Relation between Solvent and Protein Dynamics as Studied by Dielectric Spectroscopy

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We present results obtained by dielectric spectroscopy in wide frequency (10^{-2}−10^{9} Hz) and temperature ranges on human hemoglobin in the three different solvents water, glycerol, and methanol, at a solvent level of 0.8 g of solvent/g of protein. In this broad frequency region, there are motions on several time-scales in the measured temperature range (110−370 K for water, 170−410 K for glycerol, and 110−310 K for methanol). For all samples, the dielectric data shows at least four relaxation processes, with frequency dependences that are well described by the Havriliak−Negami or Cole−Cole functions. The fastest and most pronounced process in the dielectric spectra of hemoglobin in glycerol and methanol solutions is similar to the α-relaxation of the corresponding bulk solvent (but shifted to slower dynamics due to surface interactions). For water solutions, however, this process corresponds to earlier results obtained for water confined in various systems and it is most likely due to a local β-relaxation. The slowing down of the glycerol and methanol relaxations and the good agreement with earlier results on confined water show that this process is affected by the interaction with the protein surface. The second fastest process is attributed to motions of polar side groups on the protein, with a possible contribution from tightly bound solvent molecules. This process is shifted to slower dynamics with increasing solvent viscosity, and it shows a crossover in its temperature dependence from Arrhenius behavior at low temperatures to non-Arrhenius behavior at higher temperatures where there seems to be an onset of cooperativity effects. The origins of the two slowest relaxation processes (visible at high temperatures and low frequencies), which show saddlelike temperature dependences for the solvents water and methanol, are most likely due to motions of the polypeptide backbone and an even more global motion in the protein molecule.

Introduction

A protein is built up of amino acids linked to each other to form a long chain, which is formed in a well-defined three-dimensional structure. The function of a protein is closely related to its structure and involves structural changes, both in the interior and on the surface of the protein.[1] Proteins soluble in water are folded into a structure with an almost nonpolar core. Hemoglobin, the protein investigated in this study, is a globular transport protein, which consists of two pairs of polypeptide chains each conformed to a subunit. Each subunit contains a heme group where the oxygen molecules bind to the iron atom for transport to the body tissue.

The presence of water is of fundamental importance for both the structure and the function of a protein. The water molecules are bound to the protein surface by hydrogen bonds, which are formed with the main chain or the side chain functional groups. The motion of water close to the protein surface is thereby restricted and differs substantially from that of bulk water.[2,3] This retarded motion of water molecules associated with the protein surface is, however, of great biological importance since it enables protein motion and proton transfer along the protein surface, which are both necessary for biological processes. Diffusion of surrounding solvent molecules promotes the structural changes of the protein that are necessary for the function of the protein,[4,5] and the critical point for protonic percolation is close to the hydration level for enzymatic activity.[6] The function of a protein starts when the hydration level reaches approximately 0.2 g of water/g of protein,[7] and with a further increase in hydration, the protein dynamics (which is essential for function) is increased. The protein achieves full function at equal amounts of water and protein. (1 g of water/g of protein).[6] This can be explained by the plasticizing effect of water on proteins that leads to the increase in flexibility of the protein structure necessary for protein function.[8]

The water surrounding a protein is usually divided into three layers, dependent on different interactions with the surface.[9] The first hydration layer consists of water molecules strongly bound to the surface, and the second layer consists of the more loosely bound molecules, which interact with the tightly bound water. The third layer is the surrounding bulk water, which is considered to be unaffected by the protein surface. The first and second layers constitute the so-called hydration shell. It has recently been suggested[10] that the hydration shell and the surrounding bulk solvent have different effects on the protein motions. Fast β-relaxations in the hydration shell control fast local (β-like) fluctuations in the protein, whereas the cooperative α-relaxation in the bulk solvent controls the large-scale (α-like) protein motions.[10] The authors convincingly supported this idea by showing that both the fast local (β-like) fluctuations and the large-scale (α-like) motions in the protein follow the same temperature behavior as the β- and α-relaxations, respectively, of the solvent.

However, the role of water in protein function is still far from fully understood. Further experimental and theoretical studies

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are needed to clarify how and why the water molecules close to a protein surface influence the structure and dynamics of the protein. One way to elucidate why water is of such importance for biological functions is to compare how solvents of different viscosities and dynamical properties affect the protein structure and dynamics. In this study, we have, therefore, investigated the relation between solvent and protein dynamics, i.e., how the solvent dynamics is affected by the interaction with the protein surface and how the solvent influences the protein dynamics, by broad-band dielectric spectroscopy. Due to its high resolution and large frequency window, dielectric spectroscopy is a suitable method to investigate dynamic processes that involve the reorientation of dipolar units or the displacement of charge entities in the material under investigation.

It is known that alcohols can affect the stability of a protein in two ways: by destabilizing the tertiary structure of a protein with denaturation as a consequence and by stabilizing the secondary structure with an extended helical structure as a result. The denaturation ability of an alcohol is increased with increasing chain length (i.e., hydrocarbon content) and decreased with increasing branching of the hydrocarbon chain or increasing number of hydroxyl groups.[11,12] Thus, methanol and other short alcohols destabilize the tertiary structure and induce a more extended α-helical conformation. Glycerol, on the other hand, which contains three hydroxyl groups and is, thus, more hydrophilic than methanol, stabilizes the protein structure with a decreased protein volume as a result (i.e., a protein with a more compact and rigid core).[13] The effect of alcohols can be explained by the decreasing polarity of the solvent[14,15] with increasing length of the molecule. In solvents of low polarity, the hydrophobic interactions that stabilize the folded structure are weakened and, simultaneously, the intramolecular hydrogen bonds are strengthened, causing unfolding of the native protein and stabilization of the extended α-helical structure.

