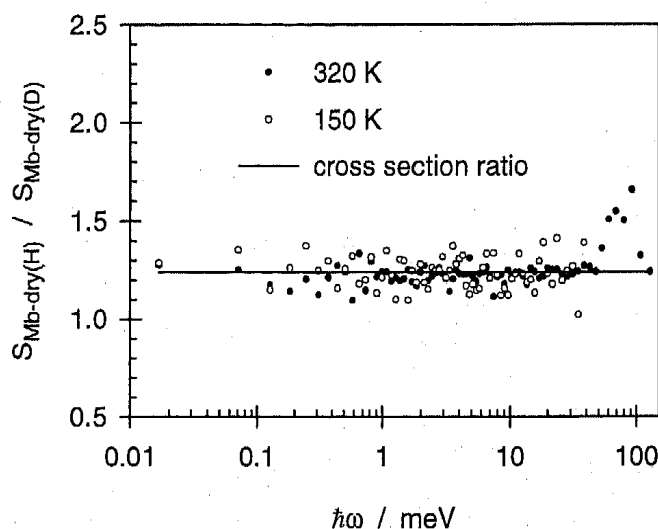


FIGURE 3 Ratio of the dynamical structure factors of Mb-dry(H) to Mb-dry(D) at 320 K and 150 K. The solid line represents the calculated ratio of the total cross sections.

## DISCUSSION

### Analysis of water-induced diffusive motions

The spectra of hydrated and dehydrated myoglobin differ considerably at 320 K (Fig. 1). However, if adjusted by a constant factor, the data superimpose in the quasielastic range. Since the experimental energy window is limited, it is not evident whether the spectra differ in spectral amplitude or linewidth. The assumption of a Lorentzian lineshape leads to the following high-frequency limit ( $\hbar\omega \gg \Gamma$ ) of the dynamical structure factor:

$$S(Q, \omega) \propto \frac{A\Gamma}{\omega^2} \quad (3)$$

The constant  $A$  denotes the amplitude and  $\Gamma$  represents the width of the spectrum. The changes that occur with increase in hydration could thus be equally well explained by larger motional amplitudes  $A$ , or a shorter relaxation time  $1/\Gamma$ . The observed lineshape, however, deviates from a Lorentzian, which excludes explanations based on a simple mechanism.

In previous work, covering a wider range in energy exchange and temperature, we found that the quasielastic spectrum of hydrated myoglobin effectively contains two components that were approximated by two power laws (Doster et al., 1990):

$$S(Q, \omega) \propto A\omega^{-1-b} + B\omega^{-1+a} \quad (4)$$

To put this result into perspective, consider the model of Brownian motion defined by large particles suspended in a solvent composed of many small particles. This system exhibits a two-component spectrum: a Lorentzian line ( $b = 1$ ) due to diffusion of the large particles, and white noise ( $a = 1$ ) approximating the much faster dynamics of the solvent. The mobile units of a globular protein—essentially the side chains—interact strongly with each other and their relative motions do not exhibit such a clear-cut separation of time scales. The resulting dynamical correlations, site heterogeneity, and the coupling to water all modify the spectral shape. Mode coupling theory of simple liquids predicts a two-component lineshape such as Eq. 4 for dense liquidlike systems and allows calculation of the parameters in simple cases (Götze and Sjögren, 1992). The basic idea of the theory attributes the dominating influence on short time dynamics to the cage effect that each particle experiences by its nearest neighbors. The two spectral components then presumably reflect fast local motions in the cage (exponent  $a$ ) and collective reorganization of the cage (exponent  $b$ ). Analysis of experimental data based on this theory (Doster et al., 1990) and computer simulations of myoglobin suggest indeed a liquidlike mobility of protein side chains (Kneller and Smith, 1994).

By interpreting our results according to the model of Eq. 4, we intend to elucidate whether water affects mainly amplitude or correlation time of protein motions. To extract these features from the data in Fig. 1 requires separation of

diffusive from vibrational modes. As discussed above, at 150 K, diffusion is too slow to contribute to the spectrum, which allows isolation of the vibrational component. For harmonic vibrations one is permitted to extrapolate the spectrum to higher temperatures using the density of states,  $g(\omega)$ , the Bose occupation factor  $n(\omega) = 1/(\exp(\hbar\omega/k_B T) - 1)$ , and the Debye-Waller factor  $f(Q, T) = \exp(-\langle x^2 \rangle Q^2)$  according to Bée (1988):

$$S_h(Q, \omega) = \frac{\hbar^2 Q^2}{2m} \times g(\omega) \times \frac{n(\omega, T)}{\omega} \times f(Q, T) \quad (5)$$

where  $m$  denotes the effective mass. Below 180 K the inelastic neutron scattering spectra of myoglobin and lysozyme at low frequency follow this equation rather well (Cusack and Doster, 1990; Doster et al., 1990). We use Eq. 5 to approximate the vibrational component at 320 K, which is subtracted from the experimental spectrum. This procedure leads to a good match of the scaled 150 K spectrum and the one at 320 K in the inelastic range above 3–4 meV, indicating harmonic behavior. At lower energy transfers, however, the spectra are not identical due to the additional quasielastic scattering that appears at 320 K. The resulting difference spectra, shown in Fig. 4 for the hydrated and dehydrated samples of myoglobin, represent the anharmonic effect. The decomposition appears meaningful for the following reasons: in the crossover region between diffusive and vibrational motions near 2–3 meV, the observed difference spectrum exhibits a Gaussian short-time behavior, which must be expected of any reasonable spec-

