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ARTICLE

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# Vibrational frequency shifts as a probe of hydrogen bonds: thermal expansion and glass transition of myoglobin in mixed solvents

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**Abstract** The contribution of hydrogen bonds to protein-solvent interactions and their impact on structural flexibility and dynamics of myoglobin are discussed. The shift of vibrational peak frequencies with the temperature of myoglobin in sucrose/water and glycerol/water solutions is used to probe the expansion of the hydrogen bond network. We observe a characteristic change in the temperature slope of the O–H stretching frequency at the glass transition which correlates with the discontinuity of the thermal expansion coefficient. The temperature-difference spectra of the amide bands show the same tendency, indicating that stronger hydrogen bonding in the bulk affects the main-chain solvent interactions in parallel. However, the hydrogen bond strength decreases relative to the bulk solvent with increasing cosolvent concentration near the protein surface, which suggests preferential hydration. Weaker and/or fewer hydrogen bonds are observed at low degrees of hydration. The central O–H stretching frequency of protein hydration water is red-shifted by 40 $cm^{-1}$ relative to the bulk. The shift increases towards lower temperatures, consistent with contraction and increasing strength of the protein-water bonds. The temperature slope shows a discontinuity near 180 K. The contraction of the network has reached a critical limit which leads to frozen-in structures. This effect may represent the molecular mechanism underlying the dynamic transition observed for the mean square displacements of the protein atoms and the heme iron of myoglobin.

**Key words** Infrared spectroscopy · Protein dynamics · Amicle bands · Preferential hydration

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## 1 Introduction

Freezing represents a convenient method to store and preserve biological materials. Ice formation, however, proved to be damaging to cells and subcellular biological structures. A possible solution to this problem, which is a strategy also followed by some organisms, is to replace the water by a glass forming solvent (Crowe et al. 1996). The liquid to glass transition occurs at constant volume and is thus less harmful to biological structures than crystallization. These solvents also expand the small temperature window available for the study of biomolecules in their native state. One important goal of low temperature work is to establish correlations between structural, dynamic and functional properties of proteins. Low temperatures however emphasize the strength of protein-solvent interactions up to the point of structural arrest at the glass transition. In this context we intend to discuss the question of how protein-solvent hydrogen bonds behave as a function of the temperature and how this affects structural flexibility and protein function. The oxygen transport and storage protein, myoglobin, serves as a model system because it is one of the few biomolecules which has been studied over a wide range of temperatures.

We first summarize some experimental results obtained when myoglobin is cooled to subzero temperatures: X-ray scattering on myoglobin crystals records a continuous contraction of the unit cell and of the molecular volume with decrease in temperature (Frauenfelder et al. 1987). Although no major changes in conformation occur, a nonuniform and anisotropic thermal expansion across the molecule was deduced from the X-ray analysis. Similar continuous but nonuniform changes have been found for the residue temperature factors (Frauenfelder et al. 1979; Parak et al. 1987).

In contrast, Mössbauer spectroscopy (Parak et al. 1982) and neutron scattering (Doster et al. 1989), methods with a built-in time resolution (140 ns and 150 ps respectively), reveal a discontinuous temperature dependence of the atomic mean square displacements: For crystals and hy-

drated powders the displacements below 180 K increase linearly with the temperature, a characteristic feature of harmonic solids. Above 180 K one observes a striking increase in the dynamic amplitudes, suggesting the excitation of new types of motion. This is supported by the simultaneous onset of quasielastic broadening in the central lines observed with both methods. Assuming a simple relaxation process which speeds up with the temperature would lead to transition temperatures which decrease with increasing time resolution of the experimental method. The observed transition temperatures however are invariably about 180 K for hydrated proteins. Furthermore, molecular dynamics simulations of hydrated myoglobin reproduce the discontinuity in the neutron scattering results (Lonchariche and Brooks 1990; Steinbach and Brooks 1993; Steinbach and Brooks 1996). Similarly, the Gaussian width of the Soret band shows a cross-over from harmonic to nonharmonic behaviour in this temperature range, suggesting the coupling of the heme group to fast motions (Cupane et al. 1995). Finally, a second transition which induces even larger displacements was observed with neutron scattering (Doster et al. 1989) and Mössbauer-Rayleigh scattering (Krupyanskij et al. 1982) with hydrated myoglobin near 250 K. Frauenfelder and collaborators have performed extensive studies on the flash-induced ligand binding to myoglobin at low temperature. Their most striking result was the observation of strongly non-exponential rebinding kinetics of $O_2$ and CO to myoglobin (Austin et al. 1975). They showed that the data could be described using a temperature invariant distribution of activation enthalpies. The combination of multiple flash kinetic results and the X-ray temperature factors (Frauenfelder et al. 1979; Parak et al. 1987) was used to connect the enthalpy spectrum to a distribution of conformational substates (Austin et al. 1975; Frauenfelder et al. 1979; Parak et al. 1987; Post et al. 1993). Essentially, the enthalpy spectrum is supposed to reflect the variance about the average structure, while each protein molecule remains arrested in a particular substate. The structural rigidity also prevents the photodissociated CO molecule from leaving the heme pocket. Softening of the structure above the glass temperature of the solvent opens channels which allow the ligand to escape to the solvent. The escape fraction was shown to depend on the viscosity of the solvent (Beece et al. 1980; Doster et al. 1995).

The dynamic transition at about 180 K was also seen with other hydrated systems such as lysozyme (Settles 1996), bacteriorhodopsin (Ferrand et al. 1993), superoxide dismutase (Andreani et al. 1995) and tRNA (Doster et al. 1992).