In this study, we compare the dynamics of hemoglobin in the three different solvents water, methanol, and glycerol at a solvent level of 0.8 g of solvent/g of protein. These three solvents exhibit different microscopic dynamics and are known to affect protein structures, as mentioned above, in different ways.

Experimental Section

Lyophilized powder of human hemoglobin ($M_w = 64.5$ kDa) from Sigma was chosen for this study. As a dry sample, powder from the bottle was used. The solvents used in this study were water, methanol, and glycerol. In each measurement, samples of 0.8 g of solvent/g of protein (which in the case of water corresponds to about 2 molecular layers) were examined. The desirable concentration was obtained by mixing the dry protein with the solvent.

The dielectric measurements were performed on a broad-band dielectric spectrometer from Novocontrol. For each experiment, the sample was placed between two Al electrodes and thereafter placed in a sample holder during measurement. The sample was first cooled to 110 K and then progressively reheated to 370, 310, and 410 K for the water, methanol, and glycerol solutions, respectively. Isothermal measurements of the complex dielectric function $\epsilon^*(f) = \epsilon'(f) - i\epsilon''(f)$ were performed at every fifth degree, with an isothermal stability of ±0.2 K, in the frequency range $10^{-2}-10^9$ Hz. Two different spectrometers were used for this study. For frequencies between 10^{-2} and 10^7 Hz, an Alpha-S high resolution dielectric analyzer was used, and for the frequency range 10^6-10^9 Hz, an Agilent 4291B radio frequency (RF) impedance analyzer was used. The imaginary part of the obtained dielectric response is well described by one (in the case of glycerol, two) conductivity term for the low-frequency response, and in all cases, several Havriliak−Negami (HN) functions[16] were used for the loss peaks, i.e.,

$$\epsilon''(\omega) = \left(\frac{\sigma}{\epsilon_0 \omega}\right)^n + \sum \mathrm{Im}\left[\frac{\epsilon_s - \epsilon_\infty}{(1 + (i\omega\tau)^\alpha)^\beta}\right] \quad (1)$$

where ϵ_s is the static dielectric constant, ϵ_∞ is the limiting value of the dielectric constant at high frequency, and $\omega = 2\pi f$ is the angular frequency. The broadening of a loss peak of the relaxing molecules is described by the two shape parameters α and β, where α parametrizes a symmetric and β parametrizes an asymmetric broadening of the loss peak. However, the special versions of the HN function with only one shape parameter, the asymmetric Cole−Davidson (CD) equation ($\alpha = 1$)[17,18] and the symmetric Cole−Cole (CC) equation ($\beta = 1$),[19] are often used to describe the main α-relaxations and the secondary β-relaxations, respectively. The conductivity contribution to and polarization effects on the dielectric spectra are visible as an increase in ϵ'' at low frequencies. The shape of the increase is determined by the exponential factor n (for pure direct current (dc) conductivity $n = 1$). For hydration water in biological systems, the conductivity is known to increase exponentially $n = 1$ with increasing water content up to the hydration level $h \approx 0.2$.[20] Above this hydration level, a two-dimensional (2D) percolation of water is induced,[21] which coincides, as mentioned above, with the threshold for the onset of water-induced functionality of biomolecules.

The temperature dependence of relaxation processes often follows either an Arrhenius (eq 2) or a Vogel−Fulcher−Tammann (VFT) behavior (eq 3)[22−24]

$$\tau = \tau_0 \exp(E_a/k_B T) \quad (2)$$

$$\tau = \tau_0 \exp\left(\frac{-DT_0}{T - T_0}\right) \quad (3)$$

where τ_0 is the relaxation time extrapolated to infinite temperature and usually corresponds to quasi-lattice or molecular vibrations on the order of 10^{-14} s. E_a is the activation energy, and T_0 is the temperature where τ goes to infinity. The constant D determines the deviation from Arrhenius temperature dependence. When the relaxation time τ of an α-relaxation process has reached a value of 100 s, the supercooled liquid or polymer behaves like a solid and is called a glass. So-called strong glass-formers, according to Angell's strong−fragile classification scheme,[25,26] have an α-relaxation time with close to Arrhenius temperature behavior (a large D-value in the VFT function, eq 3), while a fragile glass-former shows a much more rapid increase of the α-relaxation time close to the glass transition temperature T_g (i.e., a low D-value in the VFT function). The faster β-relaxation, on the other hand, normally shows Arrhenius temperature dependence.

