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ARTICLE

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Fluoroalcohol-induced structural changes of proteins: some aspects of cosolvent-protein interactions

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Abstract The conformational transitions of bovine β-lactoglobulin A and phosphoglycerate kinase from yeast induced by hexafluoroisopropanol (HFIP) and trifluoroethanol (TFE) have been studied by dynamic light scattering and circular dichroism spectroscopy in order to elucidate the potential of fluoroalcohols to bring about structural changes of proteins. Moreover, pure fluoroalcohol-water mixed solvents were investigated to prove the relation between cluster formation and the effects on proteins. The results demonstrate that cluster formation is mostly an accompanying phenomenon because important structural changes of the proteins occur well below the critical concentration of fluoroalcohol at which the formation of clusters sets in. According to our light scattering experiments, the remarkable potential of HFIP is a consequence of extensive preferential binding. Surprisingly, preferential binding seems to play a vanishing role in the case of TFE. However, the comparable Stokes radii of both proteins in the highly helical state induced by either HFIP or TFE point to a similar degree of solvation in both mixed solvents. This shows that direct binding or an indirect mechanism must be equally taken into consideration to explain the effects of alcohols on proteins. The existence of a compact helical intermediate with non-native secondary structure on the transition of β-lactoglobulin A from the native to the highly helical state is clearly demonstrated.

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Abbreviations *CD*: circular dichroism · *DLS*: dynamic light scattering · *HFIP*: hexafluoroisopropanol · *β-Lg A*: β-lactoglobulin A · *PGK*: phosphoglycerate kinase · *TFE*: trifluoroethanol

Introduction

Protein molecules adopt their native conformation in solution and within the cell only under specific environmental conditions. Studying the structural and thermodynamic response of proteins in dependence on solvent conditions is thus a pertinent way to elucidate their stability, folding pathways, and intermolecular aggregation behavior. Among different cosolvents used for this purpose, alcohols, and particularly their fluorinated derivatives, have found a wide range of applications. Alcohols mainly disrupt the native tertiary structure by weakening hydrophobic interactions and strengthening helical propensities (Thomas and Dill 1993). This special effect of alcohols as structure-inducing denaturants was already recognized several decades ago (Weber and Tanford 1958; Inoue and Timasheff 1968). Thus, alcohols are suitable to modulate the interactions between the polypeptide chain and the solvent and the interactions between different segments of the polypeptide chain as well. Therefore, fluorinated alcohols, mostly such very efficient members like trifluoroethanol (TFE) and hexafluoroisopropanol (HFIP), have been used not only in order to promote the formation of helical structure in protein fragments and peptides (Nelson and Kallenbach 1986; Sönnichsen et al. 1992; Dyson and Wright 1993; Luo and Baldwin 1997) but in many other instances such as the transformation of proteins into molten globule-like intermediates (Buck et al. 1993; Alexandrescu et al. 1994; Cort and Andersen 1997; Gast et al. 1999; Konno et al. 2000) and the

stabilization of intermediate structures of proteins (Luo and Baldwin 1998). Recent investigations have dealt with the influence of cosolvents on the folding kinetics of proteins (Lu et al. 1997; Chiti et al. 1999a; Hamada et al. 2000), the tuning of solvent conditions for studies of the amyloid formation of peptides and proteins (Barrow et al. 1992; Chiti et al. 1999b), the dissection and reassembly of amyloid fibrils (Barrow et al. 1992; MacPhee and Dobson 2000), and the α-β transition of proteins (Dong et al. 1998; Kuwata et al. 1998), which plays an essential role in various conformational diseases. Furthermore, fluoroalcohols are widely used in the field of peptide chemistry to dissolve peptide aggregates. A comprehensive overview concerning the effects of TFE and related cosolvents on polypeptide chains has been given by Buck (1998).

Despite the numerous applications and the various effects observed so far, the detailed mechanisms of how alcohols act on proteins and peptides are still not well understood, nor can the order of effectiveness of various alcohols in provoking conformational transitions be explained in a satisfactory manner. Obviously, their action results from a superposition of the effects of different properties (solvent polarity, dielectric constant, length of the carbohydrate chain, number of OH groups, degree of halogenation), which can hardly be separated adequately. In this respect, the consideration of the influence of these properties in terms of group additive contributions (Hirota et al. 1998) was an essential step forward in quantifying the effects of different alcohols on polypeptides. For some frequently used alcohols the order of effectiveness is HFIP > TFE > isopropanol > ethanol > methanol (Hirota et al. 1997). Nevertheless, the extremely high potential of HFIP and also TFE compared to other alcohols suggests the existence of additional factors. These alcohols will be considered in more detail. The tendency of HFIP and to a lesser extent of TFE to form clusters in aqueous solutions (Kuprin et al. 1995; Gast et al. 1999) has been proposed to be an important factor (Hirota et al. 1997, 1998; Hong et al. 1999). It is one purpose of this work to find out proper relationships between cluster formation and the effectiveness of HFIP and TFE in aqueous solutions.

Cluster formation is obviously a consequence of the partially hydrophobic nature of fluorinated alcohols. It has been proposed that the hydrophobicity of fluorinated alcohol molecules plays an essential role for attachment at the polypetide chain, leading to their high efficiency (Rajan and Balaram 1996). An indication that hydrophobic interactions play an important role at least for some fluoroalcohols is supported by the observation of cold denaturation of the induced structures (Andersen et al. 1996; Bhattacharjya et al. 1999).

It is still a matter of dispute whether alcohols attain their effects by direct binding to the polypeptide chain (Jasanoff and Fersht 1994; Rajan and Balaram 1996; Luo and Baldwin 1997) or by an indirect mechanism caused by alcohol-mediated changes in the solvent shell around the polypeptide (Conio et al. 1970; Cammers-Goodwin et al. 1996; Walgers et al. 1998). It is conceivable that various alcohols have very different binding affinities to peptides and therefore differ strongly in the way they substitute the normal aqueous environment of the polypeptide chain. Earlier investigations, including studies of the influence of 2-chloroethanol on β-lactoglobulin A and other proteins (Timasheff and Inoue 1968; Inoue and Timasheff 1972), suggested that preferential binding of the alcoholic component and conformational changes occur in parallel fashion. It is a further important purpose of this work to relate the preferential binding of HFIP and possibly TFE to the ability to induce conformational transitions in the investigated proteins.