The nature of this transition and its relation to functionally important motions, is still not fully understood. The forces controlling the transition may still be operative at room temperature. The common phenomenology in the temperature dependence of several experimental quantities suggests a related molecular mechanism. Below we discuss the role of protein-solvent hydrogen bonds in this context.

In our previous work we have studied the temperature dependence of the O–D stretching band of partially $D_2O$-hydrated myoglobin (Doster et al. 1986). The band is 160 $cm^{-1}$ wide and has its maximum near $\nu$ ($OD$) = 2480 $cm^{-1}$. The redshift of $\delta\nu = 40$ $cm^{-1}$ relative to bulk HO–D (2520 $cm^{-1}$) indicates a substantial increase in hydrogen bond strength. Decreasing the temperature leads to a continuous redshift reaching 2440 $cm^{-1}$ at 180 K. Below 180 K the $\nu(OD)$ shifts at a much smaller rate with the temperature. This discontinuity in the temperature slope was interpreted as the signature of a glass transition in the hydration shell. It was proposed that the structurally arrested adsorbed water immobilises the protein structure leading to a static conformational heterogeneity (Doster et al. 1986; Doster 1986). Similar conclusions were derived from pressure relaxation experiments with myoglobin in glycerol/water. Frauenfelder and collaborators proposed that the protein structural motions may be 'slaved' by the glass transition of the solvent (Iben et al. 1989). We thus extended our infrared experiments to include mixed solvents. We first discuss the correlation between O–H stretching frequency and thermal expansion.

## 2 Vibrational frequency shifts and thermal expansion

The volume expansion coefficient $\alpha$ at constant pressure P is defined by:

$$\alpha = \frac{1}{V}\frac{\partial V}{\partial T}\bigg|_P \tag{1}$$

The normal mode frequencies, $\nu(n)$, of a quasiharmonic solid decrease with increasing volume, $\nu(n) \propto V^{-\gamma(n)}$. The exponent $\gamma(n)$ is called the Grüneisenparameter of mode $n$ which typically assumes values close to unity (Ashcroft and Mermin 1981). The relative changes of the mode frequencies with the temperature are thus determined, according to Eq. (1), by the volume expansion coefficient according to:

$$\alpha = -\frac{1}{\gamma(n)\cdot\nu(n)}\frac{\partial\nu(n)}{\partial T} \tag{2}$$

This equation applies to volume modes of a quasiharmonic solid. We are however interested in molecular vibrational modes of protein solutions. For instance, low frequency modes of dehydrated purple membrane fragments give rise to a maximum in the inelastic neutron structure factor near 20 $cm^{-1}$ at 100 K which shifts to 16 $cm^{-1}$ at 300 K (Ferrand et al. 1993). Assuming the validity of Eq. (2) and $\gamma \approx 1$, leads to a volume expansion coefficient of $10^{-3}$/K, about twice that of glycerol. Unfortunately, in hydrated and solvated systems, this band interferes with quasielastic scattering and cannot be extracted easily (Doster et al. 1990; Cusack and Doster 1990; Ferrand et al. 1993).

In a series of infrared experiments on protein solutions as a function of the temperature we noticed that Eq. (2) may hold even for some localized vibrational modes. We found in particular that the O–H stretching frequency of pure glycerol $\nu(OH)$ as a function of the temperature is well

approximated by the following equation:

$$\frac{1}{\nu(OH)}\frac{\partial\nu(OH)}{\partial T} = -\alpha_l \cdot \gamma(OH) \quad (3)$$

with $\gamma(OH) \approx 1$. $\alpha_l$ denotes the linear expansion coefficient which in isotropic systems is given by $\alpha_l = \alpha/3$. At first sight this result seems surprising since the thermal expansion of glycerol is certainly not dominated by the O–H stretching vibration. The relevant modes must involve instead the intermolecular displacements of entire glycerol molecules. The force constants however, of these intermolecular modes depend on the strength of hydrogen bonds which also affects the O–H stretching vibration. This correlation may explain qualitatively why the O–H stretching frequency apparently couples to the thermal expansion in hydrogen bonded systems.

For ice I a quantitative correlation between O–H stretching frequency and the average O–O nearest neighbour separation was established: It was found that $\partial\nu(OH)/\partial R_{OO} = 1843\ \mathrm{cm^{-1}\ \AA^{-1}}$ and $\partial\nu(OD)/\partial R_{OO} = 1362\ \mathrm{cm^{-1}\ \AA^{-1}}$ for $R_{OO} = 2.76$ Å (Sceats and Rice 1982).

The frequencies of the uncoupled O–H and O–D oscillators increase with temperature; this was attributed to thermal expansion, consistent with the known increase of the lattice parameters (Luck 1973; Sceats and Rice 1982; Joesten and Schaad 1985). These results imply a Grüneisen relation according to Eq. (3) between the O–H(D) stretching frequencies and the linear thermal expansion coefficient. We abbreviate the left hand side of Eq. (3) by $\alpha_\nu(OH/D)$ which gives:

$$\alpha_\nu(OH/D) = -\gamma(OH/D) \cdot \alpha_l \quad (4)$$

This equation ignores the effect of bond linearity on the stretching vibration. A distortion of the tetrahedral angle may change the stretching frequency without affecting the volume. With the IR-data on the uncoupled O–D stretch in ice of Ford and Falk (1968) we obtain $\alpha_\nu(OD) = 80 \cdot 10^{-6}/\mathrm{K}$ which has to be compared to the macroscopic linear coefficient, $\alpha_l = 50 \cdot 10^{-6}/\mathrm{K}$. This result, cf. Eq. (4), corresponds to a Grüneisenparameter $\gamma(OD) = -1.6$ (±0.1). Moreover, it was shown for two overtone bands of ice that the frequency-distance curve extrapolates linearly into the liquid region (Luck 1973; Ford and Falk 1968). This result indicates that $\gamma(OD)$ does not change drastically at the phase transition. In the liquid phase one has a much wider distribution of nearest neighbour distances. Correspondingly, the O–H band of water comprises 160 $\mathrm{cm^{-1}}$ as compared to 40 $\mathrm{cm^{-1}}$ in ice (Doster et al. 1986).