Results and Discussion

In Figure 1, three-dimensional representations of the dielectric loss, i.e., the imaginary part of the dielectric function, versus the frequency and temperature (in the frequency range $10^{-2}-10^6$ Hz) are shown for hemoglobin in water, glycerol, and methanol. From this figure, it is clear that the water and glycerol samples show similar temperature behavior while the methanol sample is very different. The spectra can be described by several processes, both relaxations, which give rise to peaks in the

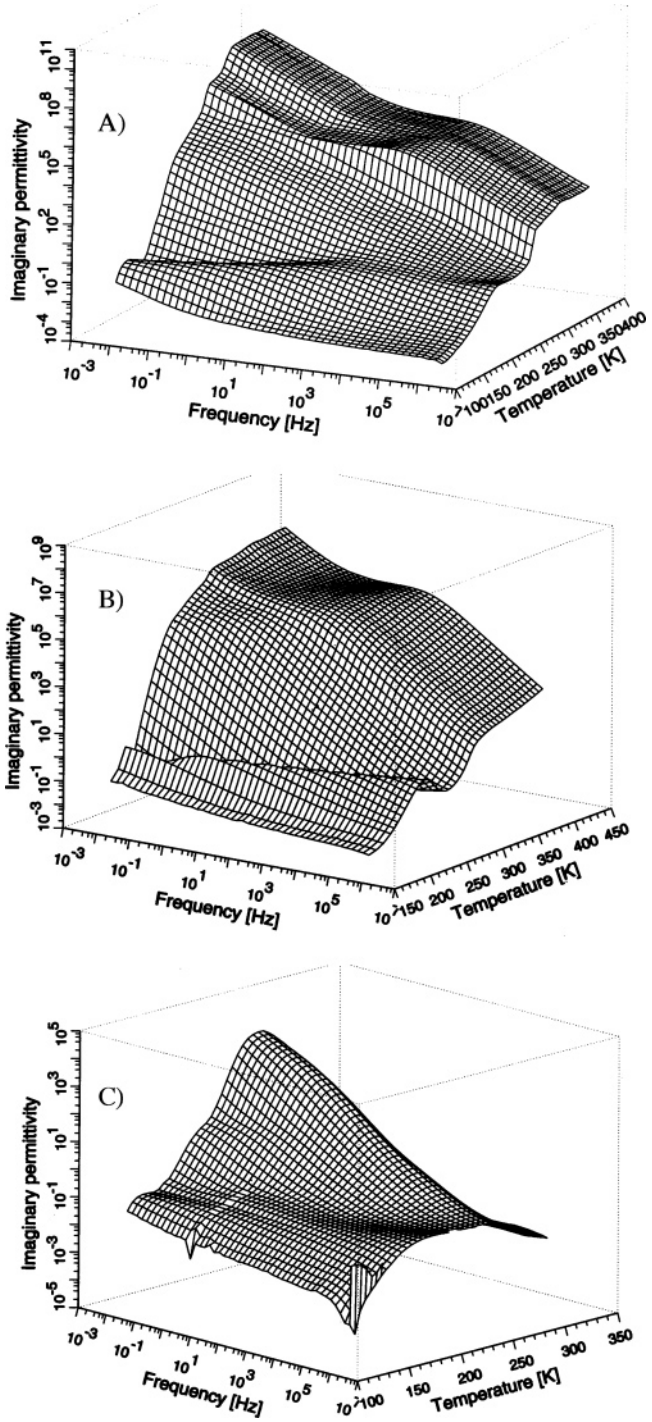

Figure 1. Imaginary part (ϵ'') of the dielectric spectra for hemoglobin in (A) water, (B) glycerol, and (C) methanol as a function of frequency and temperature.

spectrum, and polarization effects or conductivity, which both cause an increase of ϵ'' at low frequencies. The conductivity, or low-frequency dispersion, of proteins at low or moderate hydration is dominated by mobile protons and arises from long-range percolation of the charges along pathways between localized sites.[8,27]

The conductivity contribution to the dielectric spectrum is seen in Figure 1 at low frequencies, where the amplitude of ϵ'' increases dramatically with increasing temperature and decreasing frequency. The maximum amplitude of this process occurs at certain temperatures, which are somewhat different for the different solvents. This process is, as mentioned above, related to the percolation of protons along the protein surface, where the protons start to move between localized sites and thereby create charge separation and, as a consequence, polarization occurs. At the temperature where the permittivity reaches its maximum, the protons are free to move along threads of connected solvent molecules on the protein surface.[28,29]

The imaginary part of the dielectric spectrum of each protein−solvent sample was fitted to four Havriliak−Negami functions (eq 1). Figure 2 shows the so-obtained relaxation times for the water, glycerol, and methanol samples, and in Figure 3, the corresponding shape parameters are shown. The most pronounced relaxation process for all of the investigated samples is the fastest process (denoted as process I in Figure 2). For the glycerol and methanol samples, the temperature behavior of this process is well-described by the VFT equation (eq 3). The process is, furthermore, similar to the α-relaxation of the corresponding bulk solvents although it is broader (see Figure 3 for the obtained shape parameters) and shifted to slower relaxation times. As a consequence of the slowing down of this relaxation process, the glass transition temperature T_g is shifted to higher temperatures, from 182 K to ≈187 K for glycerol and from 103 K to ≈125 K for methanol. This slower relaxation time (and increase in T_g) can be explained by the fact that the solvent molecules are affected by the interaction with the protein surface. The relaxation process is more affected for methanol than for glycerol, most likely due to the fact that the methanol molecules induce conformational changes of the protein, which expose the hydrophobic part of the protein to the solvent and create more and stronger bonds to the protein.[30] For the water sample, the temperature behavior of process I is better described by the Arrhenius equation (eq 2), where both the relaxation time and the activation energy (E_a = 0.41 eV) obtained from the Arrhenius fit correspond well to water confined in a molecular sieve of pore size 10 Å,[31] clay[32] (see Figure 2A), purple membranes,[33] and other systems containing interfacial water.[34]