For our investigations, we haven chosen bovine β-lactoglobulin A (β-Lg A) and phosphoglycerate kinase (PGK) from yeast to study the conformational transitions induced by an increasing volume fraction of HFIP in 10 mM HCl, pH 2. In 10 mM HCl, β-Lg A is in the monomeric compactly folded native state (Kuwata et al. 1999), while PGK attains a highly unfolded conformation under these conditions (Damaschun et al. 1998, 1999). Accordingly, these proteins represent two totally different model systems: in the first case, HFIP interacts with an originally folded protein, while in the second case the polypeptide chain is entirely exposed to the solvent. For comparison, some experiments were also performed in the presence of TFE. The changes in secondary and tertiary structure were observed using circular dichroism (CD) spectroscopy. Cluster formation, changes of the hydrodynamic dimensions, and preferential binding to the polypeptide chains were monitored by combined dynamic and static light scattering.

The aim of this work is to give further evidence of how fluoroalcohols may act on polypeptide chains. We will demonstrate that essential structural changes of both proteins appear well before the critical concentration for cluster formation of alcohol molecules is reached. Thus, cluster formation of fluoroalcohols in aqueous solutions cannot be the primary cause of the induced structural changes. Both phenomena are independent reflections of the hydrophobicity of the particular fluoroalcohol. The high efficiency of HFIP correlates with its strong preferential interaction with polypeptide chains. Furthermore, our dynamic light scattering data directly demonstrate the existence of a compact helical intermediate state during the transition of β-Lg A from the native to the expanded helical state.

Materials and methods

Materials

HFIP (>99%, GC) and TFE (>99%, GC) were obtained from Sigma-Aldrich (Germany) and Fluka (Switzerland), respectively. The proteins, β-Lg A (bovine milk) and PGK (yeast), were purchased from Sigma-Aldrich and from Roche Diagnostics (Germany), respectively. All other chemicals were of analytical

grade. Aqueous stock solutions of proteins in 10 mM HCl, pH 2, were produced in the following way. Lyophilized samples of β-Lg A were directly dissolved in 10 mM HCl and then dialyzed against the same solvent. Ammonium sulfate precipitated PGK was dissolved in 20 mM sodium phosphate buffer (pH 6.5). Insoluble material was removed by centrifugation. The supernatant was first dialyzed against the same buffer and finally against 10 mM HCl. Protein concentrations were determined photometrically using the specific absorptions $A(0.1\%,\ 1\ \text{cm}) = 0.96$ and $A(0.1\%,\ 1\ \text{cm}) = 0.495$ at 278 nm for β-Lg A and PGK, respectively. Mixed solvents were prepared by weighing the individual components.

Methods

Circular dichroism

CD measurements were performed on a JASCO J-720 spectrometer (Japan) using 10 mm (near-UV) and 1 mm (far-UV) rectangular cells (Hellma, Germany). Protein concentrations amounted to 1–2 g/L in the near-UV and to 0.1–0.15 g/L in the far-UV regions. Mean residue ellipticities were calculated using mean residue weights of 113.3 and 107.4 for β-Lg A and PGK, respectively. The spectrometer was calibrated with (+)-10 camphorsulfonic acid at 290.5 and 192.5 nm (Johnson 1990).

Light scattering

Dynamic (DLS) and static (SLS) light scattering were measured simultaneously with one and the same instrument at a scattering angle of 90°. The laboratory-built apparatus, consisting mainly of an argon laser (Lexel 3500, Lexel Laser, USA) operating at 514.5 nm wavelength and 0.5 W output power, a thermostated cell holder, and a photon detection system, presently equipped with a high quantum yield avalanche photodiode, has been described in detail (Gast et al. 1992). The solvents and protein solutions were filtered through 100 nm pore-size Anotop filters (Whatman, UK) directly into 100 µL flow-through cells (Hellma, Germany). Relative scattering intensities and Rayleigh ratios were obtained using benzene as a reference sample. The homodyne time-autocorrelation functions of the scattered light intensity $g(\tau)$ were calculated by a 90-channel multibit multiple-τ correlator. Distribution functions of the translational diffusion coefficient D were obtained from $g(\tau)$ using the program CONTIN (Provencher 1982). The diffusion coefficients were converted into Stokes radii via the Stokes-Einstein equation $R_S = k_B T/(6\pi\eta_0 D)$, where k_B is Boltzmann's constant, T is the temperature in K, and η_0 is the solvent viscosity. Solvent viscosities and densities were measured using an Ubbelohde-type viscometer (Viscoboy 2, Lauda, Germany) and a digital density meter (DMA 58, Anton Paar, Austria), respectively. Refractive indices of the solvent mixtures were measured with an Abbe-type refractometer. Refractive index increments $(\partial n/\partial c)$ of the proteins were obtained using a differential refractometer (Optilab DSP, Wyatt Technology, USA).

Estimation of preferential binding from light scattering data

The analysis of multicomponent solutions has been treated in a comprehensive manner by Casassa and Eisenberg (1964). Here we follow the general notation, in which the principal solvent (water or 10 mM HCl in this case) is component 1, the macromolecular solute (protein) is component 2, and the cosolvent (fluoroalcohol) is component 3. The concentration of cosolvent will be given as volume fraction Φ_3, that of the protein as weight concentration c (g/cm^3), where the subscript 2 will be omitted throughout.

Preferential binding, sometimes also referred to as selective sorption (Kratochvil 1987), means that the concentration of one of the solvent components is higher within the solvation shell of the macromolecule than in the bulk mixed solvent. The thermodynamic treatment of this effect yields a quantitative measure, the coefficient of preferential binding $\Gamma_3 = (\partial C_3/\partial C)_\mu$, but no information about the binding process on a molecular level. ∂C_3 is the change in the concentration of cosolvent caused by an infinitesimal concentration change of the macromolecular solute ∂C after establishing the thermodynamic equilibrium, that is at constant chemical potential μ of components 1 and 3. In principle, this can be achieved by adding a small amount of protein to a vessel containing the mixed solvent with a volume fraction of cosolvent $\Phi_{3,0}$ and finally dialyzing this solution against a large reservoir containing the same solvent. ∂C_3 is then obtained from the concentrations of cosolvent inside the vessel before and after dialysis. The special concentration units (molal, molar, weight concentration, volume fraction Φ, etc.) entering the coefficient of preferential binding are of particular importance. A very obvious result can be obtained if weight concentrations are used because the corresponding $\gamma'_3 = (\partial w_3/\partial w)_\mu$ yields preferential binding in grams of cosolvent per grams of protein. However, the choice of units depends also on the experimental methods used. In our calculations, we will essentially apply the very transparent procedure derived by Kratochvil (1987) and the corresponding notation.

The coefficient of preferential binding, calculated by:

$$\gamma_3 = (\partial\Phi_3/\partial c) \tag{1}$$

can be easily related to measurable quantities (see below). The relation between γ_3 and γ'_3 is (Kratochvil 1987):

$$\gamma_3 = \gamma'_3\bar{v}_3\left(1 - \Phi_{3,0}\right)/(1 - c\bar{v}) \tag{2}$$

where $\bar{v}_3$ and $\bar{v}$ are the partial specific volumes of the cosolvent and the protein, respectively. $\Phi_{3,0}$ is the volume fraction of cosolvent in the bulk mixed solvent. All volume fractions in solution are considered relative to the polymer-free mixed solvent (for details, see Kratochvil 1987).