However, the thermal expansion coefficient of bulk water contains a anomalous negative component due to correlations between enhanced entropy and volume fluctuations (Speedy and Angell 1976). This anomaly does not modify the asymmetric O–H stretch which is mainly seen with infrared spectroscopy. The symmetric, in-phase, O–H stretch, observed in the Raman spectrum of bulk water around 3290 $\mathrm{cm^{-1}}$, is absent in the spectrum of hydration water (unpublished results). The decrease in the symmetric component with decrease in hydration was also seen with hydrated DNA (Tao et al. 1989). Thus for hydrogen bonded systems the correlation between the temperature dependence of the O–H stretching frequency and thermal expansion is reasonably well understood. Our initial studies were concerned with the temperature dependence of $\nu(OH)$ in pure glycerol. The temperature slope $\alpha_\nu(OH)$ showed a striking discontinuity around 183 K, the calorimetric glass temperature $T_g$ of glycerol. Moreover, we derive $\alpha_\nu(OH) = 160 \cdot 10^{-6}/\mathrm{K}$ above and $40 \cdot 10^{-6}/\mathrm{K}$ below $T_g$ which is remarkably close to densitometric results of $\alpha_l = 167 \cdot 10^{-6}/\mathrm{K}$ and $30 \cdot 10^{-6}/\mathrm{K}$ above and below $T_g$ respectively (Schulz, 1954).

The effect of thermal expansion on other molecular vibrations, in particular the amide bands of proteins in solution is much less understood. Both amide bands are affected by hydrogen bonding (Zundel 1982): Softening of C–O … H bonds enhances the peak frequency of amide I, which is dominated by $\nu_{C-O}$. Amide II, a combination of N–H stretch, ($\nu_{N-H}$), and C–N bending ($\delta_{C-N}$) shows the opposite temperature dependence, since the bending force constant decreases with softening of the N–H … O coupling. If the glass transition of the bulk solvent is transmitted to the H-bonds in the protein main chain, we should observe a discontinuity as well in the temperature dependence of the amide I/II peak frequency, $\alpha_\nu$ (amide I/II) in parallel to $\alpha_\nu(OH)$ at the glass temperature.

## 3 Materials and methods

Glycerol was obtained from Sigma Chemical (purity 99%) and used as received. Samples were prepared from salt-free lyophilized powder of horse myoglobin (Sigma Chemical Co., St. Louis, MO). The protein was dissolved at a concentration of 15 mM in 75% or 90% glycerol-water (by volume) and 0.1 M phosphate buffer at pH 7.6. The 75% glycerol-water solvent contained 15% $H_2O$ and 10% $D_2O$. To obtain the 90% solution a 10% $D_2O$-buffer was added to glycerol.

Hydrated films were prepared by drying approximately 125 µl of a 0.75 mM met-myoglobin solution (75% $H_2O$ and 25% $D_2O$) plus 2 mg of sucrose on a $CaF_2$ window. The addition of sucrose yields optically homogeneous films. Two kinds of films were prepared: For the dry sample the water was removed as far a possible using silica gel and evacuation. A residual water content of less than 10% was estimated by weighing. The hydrated sample was dried over a saturated $KNO_3$ solution containing 20% $D_2O$. The calorimetric glass temperature of this sample measured in a DSC (Perkin Elmer) was 225 (±5) K. This corresponds roughly to $T_g = 230$ K of a 80% (by weight) sucrose-water solution.

The addition of a small amount of $D_2O$ allows one to study the uncoupled O–D oscillator of water, glycerol or sucrose together with the amide bands of the protein. The strong O–H stretching band in a fully protonated solution containing 15 mM protein would saturate the detector. To

correct for the water deformation band in the amide I spectral region a solvent spectrum was subtracted from the solution spectrum at each temperature (Dong et al. 1990). The amide-spectra of the 90% glycerol-water samples were of particularly high quality because of the absence of $H_2O$. The peak position of amid I is not affected by $D_2O$. For amide II we only observe the protonated (unshifted) amide group (N–H), the N–D species is outside of our window. The sample cell consisted of two $CaF_2$ windows separated by a 10 μm teflon spacer. The cell was mounted in a helium flow cryostat (Leybold-Heraeus), wich allowed control of temperature with an accuracy of ±1 K. Spectroscopic measurements were carried out with a McPherson 218 monochromator (f/5.3) in a chopped single-beam setup using lock-in techniques and an HgCdTe detector (Infrared Associates). The signal was sampled with a step-size of 0.2 $cm^{-1}$ at a spectral resolution of 2 $cm^{-1}$. Samples were cooled from room temperature at a constant rate of 4 K/minute and equilibrated for 30 minutes at the target temperature.