The dielectric loss peak of process I for the water sample is symmetric for all temperatures ($\beta = 1$ in eq 1), which suggests that it is due to a more local β-relaxation[34] rather than the cooperative α-relaxation. This is further indicated by the fact that this process reaches a relaxation time of 100 s at $T \approx 123$ K and not at about 165 K, which was recently suggested to be the glass transition temperature for bulk water.[35−37] This would then be in conflict with the common belief that T_g for bulk water is in the range 124−136 K,[38,39] but there are indications that the α-relaxation of water is not directly observed in the present system, as well as in most other types of confined systems and solutions.[34] All fit parameters of the VFT or Arrhenius fits of the temperature dependences of relaxation process I are shown in Table 1.

Process II is attributed to local motions of polar side groups of the protein (possibly together with a small amount of bound solvent molecules), since earlier observations on hydrated proteins by dielectric spectroscopy have shown that such motions give rise to a relaxation process on the same time-scale as the second fastest process in the water sample (i.e., process II in Figure 2).[40] For all samples, this process seems to follow an Arrhenius temperature dependence up to a certain temperature (which depends on the solvent), where it appears to change its temperature dependence (see Figure 2) to high temperature VFT behavior. The activation energy of process II in the low temperature Arrhenius range is about 0.80 eV for the water and glycerol samples and 0.58 eV for the methanol sample. The lower activation energy for the methanol sample

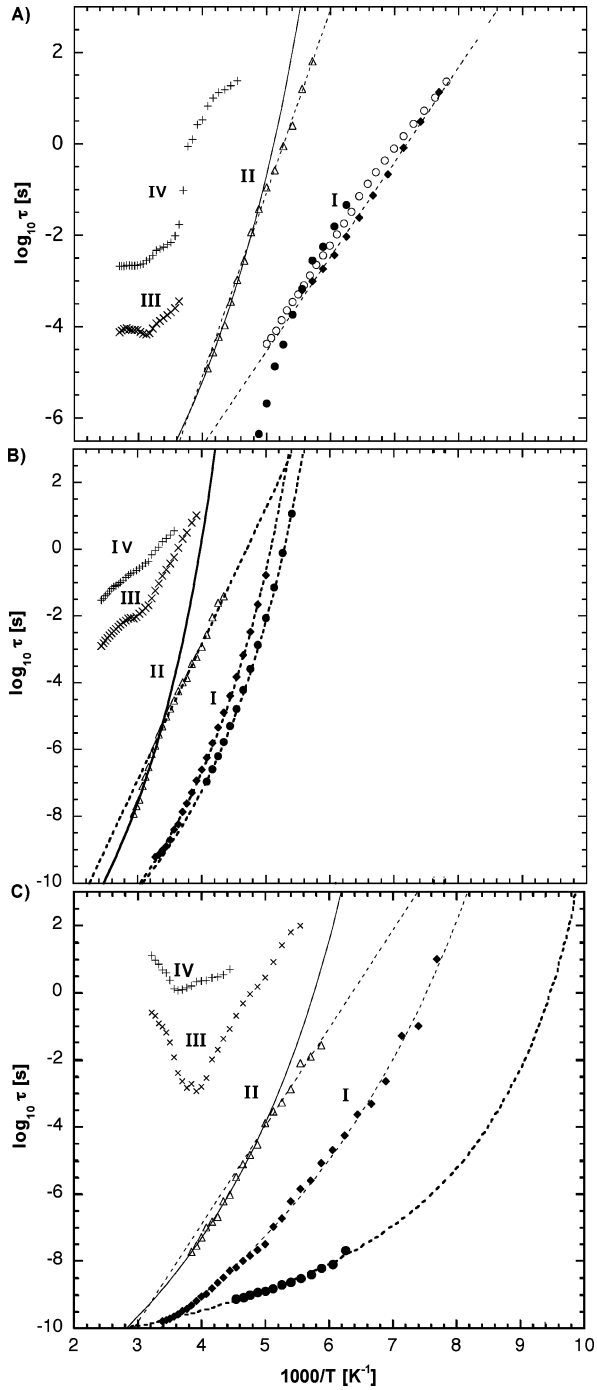

Figure 2. Temperature dependences of the relaxation times for the four clearly observed dielectric processes obtained for (human) hemoglobin (Hb) in (A) water, (B) glycerol, and (C) methanol at the concentration 0.8 g of solvent/g of protein. Also shown are the relaxation times for water confined in (●) molecular sieves and (○) clay in part A and bulk relaxation for (●) glycerol and methanol in parts B and C, respectively. The relaxations are called I, II, III, and IV from the fastest to the slowest times (marked with the symbols ◆, △, ×, and +, respectively). The dashed lines are fits to either the Arrhenius law or the VFT function, as described in the text (note that bulk methanol has not been measured to temperatures lower than 160 K due to crystallization).