γ_3 can be obtained by measuring the changes of intensive quantities like concentration, density, and refractive index in dependence on c. The refractive index increment method is particularly useful if the refractive indices of the solvent components are sufficiently different, which is the case for fluoroalcohols and water.

$$\gamma_3 = [(\partial n/\partial c)_\mu - (\partial n/\partial c)\Phi_3]/(\partial n/\partial\Phi_3)_c \tag{3}$$

can be obtained by measuring three types of refractive index increments (Kratochvil 1987): $(\partial n/\partial c)_\mu$ and $(\partial n/\partial c)\Phi_3$ are those measured at either constant chemical potential or constant concentration of cosolvent, respectively; $(\partial n/\partial\Phi_3)_c$, the change of the refractive index with cosolvent concentration at constant protein concentration, can be measured using the pure solvent ($c = 0$).

Refractive index increment measurements are particularly complicated in our case because fluoroalcohols are very volatile. For the same reason, evaporation of fluoroalcohol during the dialysis step preceding the measurement of $(\partial n/\partial c)_\mu$ can cause large errors in fluoroalcohol concentration. It has been shown (Kratochvil 1987) that some of these measurements can be circumvented using related light scattering data, which can be obtained more precisely and easier. The molecular mass of the protein M can be calculated from the measured Rayleigh ratio extrapolated to zero scattering angle R_0 by:

$$M = R_0(K'(\partial n/\partial c)^2 c)^{-1} \tag{4}$$

where K' is the optical constant and extrapolation to $c = 0$ has to be performed. In a mixed solvent, the true molecular mass M is obtained only if $(\partial n/\partial c)_\mu$ is used in Eq. 4. If the calculations are performed with $(\partial n/\partial c)\Phi_3$, one obtains an apparent molecular mass M_{app}. M_{app} and M calculated from the same Rayleigh ratio are related by $M_{app}/M = ((\partial n/\partial c)_\mu/(\partial n/\partial c)\Phi_3)^2$. Relying on the fact that the true molecular mass does not change, one can calculate $(\partial n/\partial c)_\mu$ from measurements of $(\partial n/\partial c)\Phi_3$ and R_0, yielding M_{app} by $(\partial n/\partial c)_\mu = (M_{app}/M)^{1/2}(\partial n/\partial c)\Phi_3$. Substituting this expression into Eq. 3 leads to:

$$\gamma_3 = [(M_{app}/M)^{1/2} - 1](\partial n/\partial c)\Phi_3/(\partial n/\partial\Phi_3)_c \tag{5}$$

The experimental determination of $(\partial n/\partial c)\Phi_3$ for many Φ_3 is rather expensive and the results may be affected by large errors.

Therefore, we have calculated $(\partial n/\partial c)\Phi_3$ by a well-established equation (Cassasa and Eisenberg 1964), which relates the unknown specific refractive index increment in one solvent to the known increment in another solvent. Thus, $(\partial n/\partial c)\Phi_3$ is calculated from $(\partial n/\partial c)_0$ in the absence of cosolvent by:

$$(\partial n/\partial c)\Phi_3=(\partial n/\partial c)_0-\bar{v}_3(n\Phi_3 - n_0) \quad (6)$$

where $n\Phi_3$ and n_0 are the refractive indices in the presence and absence of cosolvent, respectively.

In summary, the procedure for estimating preferential interaction includes the following steps: determination of n_0, $n\Phi_3$, and $(\partial n/\partial\Phi_3)_c$ at $c=0$ with a conventional Abbe refractometer, determination of $(\partial n/\partial c)_0$ with a differential refractometer, calculation of $(\partial n/\partial c)\Phi_3$, estimation of M_{app} from light scattering data, and calculation of γ_3 and γ'_3 using Eq. 5 and Eq. 2, respectively. It is worth remembering that an unexpected molecular mass measured by light scattering using $(\partial n/\partial c)\Phi_3$, which is the conventional way for one-component solvents, could be an indication of preferential binding.

Results

Cluster formation of HFIP and TFE in mixed water-alcohol solvent systems

An important object of our investigations was a more detailed study of the connection between cluster formation and the efficiency of the fluoroalcohols in inducing structural changes in proteins. Therefore, the first step was to analyze cluster formation in the absence of proteins. Mixed solvents containing defined volume fractions of alcohols Φ_3 were studied by static and dynamic light scattering. The light scattering intensity of 10 mM HCl was practically identical to that of pure water. Furthermore, we did not find any differences in the behavior of clusters in 10 mM HCl or in water. Thus, the relative scattering intensities compared to those in the absence of fluoroalcohol and the Stokes radii of HFIP clusters have been taken from experiments in water and 10 mM HCl as well (Fig. 1). To calculate the Stokes radii of clusters, we need an appropriate value of the solvent viscosity in the investigated systems. For the HFIP system, we have used the viscosity of water, because cluster formation appears essentially at low volume fractions ($\Phi_3 < 0.4$). This is not a reliable assumption for the TFE system, where clusters are formed mostly near $\Phi_3 = 0.5$. Thus, we have not calculated Stokes radii for TFE clusters.

The dependence of the scattering intensity and the Stokes radius on fluoroalcohol concentration (Fig. 1) was investigated at 25 °C. For HFIP, we have also measured the relative changes of the scattering intensity and the Stokes radius at $\Phi_3 = 0.25$ in dependence on temperature. In Fig. 2, the data have been normalized to that at 25 °C. It should be mentioned that in addition to the appearance of small clusters, the effects of large-scale phase separation mimicking the presence of large aggregates were observed at temperatures below 25 °C and HFIP concentrations within the range $0.12 < \Phi_3 < 0.2$.

According to the measured light scattering intensities, cluster formation is maximal near $\Phi_3 = 0.3$ for HFIP and slightly below $\Phi_3 = 0.5$ for TFE (Fig. 1), respectively.