The infrared intensity transmitted through the sample of thickness d follows from the Lambert-Beer's law:

$$I = I_0 \cdot \sum \exp(-\varepsilon_i(\nu) \cdot c_i \cdot d) \tag{5}$$

where $\varepsilon_i(\nu)$ denotes the molar extinction coefficient and $c_i$ the concentration of molecule *i*. Consider two absorption lines of identical shape $I(\nu)$ and $I(\nu+\delta\nu)$ which are shifted relative to each other by $\delta\nu$. For frequency shifts small compared to the linewidth $\Gamma$, we express the difference spectrum by the first term of a Taylor expansion:

$$I(\nu+\delta\nu) - I(\nu) = \frac{\partial I}{\partial \nu} \cdot \delta\nu \tag{6}$$

Knowing the lineshape one can calculate frequency shifts from intensity changes. For a Gaussian lineshape one gets:

$$\delta\nu = 0.35 \cdot \Gamma \cdot \frac{I_+ - I_-}{I_{\max}} \tag{7}$$

$I_{\max}$ denotes the transmitted intensity at the band maximum and $I_+ - I_-$ gives the difference between maximum and minimum of the difference spectrum. Rousseau and Shelnutt (Shelnutt et al. 1979; Rousseau 1981) have measured frequency shifts of Raman spectra down to $\Gamma/100$ with this method. This requires the lineshape to remain constant. However, small deviations have only minor effects: To transform a Gaussian line into a Lorentzian at constant $\Gamma$, modifies the factor 0.35 in Eq. (7) to 0.38. Below we calculate 'Gaussian' frequency shifts as a function of the temperature from difference spectra with respect to a spectrum taken at a reference temperature. From the error analysis, based on an instrumental resolution of 2 $cm^{-1}$ we estimate that spectral shifts $\delta\nu$ above 0.2 $cm^{-1}$ are reliably detected. The peak frequencies, however, are less well defined. They depend on the absolute frequency calibration and the problem of very broad absorption bands. Below we discuss temperature and solvent composition dependent spectral changes. The main contribution to the difference spectra comes from frequency shifts. But variations in absolute intensity and lineshape do occur and are easily detected as distortions in the originally symmetric difference spectrum.

## 4 Results

The uncoupled $\nu(OD)$ absorption band of glycerol-water solutions, at 10% deuteration, consists of a Gaussian line without any discernable structure even in the presence of protein (Fig. 1 a). The band position shifts when the temperature is varied and its intensity changes. Figure 1 b shows the corresponding temperature-difference spectra of a 75% glycerol-water solution containing 15 mM myoglobin with respect to a spectrum taken at 260 K. The increasing difference reflects the redshift of $\nu(OD)$ with a decrease in temperature. The redshift indicates stronger hydrogen bonding, O–D…O, which decreases the force constant of the O–D oscillator. Note the approximate inversion symmetry of the difference spectrum, showing that

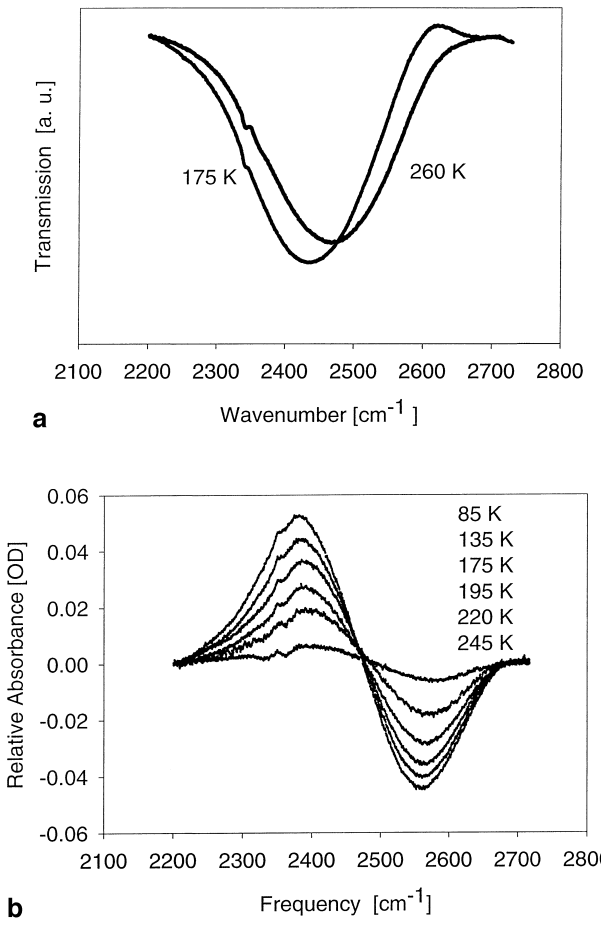


**Fig. 1** **a** Infrared transmission spectra in the O–D stretching region of a 75% glycerol/water (10% $D_2O$) solution containing 15 mM metmyoglobin (horse). **b** Temperature difference spectra in the O–D stretching region of a 75% glycerol/water solution (10% $D_2O$) containing 15 mM myoglobin relative to a reference spectrum at 260 K