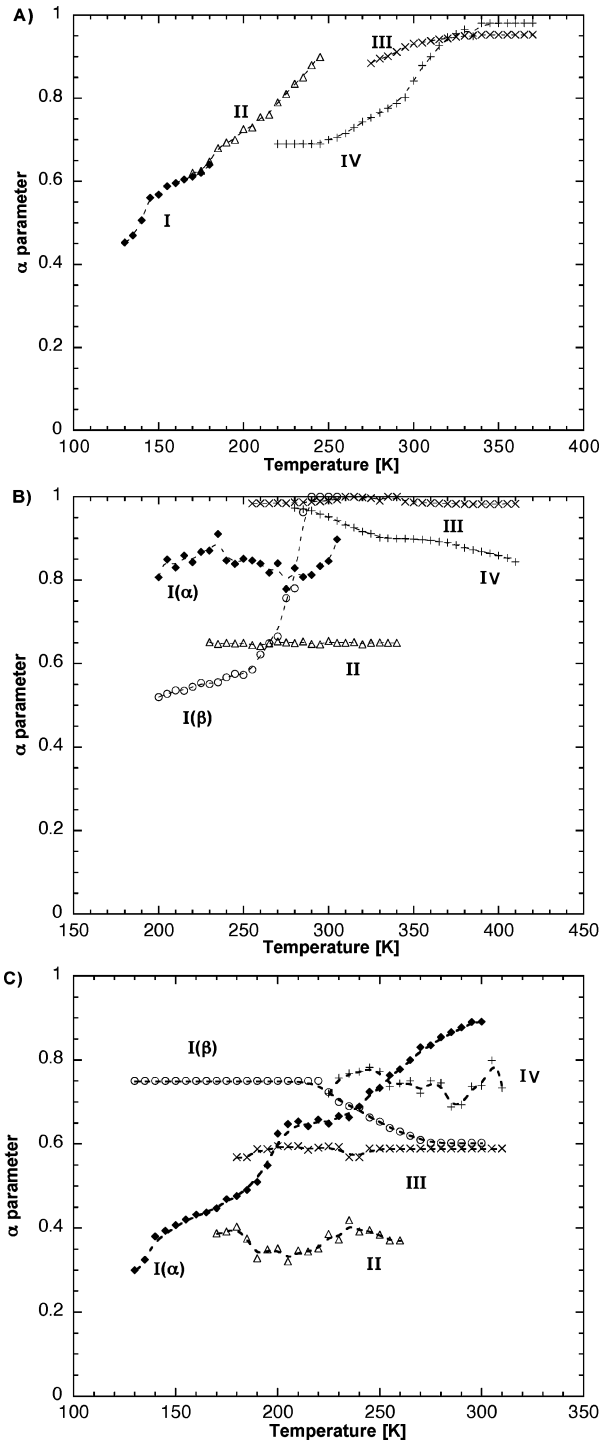

Figure 3. Shape parameters (α and β in eq 1) for the obtained relaxation processes in Figure 2, where parts A, B, and C correspond to hemoglobin in water, glycerol, and methanol, respectively. The α-parameters (in eq 1) are denoted with the same symbols as the corresponding relaxations in Figure 2, while the β-parameter for the glycerol and methanol samples is marked with an open circle (○). The corresponding parameters obtained for the bulk solvents are for glycerol $\alpha = 1$ and β varies from ≈ 0.6 to ≈ 0.8, whereas for methanol $\alpha = \beta \approx 1$ at all measured temperatures (note that the β-parameter for the methanol sample was kept fixed up to 220 K).

is most likely due to the fact that the protein molecule is more flexible in methanol, which destabilizes the tertiary structure and instead increases the number of helical structures. An extrapolation of the Arrhenius temperature dependence to an infinite temperature gives an unrealistic value of the relaxation time ($\tau_0 \approx 10^{-20}$) while an extrapolation of the high temperature data by the VFT function gives a more realistic value of τ_0; see Table 2. This suggests that this protein relaxation changes its character from a local β-like process at low temperatures to a more cooperative α-like relaxation at higher temperatures; that

TABLE 1: Fit Parameters Obtained from the VFT or Arrhenius Fits to Process I[a]

	τ_0 (s)	E_a (eV)	D	T_0 (K)
water	10^{-15}	0.41		
glycerol	10^{-15}		17	132
methanol	10^{-14}		24	75

[a] Here, the temperature behavior of the glycerol and methanol samples is given by the VFT equation, while that of the water sample is described by the Arrhenius equation.

TABLE 2: Fit Parameters Obtained from the VFT and Arrhenius Fits to Process II[a]

	τ_0 (s)	E_a (eV)	D	T_0 (K)	T range (K)
water[b]	10^{-21}	0.79			175−210
water[c]	10^{-13}		18	120	210−245
glycerol[b]	10^{-19}	0.81			230−295
glycerol[c]	10^{-15}		16	129	295−340
methanol[b]	10^{-18}	0.58			170−200
methanol[c]	10^{-14}		20	107	200−260

[a] Here, the temperature behavior at higher temperatures is given by the VFT equation, while the temperature dependence at low temperatures is described by the Arrhenius equation. [b] Fitting to the Arrhenius equation (low T). [c] Fitting to the VFT function (high T).

is, at low temperatures, the motions are small and local and, when the temperature increases, the motions become more global and cooperative in character.