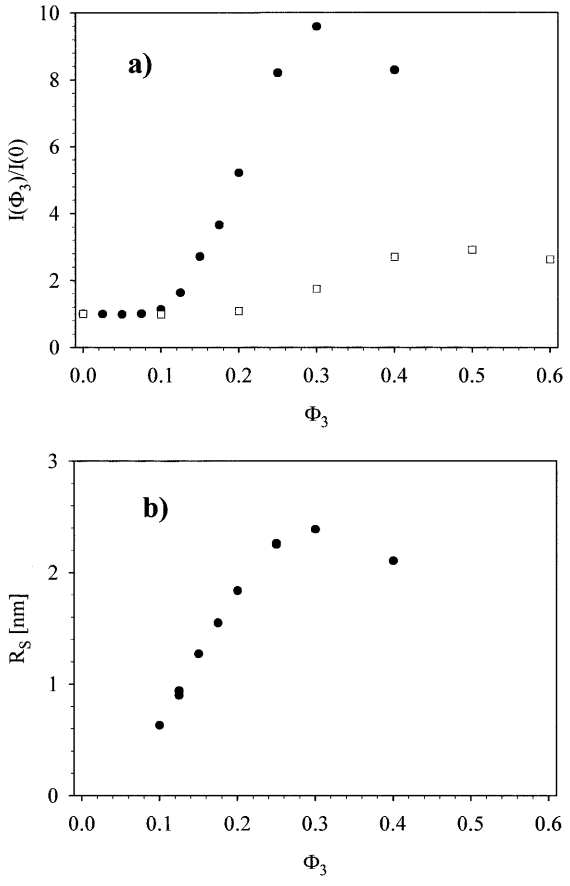

Fig. 1 **a** Relative scattering intensities of mixed solvents in dependence on the volume fractions Φ_3 of either HFIP (●) or TFE (□) at 25 °C. The principal solvent component was either water or 10 mM HCl, pH 2. **b** Corresponding Stokes radii of HFIP clusters in dependence on Φ_3 at 25 °C

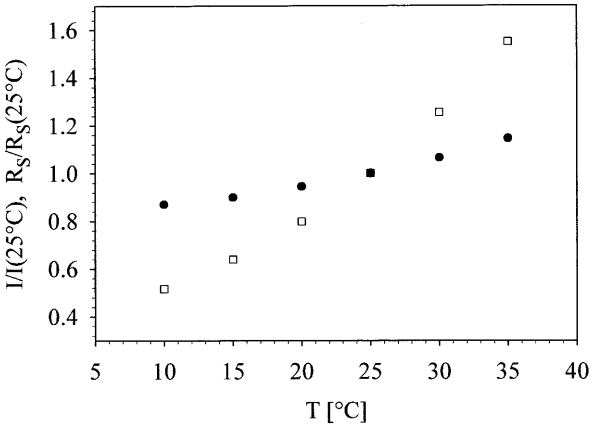

Fig. 2 Relative changes of the scattering intensity (□) and of the Stokes radius (●) in dependence on temperature for a mixed solvent containing HFIP ($\Phi_3 = 0.25$). The normalization was done with respect to the values at 25 °C

This agrees well with the results of previous X-ray scattering (Kuprin et al. 1995; Hong et al. 1999) and light scattering investigations (Gast et al. 1999). The effect is considerably more pronounced for HFIP

according to the measured maximum excess light scattering intensity, which is about 4.5 times larger than that measured for TFE. An important characteristic of cluster formation of the investigated fluoroalcohols is the existence of a critical concentration Φ_C, similar to a critical micelle concentration (cmc), above which cluster formation sets in. This critical concentration is well indicated by a remarkable increase in light scattering intensity and amounts to $\Phi_C = 0.1$ for HFIP and is slightly above 0.2 for TFE, respectively. It should be mentioned that we could not detect a change of Φ_C in the presence of proteins. The apparent size in terms of the Stokes radius R_S varies strongly with HFIP concentration and reaches a maximum value of 2.4 nm also at $\Phi_3 = 0.3$ (Fig. 1b). Hong et al. (1999) have estimated the radius of gyration R_G at concentrations $0.25 < \Phi_3 < 0.35$. The results obtained within this small concentration range suggest an essentially constant value of $R_G = 1.4$ nm. If we calculate the so-called ρ-factor $= R_G/R_S$ (Burchard et al. 1980) using an average value of $R_S = 2.3$ nm for $0.25 < \Phi_3 < 0.35$, we obtain $\rho = 0.61$; ρ is a conformation-sensitive parameter. The calculated ρ-value is even smaller than that for a homogeneous sphere, the geometric configuration with the smallest ρ-factor $= 0.775$. This excludes the assumption of non-spherical shapes having larger ρ-factors. A ρ-factor < 0.775 could be expected for micelle-like particles with an inhomogeneous density, viz. particles with a core of higher density. However, the experimentally determined ρ-value must be considered with some care because it was calculated from quantities measured at finite concentrations. The clusters cannot be considered as isolated, non-interacting particles at alcohol concentrations where they exist.

It is difficult to estimate which fraction of the total HFIP amount exists in the form of clusters. Measurements of the temperature dependence of apparent cluster size and scattering intensity (Fig. 2) show that the relative cluster size changes by a factor of 1.3 between 10 and 35 °C. However, this increase in size is not sufficient to account for the three-fold increase in scattering intensity. Thus, the fraction of molecules forming clusters is larger at higher temperatures. Both effects are obviously the result of increasing hydrophobic interactions when the temperature is raised.

HFIP-induced structural changes of β-lactoglobulin A

β-Lg A undergoes a cooperative structural transition in the presence of HFIP at $\Phi_3 = 0.045$. Characteristic CD spectra in the far-UV and in the near-UV regions before and after this transition are shown in Fig. 3a and b, respectively. According to Fig. 3b, the specific rigid environment of the aromatic amino acids of the native state is completely lost already at $\Phi_3 = 0.08$. Essential changes in the secondary structure have also occurred at this HFIP concentration. Only a moderate further increase in helical structure is reflected by the spectra at $\Phi_3 > 0.08$ (Fig. 3a).

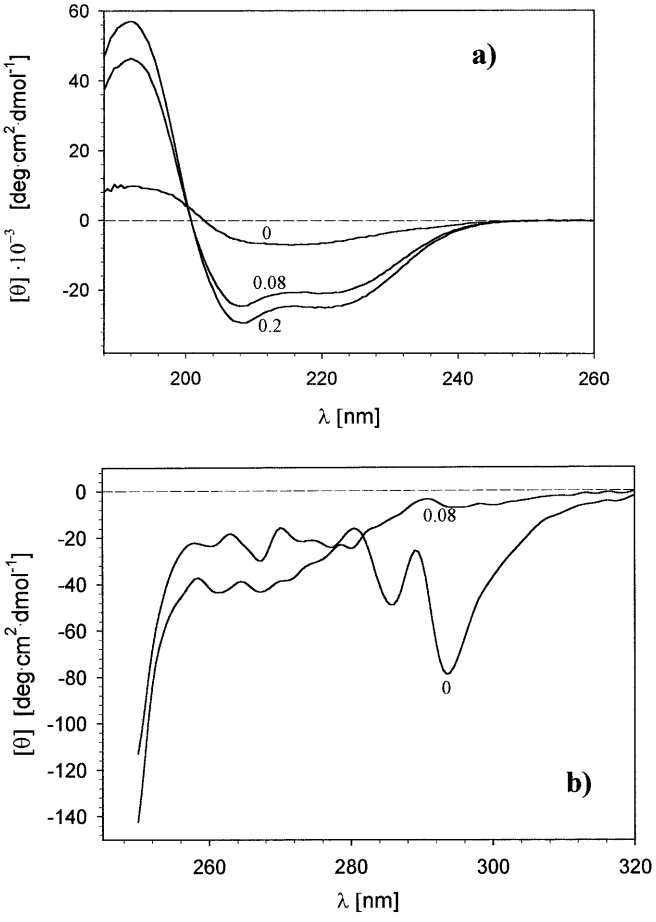

Fig. 3 Far-UV (**a**) and near-UV (**b**) CD spectra of β-Lg A at 25 °C in 10 mM HCl (pH 2) in the presence of the indicated volume fractions of HFIP