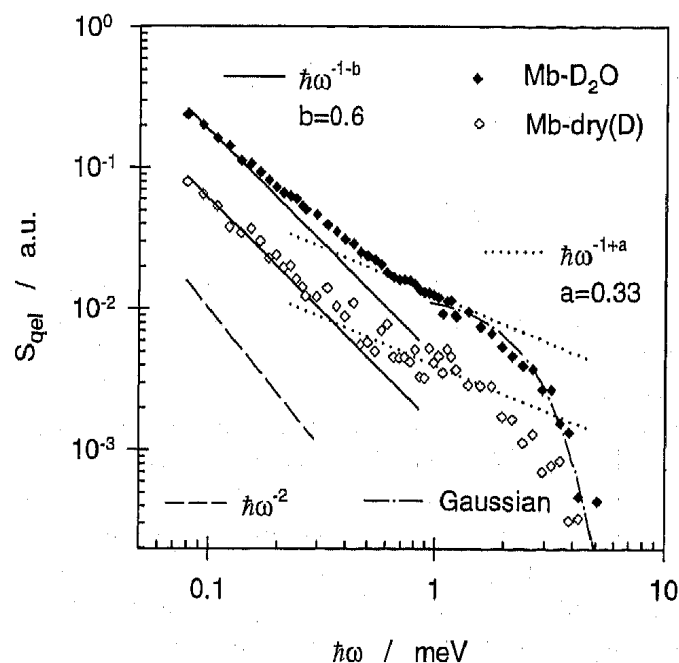


FIGURE 4 Quasielastic spectra of dry and hydrated myoglobin after subtraction of a harmonic background (Eq. 5). The dashed-dotted line validates the Gaussian shape of the high frequency tail, expected of ballistic motion. The solid and dotted lines represent the power laws of Eq. 4 that describe the two-step decay in both the hydrated and dry protein with the same exponents. The asymptotic behavior of a Lorentzian is also given for comparison (dashed line).

trum in the ballistic limit. Furthermore, the temperature-dependence of the Gaussian tail is small (Doster et al., 1989), suggesting that major components of the vibrational spectrum remain underdamped up to room temperature.

Fig. 4 also shows two lines representing the power laws discussed above. The values of the corresponding exponents  $b = 0.6 (\pm 0.1)$  and  $a = 0.33 (\pm 0.05)$  have been taken from a previous analysis of hydrated myoglobin (Doster et al., 1990). The two spectral components tend to merge at high temperatures. At 320 K the second power law corresponding to the fast process has only a small range of validity and exponent  $a$  is ill-determined. Exponent  $b = 0.6$  instead of 1.0 (Lorentzian) seems to approximate the data of both the hydrated and dehydrated sample reasonably well. Most important, the data superimpose within statistical error if the Mb-dry(D) spectrum is multiplied by a factor of 3.1 ( $\pm 0.1$ ) common for both power laws. The persistence of the spectral line shape with change in hydration implies that water primarily enhances the dynamic amplitudes and to a lesser extent the structural transition rates on a picosecond time scale. A change in the relaxation times would shift the energy of intersection—which is  $\sim 0.35$  meV—of the two power laws. This is not the case. Such a shift was observed, however, in response to changes in the temperature (Doster et al., 1990). The conclusion is based on the validity of the above decomposition procedure. In any case, the hydration dependence suggests that the observed spectra indirectly monitor the dynamics of protein-water hydrogen bonds. In the dry state, a reduced fraction of weak hydrogen bonds that open and close would result in a lower side-chain mobility and less quasielastic scattering.

### The interpretation of the water-coupled vibrational spectrum

The main intention of this study was to assess the existence of underdamped collective displacements at low frequency that were predicted for several proteins by normal mode analysis. At high temperatures strong quasielastic scattering due to structural transitions prohibits the direct observation of vibrational modes at low frequency. At low temperatures quasielastic scattering vanishes and we observe for hydrated proteins a broad maximum in the structure factor centered around  $25 \text{ cm}^{-1}$ . This feature has been observed invariably in the low-frequency neutron and Raman scattering spectra of various proteins of different structure and molecular weight (Painter et al., 1982; Martel et al., 1991). Furthermore, acid denatured myoglobin and a mixture of hydrated amino acids, composed according to the sequence of myoglobin, also showed the same feature in the inelastic neutron structure factor at low temperatures (unpublished experiments). The corresponding vibrations are thus not only independent of conformation and packing of the main chain, but also of the chemical linkage of the monomers. This suggests an interpretation in terms of water-coupled side-chain librations. The observed sensitive dependence of the

protein spectra on the degree of hydration supports this conclusion.