The change in temperature behavior occurs at different temperatures for the different solvents. The crossover temperatures are located around 210, 295, and 200 K for the water, glycerol, and methanol samples, respectively, and the crossover is most pronounced for the glycerol sample, which has the highest viscosity. Interestingly, for all of the hemoglobin samples the crossover occurs at about the same temperature as anharmonic motions appear on a time-scale of about 0.1 ns.[41] In analogy with that, the glass transition temperature of a glass forming liquid is commonly defined as the temperature where its α-relaxation time reaches ca. 100 s. This process can be considered to be frozen at $T_{100} = 170$ K for the water sample. This finding is in good agreement with earlier results obtained by differential scanning calorimetry (DSC), which have shown an onset of a broad glass transition (T_g) around 170 K in hydrated methemoglobin.[42] It was shown[42] that the onset of T_g was almost independent of the hydration level (for hydration levels in the range 0.4−0.7 g of H_2O/g of protein), whereas the width of the transition decreased with an increased hydration level. This suggests that the onset temperature of local β-like motions of the protein is only weakly dependent on the hydration level, in contrast to the slower large-scale motions, which have a strongly hydration level dependent onset at higher temperatures. For the glycerol and methanol samples, T_{100} is shifted to higher ($T_{100} \approx 190$ K) and lower ($T_{100} \approx 140$ K) temperatures, respectively, compared to the water sample; see Table 3. In the frequency domain, this relaxation process exhibits a symmetric broadening (i.e., it is described by the CC equation with $\beta = 1$ in eq 1) for all samples and temperatures. For the glycerol and methanol samples, the symmetric shape parameter, α, is almost constant over the whole temperature range, with values around 0.65 and 0.35, respectively. For the water sample, there is a decrease in width ($\propto 1/\alpha$) with increasing temperature, but interestingly, there is no change in slope at the above-discussed crossover in temperature dependence of the relaxation time (see Figure 3).

A comparison between processes I and II for all the investigated samples is shown in Figure 4. From this figure, it is clear that there is a relation between the protein and the solvent dynamics, as seen by a shift of process II to slower dynamics with increasing viscosity of the solvent. At the temperature where process II shows a crossover from Arrhenius to VFT temperature dependence (see Figure 2), process II is 3−4 orders of magnitude slower than process I for all the samples. However, from Figure 4, it is evident that the ratio between their respective relaxation times τ_I and τ_{II} is not constant over the entire experimental temperature range, as also seen in Figure 5. Possible reasons for this will be discussed below.

TABLE 3: Characteristic Temperatures Obtained for Processes I and II, All Given in Kelvin[a]

	process I		process II	
	T_{100}(bulk)	T_{100}(sample)	$T_{crossover}$	T_{100}
water		123	≈210	≈170
glycerol	182	187	≈295	≈190
methanol	103	125	≈200	≈140

[a] T_{100}(sample) corresponds to the value obtained in the investigated samples for process I. For water, T_{100}(sample) corresponds to the β-relaxation rather than the α-relaxation as for glycerol and methanol. Note that T_{100}(bulk) is not known for water.

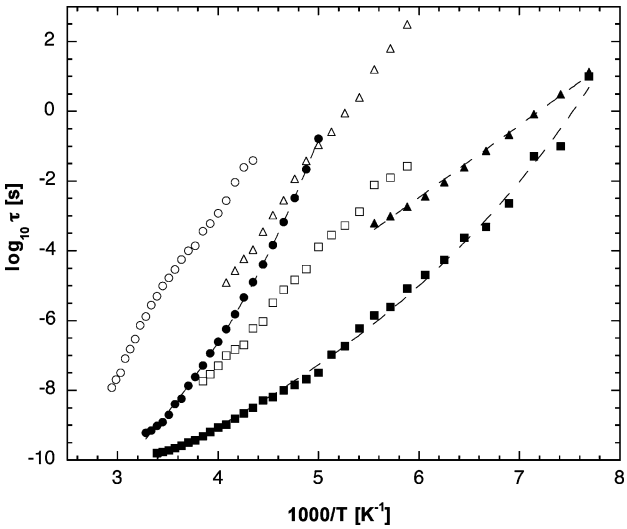

Figure 4. Comparison of the relaxation times obtained for the fastest and the second fastest relaxation processes. Triangles, circles, and squares correspond to the water, glycerol, and methanol samples, respectively. Filled symbols refer to process I, and open symbols refer to process II.

The two slowest processes (III and IV in Figure 2) visible at high temperatures show unusual temperature behaviors, particularly in the case of water and methanol as the solvents. At low temperatures, the relaxations become faster with increasing temperature, as expected, but at a certain temperature (which is different for the water and methanol samples), the relaxation times increase with further increase in temperature. This anomalous saddlelike temperature dependence is most pronounced for the methanol sample. The temperature where these processes reach their fastest relaxation times is moved to higher temperatures with increasing viscosity of the solvent; see Figure 2.