In order to monitor the changes in the molecular dimensions of the protein molecules, we have performed DLS investigations. The relative scattering intensities obtained simultaneously in the same experiments were used to calculate apparent molecular masses M_{app}. Because of substantial repulsive electrostatic intermolecular interactions at pH 2 and low ionic strength, measurements at different protein concentrations c had to be performed. This enables one to extrapolate the measured translational diffusion coefficients $D(c)$ and the apparent molecular masses $M_{app}(c)$ to their values at zero protein concentration. At HFIP concentrations $\Phi_3 > 0.1$ the calculated distribution functions of the diffusion coefficients D contained an additional peak due to the presence of clusters of pure HFIP, which could be easily separated in most cases from the peak resulting from protein molecules, $D(c)$. The concentration dependence of $D(c)$ for particular amounts of HFIP is shown in Fig. 4. For $1/M_{app}$ a similar positive slope as for $D(c)$ was observed. The Stokes radii R_S at different HFIP concentrations calculated from $D(c = 0)$ are shown in comparison with the relative changes of the CD at 222 nm and 293.5 nm in Fig. 5. The Stokes radii are not

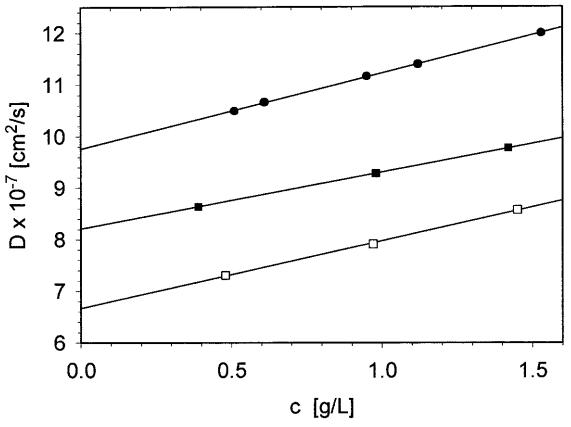

Fig. 4 Concentration dependence of the translational diffusion coefficient D of β-Lg A in 10 mM HCl (pH 2) for particular volume fractions (● 0; ■ 0.08; □ 0.125) of HFIP at 25 °C

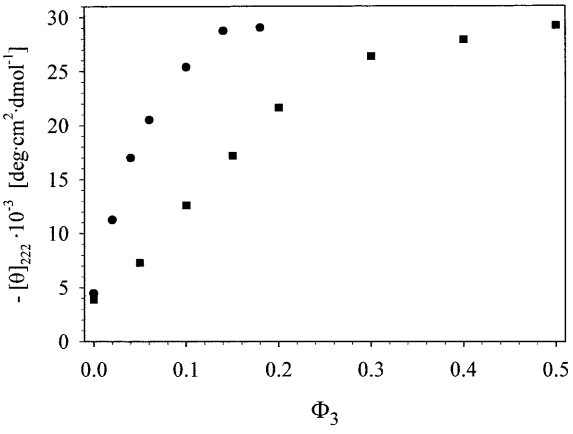

Fig. 6 Transition from the unfolded to the helical state of PGK in 10 mM HCl (pH 2) at 25 °C as monitored by the changes in the mean residue ellipticity $[\theta]_{222}$ induced by various volume fractions of HFIP (●) or TFE (■)

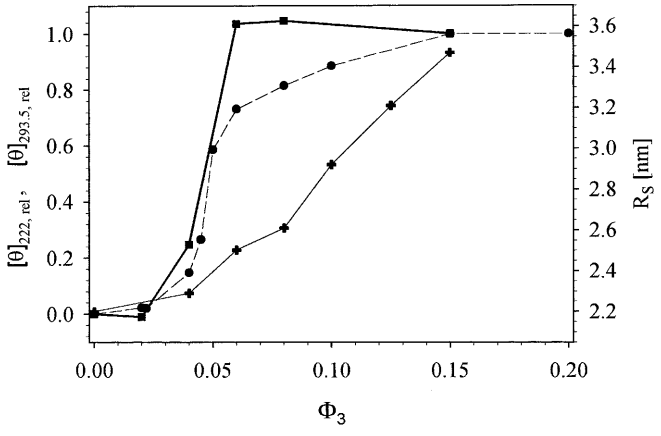

Fig. 5 Relative changes of the molar residue ellipticities measured at 222 nm (●) and 293.5 nm (■) and changes of the Stokes radius (✚) for β-Lg A at 25 °C in 10 mM HCl (pH 2) in dependence on the volume fraction of HFIP. The ellipticities were normalized according to $([\theta](\Phi)-[\theta](0))/([\theta](\Phi_{max})-[\theta](0))$

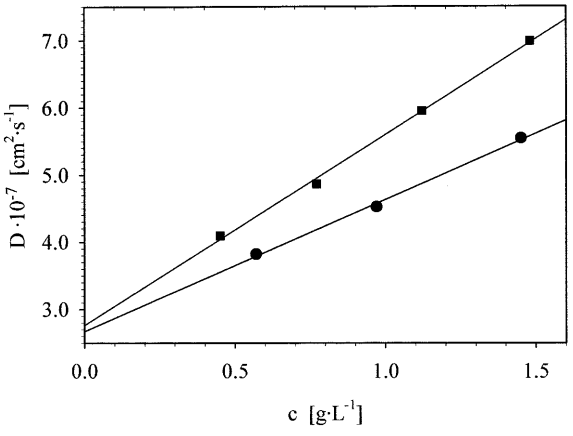

Fig. 7 Concentration dependence of the translational diffusion coefficients D for PGK in 10 mM HCl (pH 2) at 25 °C in the presence of volume fractions of either 0.15 HFIP (●) or 0.5 TFE (■)

normalized in the same manner as the CD data and have not been measured at $\Phi_3 > 0.15$ because of the decreasing scattering contrast at higher HFIP concentrations (see below). However, we expect that $R_S = 3.47$ nm in 15% HFIP is practically the Stokes radius in the helical state, because it is close to $R_S = 3.42$ nm in 50% TFE and the ellipticities level off at the corresponding concentrations of fluoroalcohol.