the main change with temperature consists in a frequency shift and not in a distortion of the lineshape. A slight asymmetry between the positive and the negative side becomes apparent below 180 K and reflects an increase in absorption strength. A similar effect was observed in pure glycerol. In Fig. 2 we display the temperature dependence of $\nu(OD)$ for a 75% and a 90% glycerol-water solvent with and without 15 mM myoglobin analysed according to Eq. (7). All samples show a transition in the slope $\alpha_\nu(OD)$ within five degrees of the glass temperature of the solvent. Approximating the data by two lines yields $\alpha_\nu = 150 - 160 \cdot 10^{-6}$/K on the liquid side and $40 - 50 \cdot 10^{-6}$/K in the glass. About the same values were obtained for pure glycerol as mentioned above. These numbers and in particular the step in $\alpha_\nu(OD)$ at the glass temperature support the validity of Eq. (4). The corresponding mode Grüneisen-parameter $\gamma(OD)$ appears to be close to unity. In spite of the high protein concentration of 15 mM in 75% glycerol-water there is little influence of the protein on the frequency shifts. However, as compared to 75% glycerol, $\nu(OD)$ of 90% glycerol is red-shifted by 25 $cm^{-1}$ indicative of stronger hydrogen bonding. Moreover, the O–D band of the protein solution is blue-shifted relative to the solvent. This protein-induced shift of about 5 $cm^{-1}$, reflects the partial disruption of the network at high cosolvent concentration. Such an effect is to be expected if the cosolvent molecules are excluded from the protein domain leading to preferential hydration. Thermodynamic experiments have indeed shown that preferential hydration represents the main cause of the stabilizing effect of glycerol and similar cosolvents on proteins (Gekko and Timasheff 1981; Timasheff 1993). It is interesting to compare these results with frequency shifts of the amide bands using the same samples. Figure 3 displays the peak frequency of amide I as a function of the temperature. Softening of C–O…H bonds enhances the frequency of the C–O oscillator of amide I. The data clearly show a discontinuity in $\alpha_\nu$ at the glass temperatures of the two solvents, 170 and 182 K for 75 and 90% glycerol/water respectively. Figure 4 shows corresponding data for amide II. In this case softening of hydrogen bonds decreases the peak frequency. Thus both $\nu(OD)$ and the amide bands seem to consistently monitor the thermal expansion of the hydrogen bond network including a discontinuity of $\alpha_\nu$ at $T_g$ of the solvent.

There is, however, a substantial difference between the spectra of amide II and the O–D stretching band. The latter exhibits an approximately temperature-invariant Gaussian lineshape. Amide II in contrast displays a strongly asymmetric difference spectrum. This is shown in Fig. 5 for a hydrated myoglobin sample at h = 0.36 g/g. The high-frequency wing of the 1550 $cm^{-1}$ band varies more strongly with the temperature than its low frequency side. The strong bonds become even stronger with a decrease in the temperature while little change is observed in the low frequency wing reflecting NH groups which are less constrained by H-bonds. This result suggests that

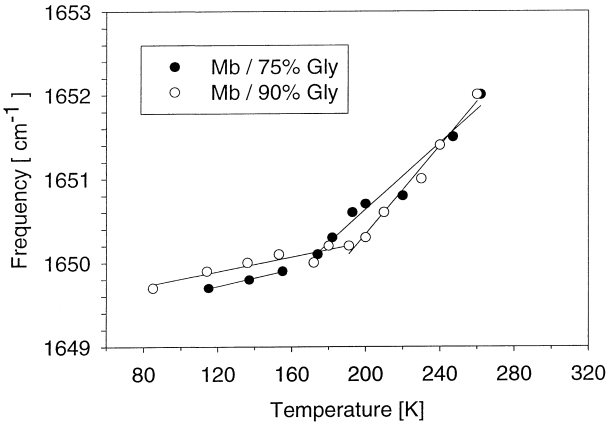


**Fig. 3** Amide I peak position shifts versus the temperature of 15 mM myoglobin in 75 and 90% glycerol-water solvent determined from difference spectra and Eq. (7)

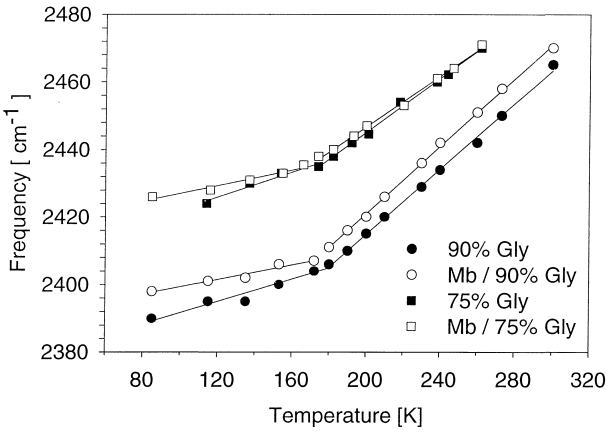


**Fig. 2** The O–D stretching frequency shifts versus the temperature determined from difference spectra and Eq. (7). 75% and 90% glycerol-water solutions with and without protein (15 mM Mb) are compared

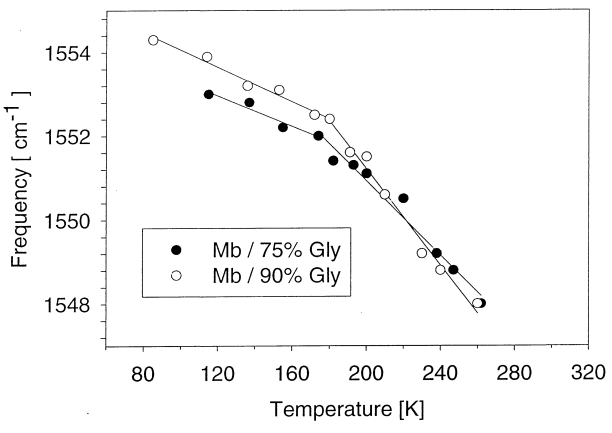


**Fig. 4** Amide II peak position versus the temperature of 15 mM myoglobin in 75 and 90% glycerol/water using the high frequency side of the asymmetric difference spectrum (Fig. 5) and Eq. (7)