In the case of process III, the water sample shows this saddle point (i.e., its fastest relaxation time) at about 325 K, which roughly corresponds to the denaturation temperature of hemoglobin in water (DSC measurements indicated that complete denaturation and aggregation occur at about 340 K). This fact suggests that this motion is of more global character and strongly dependent on the structure of the protein. The time-scale of this motion is in agreement with a previous study,[43] where the

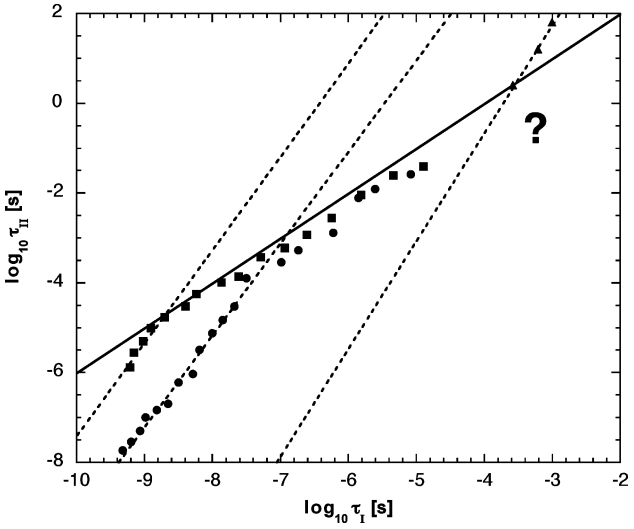

Figure 5. Logarithm of the relaxation times obtained for process II plotted as a function of the logarithm of the relaxation times for process I. The symbols ▲, ●, and ■ denote the water, glycerol, and methanol samples, respectively. The solid line is a guide to the eye and shows a slope = 1 (i.e., a linear dependence of the relaxation times with process I 4 orders of magnitude faster than process II). The dotted lines describe the quadratic dependence for the water sample at low temperatures and the high temperature dependence for the glycerol and methanol samples, respectively. The question mark indicates two possible scenarios for the water relaxation (process I). The first one (described by the dotted line) is that the relaxation follows Arrhenius temperature behavior over the whole temperature range. The second case, and the most likely one, is that there will be an increase in slope of process I, which will also result in a more linear relation (solid line) between the relaxation times of processes I and II for the water sample.

process observed in the kilohertz region was interpreted to arise from motions of the whole polypeptide backbone. This interpretation is consistent with the present finding that an aggregation of the protein molecules slows down its dynamics. The shape parameter α of process III remains almost constant in the temperature range in which it is observable. For the water and glycerol samples, the value is close to 1.0 (i.e., it shows Debye behavior), while in the case of methanol as the solvent the width of the process is considerably broader ($\alpha \approx 0.6$); see Figure 3. A possible explanation for this may be that the presence of methanol gives rise to a larger size distribution of α-helices, which in turn produces a broader distribution of relaxation times with an increased width of the relaxation process as a consequence.

The width of process IV is somewhat different, at least for the water sample, where it increases with increasing temperature from a value of $\approx$0.7 to almost 1.0 at 350 K. For the glycerol and methanol samples, the shape parameter stays almost constant at $\approx$0.9 and $\approx$0.75 (see Figure 3). The amplitude of this process increases with increasing temperature up to a certain temperature, and thereafter, the amplitude remains almost constant for the water and glycerol samples, whereas it decreases for the methanol sample. This suggests that the number of relaxing units involved in this process and/or the amplitude of the motion first increases with increasing temperature (for all the samples) and thereafter remains basically temperature independent for the water and glycerol samples and decreases for the methanol sample. The decrease in amplitude for the methanol sample may be a result of the complete denaturation and aggregation of the protein molecules in methanol, which is likely to reduce the strength of this slow motion. The origin of the relaxation processes is, however, not totally clear, but due to its slowness and close relation to process III, it is most likely a large-scale motion of almost the whole protein molecule.

As mentioned above, the role of solvent dynamics for protein motion is a very important issue to elucidate. It is still debated whether protein motions are directly determined by solvent motions (i.e., whether the protein motions are solvent-slaved) or not. In our case, there are only one or two molecular layers surrounding the protein molecules, which means that we are unable to elucidate the effect of the bulk solvent on the protein dynamics. However, we should be able to determine whether any of the protein processes are related to motions in the "hydration shell". In the case of the two slowest protein processes (III and IV), it is clear that they exhibit very different temperature behaviors compared to the solvent relaxations (process I). Thus, there is no evident relation in these cases, although one should note that processes III and IV are shifted to higher temperatures with increasing viscosity of the solvent.

In the case of the fastest (observed) protein process (II), its relation to process I has been elucidated by plotting the logarithm of the relaxation times obtained for process II vs the logarithm of the relaxation times for process I; see Figure 5. In Figure 5, it can be seen that there are basically two different types of relations between the two processes. Either the two processes are linearly proportional (giving a slope of 1 in the graph) or the relaxation time of process II, τ_{II}, increases significantly faster (quadratically) than that of process I, τ_I, with decreasing temperature (a slope of about 2 in the graph). The linear dependency is observed at low temperatures for the glycerol and methanol samples and indicates that processes I and II are correlated (but not coupled in the sense that they occur on the same time-scale) to each other. Such a relationship is only expected for processes having the same temperature dependence, which is not the case for process I and II in these samples. However, in a limited temperature range, the two processes exhibit approximately the same temperature dependence and it is in this temperature range that a linear dependency is observed. At the crossover temperature of process II (i.e., 200 K in the case of methanol and 295 K for glycerol), the almost linear dependence is lost and the relation becomes quadratic. The loss of this linear dependence is a natural result since processes I and II are likely to merge at very high temperatures when the protein and solvent motions are expected to occur on the same time-scale. Quadratic behavior is also observed for water at the few temperatures for which both processes I and II can be observed; see Figure 5.