Structural transitions of PGK in the presence of HFIP and TFE

PGK is highly unfolded at pH 2 and undergoes a coil-to-helix transition with increasing concentrations of HFIP and TFE. This is well reflected by the changes in the molar residue ellipticities at 222 nm (Fig. 6). This transition sets in at low fluoroalcohol concentrations and proceeds in a rather non-cooperative manner. The ellipticity at 222 nm levels off at sufficiently high fluoroalcohol concentrations, is identical for HFIP and TFE, and yields a helical content of 89% according to the method of Chen et al. (1972). The difference in the effectiveness of HFIP and TFE in inducing the conformational transition is clearly demonstrated. In the case of TFE, nearly a three times higher concentration is needed to bring about the same effect.

We have measured the hydrodynamic dimensions of PGK after the transition to the helical state, viz. at $\Phi_3 = 0.15$ for HFIP and $\Phi_3 = 0.5$ for TFE, respectively. The measured diffusion coefficients used for an extrapolation to zero protein concentration are shown in Fig. 7. The Stokes radii obtained from $D(c = 0)$ are given in Table 1.

To check whether hydrophobic interactions are involved in the formation of helical structure in PGK at low pH and in the presence of fluoroalcohols, we have studied the temperature dependence of the helix

Table 1 Stokes radii (in nm) of β-Lg A and PGK in 10 mM HCl in the absence of fluoroalcohol and at volume fractions Φ_3 of HFIP and TFE, at which the transition to the highly helical state is essentially complete

Fluoroalcohol concentration	β-Lg A	PGK
$\Phi_3 = 0$	2.20 ± 0.04	7.42 ± 0.15
HFIP, $\Phi_3 = 0.15$	3.47 ± 0.06	8.02 ± 0.15
TFE, $\Phi_3 = 0.50$	3.42 ± 0.06	7.76 ± 0.15

promoting effect by CD. The experiments were performed at low concentrations of HFIP ($\Phi_3 = 0.02$) and TFE ($\Phi_3 = 0.08$), where the increase in helical structure with increasing alcohol concentration is very steep and amounts to about one third of the final helical content. In the presence of HFIP, the temperature dependence of the molar residue ellipticities at 222 nm (Fig. 8) clearly shows the phenomenon of cold denaturation at temperatures below 23 °C. Cold denaturation is not detectable in the presence of TFE at temperatures above 0 °C.

Apparent molecular mass and preferential binding in the presence of fluoroalcohols

Molecular masses extrapolated to zero protein concentration have been determined for both proteins in the absence and presence of HFIP and TFE. For the molecular mass in the absence of fluoroalcohol we obtained $17{,}000 \pm 2000$ g mol^{-1} and $47{,}600 \pm 4000$ g mol^{-1} for β-Lg A and PGK, respectively. The experimentally determined refractive index increments of the proteins in the native state are 0.198 ± 0.003 cm^3 g^{-1} and 0.187 ± 0.003 cm^3 g^{-1} for β-Lg A and PGK, respectively. For the calculation of apparent molecular masses in the presence of fluoroalcohols, we have taken values of $(\partial n/\partial c)$ calculated according to Eq. 6 using $(\partial n/\partial c)_0 = 0.19$. The relative apparent molecular masses of β-Lg A in dependence on HFIP concentration Φ_3 are shown in Fig. 9. The decrease in M_{app} is unusual on first sight because degradation of protein molecules in the presence of HFIP can be excluded. This decrease is the consequence of preferential binding of HFIP molecules to the protein. The refractive index of proteins is larger ($\sim$1.6), while HFIP has a refractive index which is smaller (1.275 at 25 °C and 589.3 nm wavelength) than that of water (1.333). Thus, preferential binding of HFIP to the protein matches the difference in the refractive indices between bulk solvent and HFIP-loaded protein molecules. In this respect, the extraordinary low refractive index of HFIP is very useful in detecting and measuring preferential interactions, particularly when an actual decrease in the true molecular mass can be excluded. Cosolvents with refractive indices larger than that of water increase the apparent molecular mass. This effect has to be distinguished from possible molecular association. On the other hand, the decreasing scattering contrast at higher HFIP concentrations ($\Phi_3 \geq 0.2$) complicates DLS measurements. The scattering from pure HFIP clusters exceeds that of the protein and renders a proper estimation of $D(c)$ difficult.

For PGK, we have measured the influence of HFIP on M_{app} only at $\Phi_3 = 0.15$. The relative decrease is practically the same within the experimental error. Interestingly, a decrease in M_{app} is not detectable in the presence of TFE at characteristic concentrations (Fig. 9).

It is further interesting to compare the Stokes radii of both proteins in the helical state at equivalent fluoroalcohol concentrations. The results for HFIP with $\Phi_3 = 0.15$ and TFE with $\Phi_3 = 0.50$ are shown in Table 1.

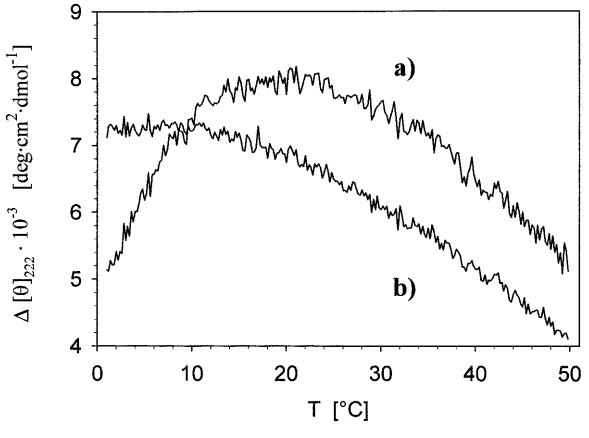

Fig. 8 Temperature dependence of the difference of mean residue ellipticities $\Delta[\theta]_{222} = [\theta]_{222}(\Phi_3 = 0) - [\theta]_{222}(\Phi_3 = x)$ of PGK in 10 mM HCl (pH 2) in the presence of *a* HFIP with a volume fraction $x = 0.02$ and *b* TFE with $x = 0.08$. The correction with respect to the CD in the absence of fluoroalcohol is necessary because $[\theta]_{222}$ of the unfolded protein changes from –3000 to –5000 deg cm^2/dmol when the temperature is raised from 1 to 50 °C