At low hydration the band maximum has shifted to  $16 \text{ cm}^{-1}$  and has become more pronounced. The neutron scattering spectra of purple membrane, which contains bacteriorhodopsin (BR), also exhibits a band near  $20 \text{ cm}^{-1}$  at low temperature even at full hydration (Ferrand et al., 1993). BR is partially shielded from the solvent by lipids, which may result in properties similar to those of dehydrated globular proteins. Furthermore, a maximum in the neutron structure factor at  $15\text{--}20 \text{ cm}^{-1}$  was derived in a simulation of myoglobin in vacuo (Smith et al., 1990). Steinbach et al. (1991) have performed extensive low-temperature simulations of myoglobin at different degrees of hydration. In their Fig. 2 *a* they display the simulated incoherent dynamical structure factors of myoglobin at 100 K at different water contents: it shows a vibrational band near  $8 \text{ cm}^{-1}$  which decreases in magnitude, shifting to higher frequencies, with increasing degree of hydration. The authors conclude that low-frequency modes are depressed by protein-water interactions.

Taken together these findings suggest that correlated librational modes of side chains, in particular those of polar side chains interacting with water, lead to the spectral feature observed between 10 and  $30 \text{ cm}^{-1}$ . The observed invariance of the vibrational spectrum to H/D exchange indicates that in the dry state polar and nonpolar residues exhibit similar dynamics. The link of polar groups to the surrounding H-bond network of water removes the degeneracy and leads to modified force constants and viscous damping. In this view, dehydration is expected to decrease the number and strength of protein-water H bonds. The smaller force constants will lower the peak frequency and increase the vibrational amplitude. The data in Fig. 2 support this conclusion. In quantitative terms, Eq. 5 for the dynamical structure factor  $S(Q, \omega)$  yields in the high temperature limit  $\hbar\omega \ll k_B T$

$$S(Q, \omega) \propto \hbar Q^2 \times g(\omega) \times \frac{k_B T}{2m\omega^2}. \quad (6)$$

A downshift in frequency by a factor of 1.5 should about double the inelastic intensity. From the data at 2 meV in Fig. 2 we estimate an increase by a factor of 1.4, which is somewhat smaller. The difference could result from a hydration-independent background due to librations of nonpolar side chains.

The above tentative assignment accounts qualitatively also for the plasticizing effect of water on the protein structure: the shear modes of water coupled to side chains can reach a critical size where the H bond flips. This process results in irreversible displacements, giving rise to quasielastic scattering. The number of such short-lived excursions, and thus the dynamical amplitude, increases with the temperature. Such a mechanism can be quantified in terms of an asymmetric two-state model which we have proposed earlier (Doster et al., 1989).

The neutron and Raman scattering spectra of bulk water display a broad band centered at  $50 \text{ cm}^{-1}$ . It has been assigned to flexing modes of three water oxygens hydrogen-bonded to each other (Sceats and Rice, 1980). This band still exists for protein-adsorbed water as we have recently shown (Settles and Doster, 1996). The protein and the water band overlap and exhibit a similar temperature dependence. This correlation indicates that sidechain-water flexing modes contribute to the density of states in the range of 20 to  $30 \text{ cm}^{-1}$ . As for bulk water the corresponding correlation length should be rather small, comprising typically a water molecule and two side chains interconnected by hydrogen bonds.

Normal mode analysis yields collective excitations with frequencies in the range of 6 to  $12 \text{ cm}^{-1}$  for myoglobin (Seno and Go, 1990) and BPTI (Smith et al., 1986). In the hydrated case, our experiments provide no conclusive evidence of such large-scale displacements. But at low hydration, the spectra of both proteins, Mb-dry(D) and Lys-dry(D), in Fig. 2 display additional intensity below  $10 \text{ cm}^{-1}$ . This result may encourage further studies on proteins at low hydration in the range of ultra-low frequency. It also suggests that membrane proteins that are less exposed to water than globular proteins are more likely to exhibit underdamped collective modes at physiological conditions. In fact, such modes have been reported to persist for some picoseconds even at ambient temperature in a membrane protein (Vos et al., 1993). The interaction with water in contrast causes not only the intermediate and high-frequency vibrations to experience a rigidified, gellike system, but also the softer modes to exchange energy with diffusive modes of water. This leads to strong damping of modes whose period exceeds the average life time of a hydrogen bond. The crossover frequency connecting the two regimes appears to be near  $25 \text{ cm}^{-1}$ , corresponding to a vibrational period of 1.3 ps. This value is close to the fastest relaxation time observed by neutron scattering in bulk water (Bellissent-Funel et al., 1992). A quantitative analysis requires the concept of viscoelasticity and a frequency-dependent damping coefficient of protein vibrations (McCammon and Wolynes, 1977; Doster, 1983; Schlitter, 1988; Götze and Sjögren, 1992).

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