For solvent-slaved processes (i.e., both processes have the same temperature dependence), a linear relation is, as mentioned above, expected. Since this is seen for the glycerol and methanol samples at low temperatures, it is possible that process II in these solvents shows slavinglike behavior in this temperature range, i.e., that the motions of the polar side groups of the protein are determined by the viscosity related α-relaxation of the solvent, despite the fact that hemoglobin is not a working protein in these solvents. These findings would then suggest that solvent slaving may be a general phenomenon for macromolecular motions in certain solutions. Thus, "slaving" may exist even for nonfunctional proteins and other macromolecules, which implies that other explanations for protein function might also be of importance.

For the water sample, we were not able to follow the process because of the water dynamics (process I) to high temperatures and, consequently, we are not able to determine the relation between τ_{II} and τ_I at high temperatures. However, in other systems,[44] process I shows a crossover in temperature depen-

dence around 200 K from low temperature Arrhenius behavior to high temperature VFT dependence. This crossover in temperature behavior is most likely due to a merging of the local β-relaxation with an α-relaxation that is experimentally absent below the crossover temperature. A similar crossover for the water close to the protein would result in a decreased slope from an almost quadratic to a more linear dependence for τ_{II} vs τ_I also for the water sample; see Figure 5. The behavior for the water sample would then be similar to that of the other solvents and, consequently, would be an indication that process II is also solvent-slaved in this case, once the temperature is high enough for the α-relaxation of water to be present. However, irrespective of whether this scenario is correct or not, it is evident that the β-relaxation of the solvent has basically no influence on the motions of the polar side groups and, hence, it is the viscosity related α-relaxation that mainly controls the motions, giving rise to process II.

Thus, our findings support earlier molecular dynamics (MD) simulations by Tarek and Tobias[4] showing that, at least, relatively global protein relaxations are determined by translational motions in the solvent. However, in this context, it should be noted that from quasi-elastic neutron scattering experiments it is evident that anharmonic protein motions appear on a time-scale of about 0.1 ns already at about 220, 270, and 180 K for the water, glycerol, and methanol samples, respectively.[41] This implies that considerably faster, and more local, protein motions exist than could be observed in the present dielectric measurements. Hence, these local motions may give a weak contribution to process I in the dielectric data. Since the α- and β-relaxations of the solvent have merged to a single process at the temperature where the fast protein motions appear on a subnanosecond time-scale, it is not possible for us to determine whether these protein motions are related to the α- or β-relaxations of the solvent. This implies that we are unable to verify (or contradict) the recent suggestion by Fenimore et al.[10] that the local β-relaxations in the solvent control the most local (β-like) protein motions.

Conclusions

The dielectric spectra of hemoglobin in the three different solvents water, glycerol, and methanol at a solvent level of 0.8 g of solvent/g of protein show at least four dielectric relaxations and a conductivity which is mainly due to percolation of protons along the surface. The fastest process is concluded to be due to solvent relaxation, which in the case of the samples with glycerol and methanol corresponds to the cooperative α-relaxation. Due to interactions with the protein surface, this relaxation is both slower and more stretched in time compared to the α-relaxation in the corresponding bulk solvents. For water, this process is almost identical to a water relaxation (in the supercooled regime) that seems to be universal for all systems containing confined or hydration water.[31−33] Recently, this process was assigned to a local β-relaxation of the confined water[34] rather than the cooperative α-relaxation.

The second fastest process, process II in Figure 2, is attributed to the dynamics of polar side groups on the protein, possibly together with a small amount of tightly bound solvent molecules. This process shows a crossover in its temperature dependence from low temperature Arrhenius behavior to high temperature VFT behavior. The temperature where this crossover occurs depends on the viscosity of the solvent, and interestingly, this temperature is roughly the same as the lowest temperature for which anharmonic protein motions can be observed by quasi-elastic neutron scattering[41] on a time-scale of 0.1 ns. The crossover suggests that the protein motion giving rise to this relaxation changes its character from a local β-like motion at low temperatures to a more cooperative α-like process at higher temperatures. It should also be noted that in the case of the two solvents glycerol and methanol there is an almost linear relationship between τ_I and τ_{II} in a given temperature range. Such a relationship is only expected for solvent-slaved processes, which suggests that protein process II is to a great extent determined by viscosity related motions in these solvents, despite the fact that the protein is structurally very different and biologically inactive in the two solvents. However, in the case of the biologically more relevant water containing sample, we are not able to follow process I to higher temperatures, and hence, we can only observe both processes for a few temperatures. In this temperature interval, there is no similar linear relation between τ_I and τ_{II} observed, probably because process I for water is due to a local β-relaxation, which is not related to the viscosity or the translational diffusion of water molecules in the hydration-shell. However, a likely change in the temperature behavior due to a merging of the α- and β-relaxations would result in a more linear dependence for processes I and II at higher values of T. An important conclusion from this study is, therefore, that solvent-slaving may be a more general phenomenon for macromolecular motions, which, per se, is not sufficient for protein function.

The two slowest processes show an anomalous temperature dependence, which is most pronounced for methanol as the solvent. The fastest of these processes, process III in Figure 2, is most likely a result of backbone motions of the protein, and the slower one (process IV) is probably due to large-scale motions of almost the entire protein molecule.

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