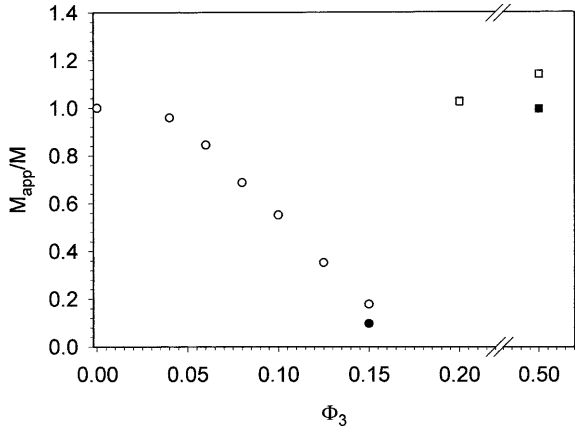

Fig. 9 Relative changes of the apparent molecular mass of β-Lg A in dependence on the volume fraction of HFIP in 10 mM HCl (pH 2) at 25 °C (○). The values for M_{app} were taken from the extrapolation to zero protein concentration (Fig. 4). For comparison, there are also shown the corresponding results for β-Lg A in the presence of TFE (□) and for PGK in HFIP, $\Phi_3 = 0.15$ (●), and in TFE, $\Phi_3 = 0.5$ (■)

Discussion

HFIP- and TFE-induced structural transitions of β-Lg A and PGK

The structural changes occurring with increasing fluoroalcohol concentration have been monitored by the changes in secondary structure according to far-UV CD, the loss of the specific tertiary structure according to near-UV CD in the case of β-Lg A, and the changes in the molecular dimensions as revealed by the hydrodynamic Stokes radius R_S. Most of the experiments were performed with HFIP. The data for TFE are discussed only for comparison or if essential additional information can be deduced. Both proteins reveal rather different transitions. This results mainly from the opposite starting conformations of both proteins under identical solvent conditions.

At pH 2, PGK (415 amino acid residues, $M =$ 44,600 g mol^{-1}, no disulfide bonds) is in an extended random coil conformation, which has been described in detail previously (Damaschun et al. 1998, 1999). The transition to the helical state sets in at very low HFIP concentrations and appears to be not very cooperative. At $\Phi_3 = 0.02$, there is already a remarkable increase in the ellipticity at 222 nm. The transition is complete above $\Phi_3 = 0.1$ for HFIP or above $\Phi_3 = 0.4$ for TFE, respectively. Despite the strong alterations in the chain configuration, the Stokes radius only slightly increases on the transition from the unfolded state ($R_S = 7.42 \pm$ 0.10 nm) to the helical state ($R_S = 8.02 \pm 0.16$ nm).

In the case of β-Lg A (260 amino acid residues, $M = 18{,}370$ g mol^{-1}, two disulfide bonds), we start from the natively folded conformation, which comprises essentially antiparallel β-sheet structure and only a little helical structure (Brownlow et al. 1997; Kuwata et al. 1999). According to our data, the transition is not simply two-state. Substantial conformational changes are observed only at $\Phi_3 \geq 0.04$ in a very cooperative manner. The disruption of the native tertiary structure precedes slightly or takes place simultaneously with essential changes in the secondary structure. This supports the view that the fluoroalcohol-induced transitions of native proteins are essentially controlled by the stability of the protein against denaturation by the particular alcohol. This cooperative transition is essentially complete at $\Phi_3 = 0.07$. According to our CD data, between 70 and 80% of the total change in secondary structure is attained at this HFIP concentration. Therefore, it is not surprising that in some previous investigations the entire transition from the native to the helical state was considered as a two-state transition (Hirota et al. 1997; Hong et al. 1999). Other authors (Uversky et al. 1997; Kuwata et al. 1998; Mendieta et al. 1999) have provided evidence for the existence of an intermediate on the transition from the native to the so-called highly helical state. Using different organic solvents, Uversky et al. (1997) have inferred the existence of a compact intermediate from the non-coinciding transitions of different optical probes and the changes of the affinity to bind the hydrophobic dye 8-ANS. The existence of a compact intermediate is now directly demonstrated by the changes of R_S (Fig. 5). At $\Phi_3 = 0.07$, R_S is 15% larger than in the native state. This is much less compared to the highly helical state at $\Phi_3 = 0.15$, where the increase in R_S amounts to 58%. This compact intermediate state with a considerable amount of helical structure is of general interest for folding investigations because it resembles the kinetic intermediate observed during refolding of β-Lg A (Hamada et al. 1996; Kuwajima et al. 1996). NMR investigations (Kuwata et al. 1998) of the TFE-induced transition of β-Lg A yielded preliminary insights into the structure of the intermediate state occurring at 15% (v/v) TFE. The results suggest that the part of the protein belonging to the C-terminal core sheet (β_E-β_H) and to the β_I strand is in a native and the rest of the molecule is in a non-native conformation. The N-terminal part has a stronger preference for helical structure and is easier transformed to the helical conformation.

The question arises of whether or not the compact helical intermediate can be considered as a typical molten globule (MG) (Kuwajima 1989; Ptitsyn 1995). The intermediate shares many features of the MG, but differs from a typical MG because of the non-native secondary structure. The transition from the compact helical state to the native state is not simply the formation of the fixed specific tertiary structure accompanied by slight rearrangements of the secondary structure, but involves a total switch in secondary structure caused by non-local interactions. It should be considered to preserve the term MG for those compact intermediates which have an essentially native-like secondary structure. It must be mentioned, furthermore, that the compact helical intermediate of β-Lg A differs from the MG found in the TFE-induced transition of bovine α-lactalbumin (Gast et al. 1999). This MG is more compact (R_S is only 10% larger than in the native state), has an essentially native secondary structure according to far-UV CD data, and exhibits stronger attractive intermolecular interactions according to the concentration dependence of the diffusion coefficient. Similarities and differences between the MG and the kinetic folding intermediate of β-Lg A have been discussed by Kuwajima et al. (1996). It is still a matter of dispute whether folding of β-Lg A is hierarchic or non-hierarchic (Forge et al. 2000).

Figure 5 shows that the moderate further increase in helical secondary structure occurring on the transition to the highly helical state is accompanied by a considerable increase in R_S. The total increase in R_S upon alcohol denaturation is very different for individual proteins but seems not to depend very much on the alcohol used (Table 1). Obviously, it depends on the number and position of disulfide bonds and the net charge of the protein. The relative increase compared to the native state is largest for PGK at pH 2 (2.7 in HFIP and 2.6 in TFE), somewhat smaller for β-Lg A (1.58 in HFIP and

1.57 in TFE), and least for ribonuclease A and bovine α-lactalbumin (about 1.2 in TFE) (Gast et al. 1999).