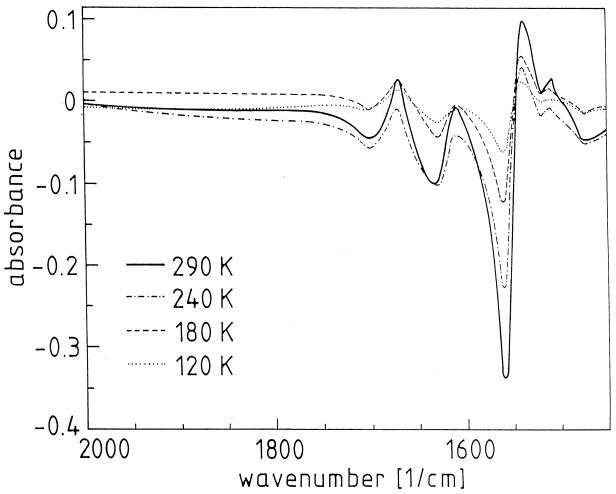


**Fig. 5** Temperature difference spectra of a hydrated myoglobin film at h = 0.36 g/g, in the amide I/II region relative to a reference temperature of 100 K. Note the strongly asymmetric amide II difference spectrum at 1550 $cm^{-1}$

amide II consists of sub-bands, modified by either protein-water coupling or intramolecular bonds. Most NH groups form only a single hydrogen bond (Baker and Hubbard 1984). This fact suggests two categories of NH groups: Those involved in hydrogen bonds with carbonyls in $\alpha$-helical structures and those in disordered regions (turns) which couple to water molecules. The striking temperature dependence of the high frequency, strong coupling side of amide II indicates that NH interacts more strongly with water oxygens than with carbonyl groups. Intramolecular bonds in contrast seem to depend very little on the temperature.

Figure 6 compares the $\nu(OD)$ of myoglobin in 80% sucrose/water with a dehydrated 90% sucrose sample. The calorimetric $T_g$ of the hydrated film was 220 K, while the dried sample had a glass temperature well above 300 K. The frequency expansion coefficient $\alpha_\nu$ shows a transition in the hydrated sample at about 215 K and a continuous change below 300 K in the dried system consistent with the calorimetric results. The peak frequencies of the dry and hydrated system are similar at low temperatures, but this should not be taken too literally in view of the large band width. The discrepancy between the corresponding O–D frequencies at high temperatures in Fig. 6, however, is significant. Our results suggests that those hydrogen bonds which are strong at low temperatures for both the dry and the hydrated system remain so up to high temperatures at reduced hydration. This is just another facet of the general correlation between H-bond strength, glass temperature and structural flexibility. The amide I and II peak frequencies, as shown in Figs. 7 and 8, exhibit the same temperature dependence. The step in the $\alpha_\nu$ occurs at 210–220 K. Reducing the water content leads to a significant increase of the amide I frequency, in particular at lower temperatures, which is probably the result of fewer or more weakly hydrogen bonded carbonyls. Amide II decreases upon dehydration which also suggests softening of constraints imposed by H-bonds.

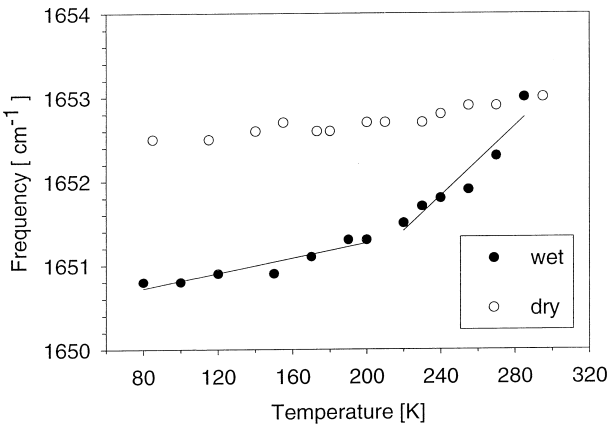


**Fig. 7** Amide I peak frequency shifts of 10 mM myoglobin in 80% (wet) and 90% (dry) sucrose-water

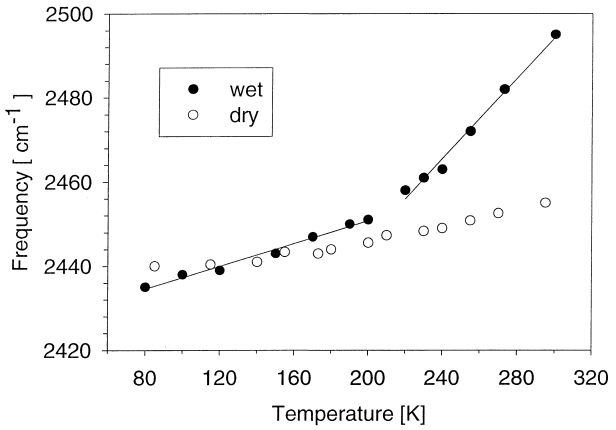


**Fig. 6** O–D stretching frequency of 10 mM myoglobin in 80% (wet) and 92% (dry) sucrose-water solvents. The sample containing 90% sucrose is a glass at room temperature

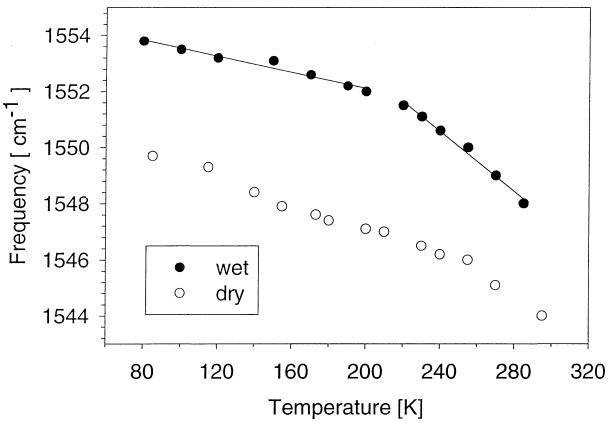


**Fig. 8** Amide II peak frequency versus the temperature of 10 mM metmyoglobin in 80% (wet) and 90% (dry) sucrose-water

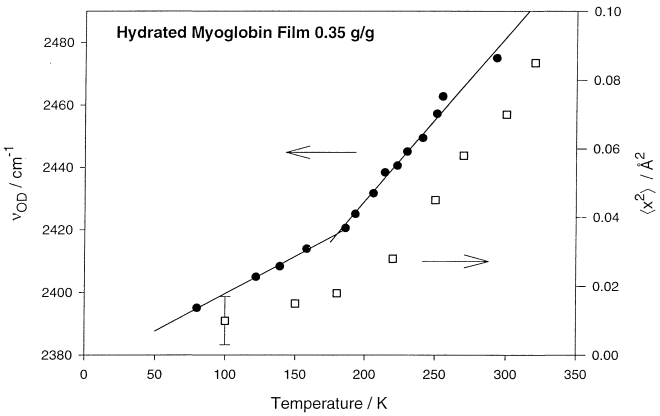


**Fig. 9** *Solid circles*: O–D stretching frequency of a hydrated myoglobin film at h = 0.35 g/g versus the temperature calculated with Eq. (7) and the spectra in Fig. 5 of Doster et al. (1986). The lines represent least square fits. Open squares: Average mean square displacements of nonpolar protons in $D_2O$-hydrated myoglobin (0.35 g/g) derived from energy-resolved neutron scattering spectra (IN6 at the ILL in Grenoble) (Doster et al. 1989; Settles and Doster 1996). The displacements correspond to motions within a time window of 15 ps

Finally in Fig. 9 we display the O–D stretching peak frequency of a myoglobin film hydrated to 0.35 g/g (Doster et al. 1986). This sample which did not contain any sucrose, corresponds to those used in previous neutron scattering experiments (Doster et al. 1989). By neutron spectroscopy we determined the average mean square displacements of the nonexchangeable myoglobin protons, also shown in Fig. 9, for a given time window of 15 ps. The comparison reveals a striking parallelism in the temperature dependence of both quantities. This observation could be interpreted to mean that the onset of fast anharmonic protein motions observed above 180 K correlates with an enhanced thermal expansion of the protein-solvent H-bond network.

## 5 Discussion and conclusion

Few measurements of thermal expansion coefficients connected with proteins have been performed: Using densitometry an apparent volume expansion coefficient of lysozyme in solution was determined, which increases with decreasing temperature (Hiebl and Maksymiv 1991). This puzzeling result was explained by assuming that the thermal expansion coefficient of protein hydration water does not contain the anomalous negative component present in the bulk. After correction for this effect a protein-intrinsic expansion coefficient of $200-300 \cdot 10^{-6}$/K depending on the pH was obtained. X-ray studies of tetragonal lysozyme crystals in the range between 100 and 298 K suggest a smaller $\alpha \approx 90 \cdot 10^{-6}$/K (Young et al. 1994). The thermal expansion of myoglobin was investigated by analysis of the refined crystal structures at 80 and 255–300 K (Frauenfelder et al. 1987). It was found that the main expansion is due to the increase of small, subatomic free volumes between atoms. An apparent linear expansion coefficient of $50 \cdot 10^{-6}$/K was estimated. This value is close to our result $\alpha_v$ (amide II) $= 30 \cdot 10^{-6}$/K which is approximately independent of the solvent. The X-ray data were taken at only three temperatures and provide important structural information, but cannot show temperature effects on $\alpha_l$. The expansion of hydrated, cross-linked protein films were recorded directly using a microscope. The authors found a step in $\alpha_l$ near 200 K, the characteristic feature of a glass transition (Morozov and Gevorkian, 1985). They obtain an $\alpha_l$ of $43 \cdot 10^{-6}$/K above $T_g$ depending little on humidity, cross-linking and type of protein.

We find that the values $\alpha_v(OD)$ of glycerol and glycerol/water solutions agree approximately with the linear expansion coefficient including the step at the glass temperature. This result indicates that the interoxygen distance and not the distortion of the H-bond geometry dominates the frequency shift. The discontinuity in $\alpha_v(OD)$ at the glass temperature shows that the interoxygen distance has reached a critical lower limit leading to glass formation. Note that water shows the opposite behaviour: Ice has the lower density and thus longer interoxygen distances than liquid water (Stillinger 1980).