Cluster formation and the alcohol-induced transition

In the previous section we have demonstrated that essential structural changes in both proteins occur at HFIP concentrations below $\Phi_3 = 0.1$, which is the critical concentration for cluster formation. Thus, HFIP clusters are not involved in such remarkable structural transitions as the disruption of the native tertiary structure and the formation of an essential degree of non-native helical structure. Similar observations have been made for TFE at concentrations below $\Phi_3 = 0.2$. Although the existence of clusters is unnecessary for most of the observed structural changes, the ability to form clusters and the efficiency to induce structural changes are correlated, since both phenomena are obviously based on the same effect, the hydrophobic interactions between halogenated alcohol molecules either with itself or with hydrophobic groups of the polypeptide chain. It is an open question whether HFIP interacts exclusively with those amino acids which are classified as hydrophobic ones. That there is indeed a strong interaction of HFIP molecules with the polypeptide chain will be discussed now.

Preferential interactions of fluoroalcohols with the polypeptide chain

A sensitive but poor quantitative indicator of preferential interactions are the relative changes of M_{app} in dependence on fluoroalcohol concentration (Fig. 9). This effect is very pronounced for HFIP and is manifested at HFIP concentrations where β-Lg A starts to unfold. The maximum of preferential interaction could not be determined because of the strongly decreasing scattering contrast of the protein-HFIP complex and the interfering scattering from pure HFIP clusters. M_{app}/M and the corresponding refractive increments were used to calculate the coefficients of preferential binding at chosen HFIP concentrations (Table 2) using Eqs. 2 and 5. In the helical state, at $\Phi_3 = 0.15$, the coefficient of preferential interaction γ' approaches an unexpected large value of about 4 g(HFIP)/g(protein). However, this might become plausible if one takes into consideration that the hydrodynamic volume increases by a factor of 3.9 on the transition from the native to the helical state. For the compact helical intermediate, γ' is about 1 g(HFIP)/g(protein). Surprisingly, preferential interactions could not be detected in the case of TFE. Preferential interactions were expected because TFE has also a considerable structure-transforming efficiency and tends to form clusters in aqueous solutions. Additional experiments including more proteins are needed to clarify whether there is only a weak or no contribution of preferential interactions concerning the effects of TFE on proteins. The results for HFIP clearly demonstrate that preferential interactions are the most important factor for the remarkable efficiency by increasing the local fluoroalcohol concentration at the protein surface compared to the bulk solvent. Therefore, one must be careful in correlating average mixed solvent properties, e.g. the dielectric constant, with the observed structural transitions in cases where preferential binding can be expected. It should be remembered that preferential interactions are related only to the excess concentration of the fluoroalcohol at the protein surface compared to the bulk solvent. At the present stage of the investigations we will not undertake attempts to relate preferential to absolute interactions, which can be better correlated with the observed conformational changes. This has been done by Inoue and Timasheff (1972) using their experimental data on the 2-chloroethanol-induced structural transition of β-Lg A (Inoue and Timasheff 1968). However, even the preferential binding data yield important insights into the mode of action of such fluoroalcohols like HFIP. Undoubtedly, preferential binding of HFIP is mostly driven by hydrophobic interactions. This is also supported by the observation of cold denaturation of PGK in the presence of low HFIP concentrations (Fig. 8). With TFE, cold denaturation is not observed or happens at temperatures below 0 °C, which are not easily attainable. These marked differences are consistent with the differences in the preferential binding between HFIP and TFE.

Table 2 Preferential interaction parameters for HFIP derived from the apparent molecular mass of β-Lg A

Φ_3	M_{app}/M	γ (cm^3/g)	γ' (g(HFIP)/g(protein))
0.04	0.96	0.14	0.24
0.06	0.85	0.48	0.83
0.08	0.69	0.88	1.55
0.15	0.18	2.3	4.3

Preferential interactions and hydrodynamic dimensions

The existence of strong preferential interactions in the presence of HFIP raises the question of whether the increase in hydrodynamic dimensions is indeed caused by a substantial extension in the geometric dimensions of the protein itself or is considerably influenced by an expansion of the solvation shell. Furthermore, it is surprising that the hydrodynamic radii obtained for the highly helical state in TFE are nearly as large as in HFIP. This shows that a considerable solvation of proteins must take place in mixed alcohol-water solvents in general. In the case of TFE, the compositions of the bulk solvent and the bound solvation shell are not very different, according to our static light scattering data. The characteristic differences in the relative increase of

the Stokes radius on the transition to the helical state, which is 2.7-fold for PGK and 1.2-fold for ribonuclease A, suggest an essential change in the geometric dimensions. However, it is more instructive to refer to radii of gyration R_G obtained from small-angle X-ray scattering (SAXS) measurements under comparable conditions. Kamatari et al. (1999) have studied several proteins, including β-Lg A, in methanol-water solvent and have reviewed previous results obtained for the helical state. The increase in R_G expressed by $R_{G,H}/R_{G,N}$ is between 1.5 (for α-lactalbumin) and 2.2 (for β-Lg A), where H and N refer to the helical and native states, respectively. According to the measured values of R_G, the dimensions of the proteins in the helical state are much closer to those in the highly unfolded state (5 M urea) than to those in the native state, indicating that there is in fact a remarkable geometric expansion. Kratky plots of SAXS data indicate that the proteins have a chain-like conformation (Kamatari et al. 1999), where the chain segments are mostly formed by helices. If we calculate the ρ-factor for β-Lg A in the helical state, taking $R_G = 3.95$ nm measured in 60% methanol (Kamatari et al. 1999) and the average of the Stokes radii in HFIP and TFE ($R_S = 3.45$ nm), we obtain 1.14. This is less than the ρ-factor of random chains in a theta solvent (1.6) and the experimentally determined values for cold denatured PGK (1.57) and acid denatured PGK (1.57) (Damaschun et al. 1998). The smaller $\rho = 1.14$ could be a consequence of strong solvation in alcohol-water mixed solvents. A strongly solvated chain consisting of different helical segments is, therefore, the most probable conformation in the highly helical state. This structure is at best described by the broken-rodlike (BR) chain model as suggested by Muroga (2000) for polypeptide chains in helicogenic solvents. In this model, several helical rods are joined by flexible random coils.

The fact that HFIP is strongly preferentially bound and TFE practically not and the comparable degrees of solvation in both cases are interesting with respect to basic views concerning the action of alcohols on proteins. One opinion (Jasanoff and Fersht 1994; Rajan and Balaram 1996; Luo and Baldwin 1997) favors direct interactions, while the other (Conio et al. 1970; Cammers-Goodwin et al. 1996; Walgers et al. 1998) assumes an indirect mechanism. According to our experimental data, a direct mechanism can be anticipated for HFIP and an indirect mechanism is more probable in the case of TFE. This means that both basic mechanisms must be taken into consideration, depending on the particular alcohol. Replacement of water molecules by alcohol molecules is obviously the most important process, regardless of whether this is due to preferential interactions or due to a modification of the solvation shell.

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