The corresponding discontinuity observed in the temperature derivatives of the amide band frequencies at the glass temperature shows that the glass formation of the solvent is transmitted to the main chain and most likely to polar side chains on the protein surface. The quenching effect on the intramolecular displacements in the protein structure can be understood based on the large number of protein-solvent H-bonds: Nearly all polar groups in proteins form hydrogen bonds either with each other or with water. The C=O group has, in contrast to N–H, a strong preference to engage in two or more H-bond (Luck 1973). About 180 hydrogen bonds between side chains and water were found in the X-ray and neutron crystallography of myoglobin (Baker and Hubbard 1984; Cheng and Schoenborn 1990). The main chain N–H and C=O groups form 38 and 145 H-bonds to water respectively. 236 intramolecular bonds between main chain atoms were detected. The bonds of the main chain to water are thus comparable in number to those of the polar side chains on the surface. Taken together it follows that the number of intermolecular interactions mediated by hydrogens exceeds the intramolecular bonds approximately by a factor of 1.5. Finally, about 70% of water-protein H-bonds involve protein oxygen atoms rather than nitrogens, partially due to the asymmetry in the hydrogen bond capacity mentioned above. The O–H/D stretching band of adsorbed water thus represents a sensitive monitor of protein-water interactions in hydrated systems (Fig. 9).

With hydrated myoglobin we observe a redshift by 40 cm$^{-1}$ of the O–D stretching vibration relative to bulk water, indicating stronger hydrogen bonding at the protein-solvent interface. The corresponding difference in enthalpy could be as large as 6 kJ/mole, estimated using the empirical Bauer-Badger rule which suggests an enthalpy shift of

0.16 kJ/cm (Pimentel and McClellan 1960; Luck 1973). Assuming a corresponding increase in activation energy would reduce the mobility of water by a factor of ten at 300 K. A comparable decrease is actually observed for rotational as well as for translational diffusion (Pethig 1992; Steinbach and Brooks 1993; Usha and Wittebort 1989; König et al. 1994, Settles 1996). The $\nu(OD)$ in protein solutions reflects to a large extent the bulk solvent. However, owing the high protein concentration of 15 mM a significant fraction of the solvent molecules must be within three hydration layers of the protein surface. It follows that about 1000 water molecules per protein are perturbed by protein-solvent coupling (Doster et al. 1986). The water concentration is 55 M. This leads to a fraction of 15/55 of all water molecules which interact with the protein surface. Strong disturbances at the surface are thus likely to affect the average O–D stretching frequency: In glycerol-water protein solutions we observe a noticeable blueshift in $\nu(OD)$ above a glycerol concentration of 75% (Fig. 2). The protein surface thus reduces the strength of the H-bond network relative to the bulk in response to the addition of cosolvent. Glycerol and sucrose are well known as stabilizers of native protein structures. We found a denaturation temperature of 378 K for myoglobin in 92% sucrose/water compared to about 333 K in water at pH 7. Timasheff and collaborators have attributed this effect to cosolvent exclusion from the protein domain (Timasheff 1993). The protein sites compete for water with the glycerol molecules. The preferentially hydrated sites exhibit weaker bonds than the more viscous bulk solution.

Reducing the water content of a sucrose solution (Fig. 6) leads to stronger bonds and finally to glass formation at room temperature. The protein-solvent coupling is however diminished: The frequency shifts of the amide bands suggest less hydrogen bonding either by a reduction in number or by softer bonds. This result may reflect again the preferential hydration of the protein.

The main goal of our study was to derive a molecular interpretation of the apparently discontinuous temperature behaviour of molecular motions in protein solutions. Kinetic studies, neutron scattering, Mössbauer spectroscopy and molecular dynamics (Doster et al. 1989; Doster et al. 1990; Ferrand et al. 1993; Andreani et al. 1995; Parak et al. 1982; Parak et al. 1988; Parak and Frauenfelder 1993; Smith et al. 1990; Steinbach and Brooks 1993; Steinbach 1996) have established the general relevance of a dynamic transition around 180 K in several hydrated proteins. These experiments indicate that below 180 K the behaviour of atomic motions is equivalent to those in harmonic solids, whereas above this temperature increasingly anharmonic structural flexibility gives rise to short-range, liquid-like motions. Our study shows that a corresponding discontinuity exists in the temperature dependence of the average hydrogen bond length (Fig. 9). This suggests that the observed anomalies essentially reflect the anharmonicity of the solvent interacting with the protein surface.

The bond length determines the bond energy as discussed above. The bond is too strong at low temperatures to be broken by thermal excitations and only vibrational motions about the minimum of the potential occur. The thermal expansion then reflects the anharmonicity of the potential near its minimum. At higher temperatures activated excursions become more frequent, which increases the fraction of longer and weaker bonds. This fast librational process gives rise to an excess expansion and is seen as a nonlinear contribution to the temperature dependence of the atomic mean square displacements. This fast local motion with a time constant of about 0.5 ps, was first observed in the quasielastic neutron scattering spectra of hydrated myoglobin (Doster et al. 1989; Doster et al. 1990; Settles and Doster 1996). It is also prominent in the neutron scattering spectra of hydration water (Settles and Doster 1996).

The dynamic softening of the H-bond network when the temperature increases, given rise to further motions which are more collective in nature. This leads to an extra increase in the displacements observed above 250 K (Doster et al. 1989). The time scale of those long range diffusive processes varies with the average hydrogen bond energy (Sciortino et al. 1990). This can be understood using percolation theory of H-bonded clusters of water (Stillinger 1980; Stanley and Teixera 1980; Lamanna and Cannistraro 1992; Lamanna et al. 1994). The H-bond dynamics will be discussed in a forthcoming publication. Apart from the shift of the peak frequency, which we have discussed, one has to consider the inhomgeneous broadening of the O–H bands. The range of realized H-bond geometries in the liquid, as reflected by the 160 $cm^{-1}$ wide O–H stretching band, presumably contributes to the structural and energetic disorder of proteins at room temperature.

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