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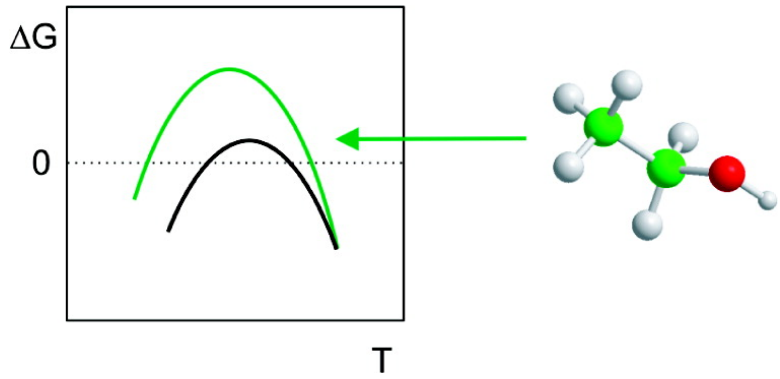

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Cold Denaturation of Yeast Frataxin Offers the Clue to Understand the Effect of Alcohols on Protein Stability

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Abstract: Although alcohols are well-known to be protein denaturants when present at high concentrations, their effect on proteins at low concentrations is much less well characterized. In this paper, we present a study of the effects of alcohols on protein stability using Yfh1, the yeast ortholog of the human protein frataxin. Exploiting the unusual property of this protein of undergoing cold denaturation around 0 °C without any ad hoc destabilization, we determined the stability curve on the basis of both high and low temperature unfolding in the presence of three commonly used alcohols: trifluoroethanol, ethanol, and methanol. In all cases, we observed an extended temperature range of protein stability as determined by a modest increase of the high temperature of unfolding but an appreciable decrease in the low temperature of unfolding. On the basis of simple thermodynamic considerations, we are able to interpret the literature on the effects of alcohols on proteins and to generalize our findings. We suggest that alcohols, at low concentration and physiological pH, *stabilize* proteins by greatly widening the range of temperatures over which the protein is stable. Our results also clarify the molecular mechanism of the interaction and validate the current theoretical interpretation of the mechanism of cold denaturation.

Introduction

Unfolding of mesophilic proteins occurs both at temperatures higher and lower than room temperature: the high temperature transition is generally referred to as "heat denaturation" whereas that at lower temperatures is known as "cold denaturation". The two transitions arise because the free energy difference between the native and denatured states has a convex dependence on temperature,[1] with a maximum at T_S, the temperature corresponding to the maximum of the stability curve and at which the difference in entropy between the denatured and native states is zero. Since T_S is generally close to room temperature, destabilization of the native state occurs as the temperature varies from room temperature in either direction, toward the two transition points.[2]

Accurate analysis of both the heat and cold denaturation transitions of several proteins is very important since, through these studies, we expect to understand better these processes and unveil new aspects of protein stability and dynamics. To do so, it would be necessary to examine several cases of cold denaturation before drawing conclusions on the mechanism of the transitions and on the influence on them of environmental conditions. However, while heat denaturation has been studied in detail for many different examples, full access to the cold denatured state is normally limited since, for most proteins, this occurs well below 0 °C, the freezing point of water. The most common way to circumvent this difficulty has been the use of destabilizing conditions, such as extreme pH values and very high pressures,[3] the addition of chemical denaturants or of cryosolvents,[4,5] and/or the insertion of ad hoc point mutations that lower protein stability. The use of alcohols as denaturing agents seemed particularly appealing since, in addition to decreasing the stability of the protein, they lead to cryoscopic lowering of the freezing temperature of the solution below 0 °C. Hatley and Franks[6] claimed that methanol could allow measurements at subzero temperatures and, at the same time, shift the temperature of cold denaturation toward zero. However, it was soon discovered that, in general, the addition of alcohols also causes a parallel decrease of the cold denaturation temperature.[1]

In a thorough calorimetric study of the thermal denaturation of lysozyme in alcohol-water mixtures, Velicelebi and Sturtevant[7] showed that the main effect of alcohols is to significantly decrease the difference in heat capacity between the folded and denaturated states (ΔC_p). This difference is the key thermodynamic parameter that influences cold denaturation.[1] The large influence of alcohols on the ΔC_p of lysozyme[7] appears, however, an exception with respect to other agents that modify protein stability: calorimetric studies on small monomeric globular

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(1) Privalov, P. L. *Crit. Rev. Biochem. Mol. Biol.* **1990**, *25*, 281–305.
(2) Caldarelli, G.; De Los Rios, P. *J. Biol. Phys.* **2001**, *27*, 229–241.
(3) Jonas, J. *Biochim. Biophys. Acta* **2002**, *1595*, 145–159.
(4) Griko, Y. U.; Privalov, P. L.; Sturtevant, J. M.; Venyaminov, S. Yu. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 3343–3347.
(5) Chen, B.; Schellman, J. *Biochemistry* **1989**, *28*, 685–699.
(6) Hatley, R. H. M.; Franks, F. *Eur. J. Biochem.* **1989**, *84*, 237–240.
(7) Velicelebi, G.; Sturtevant, J. M. *Biochemistry* **1979**, *18*, 1180–1186.

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proteins have shown that ΔC_p is specific for a given protein and does not vary significantly upon changing the environmental conditions.[8,9] Further studies are thus necessary to observe the influence of alcohols on cold denaturation without the need of adding other denaturants to raise the melting temperature of its cold denaturation above 0 °C.

Looking for a way to study cold denaturation of a natural protein without the help of destabilization, we have recently identified a protein, Yfh1, whose cold denaturation occurs at accessible temperatures close to 0 °C and under physiological conditions at pH 7; that is, without the need to add denaturants.[10] Yfh1 is a small mitochondrial protein and the yeast ortholog of frataxin, the human protein responsible for Friedreich's ataxia, a severe neurodegenerative disease. These features, together with the fact that Yfh1 is a full-length protein from natural sources rather than an ad hoc designed mutant, makes it a system uniquely suited for an extensive characterization of the cold transition and of the factors influencing its stability as a function of temperature. Yfh1 seems particularly well suited for studies of cold denaturation in water−alcohol mixtures because, starting from a cold denaturation melting temperature (T_c) above zero, it should still be possible to observe a substantial part of the denaturation curve even if the addition of alcohols shifts T_c to temperatures lower than 0 °C.

In the present paper we describe the influence of three alcohols on the cold and heat unfolding of Yfh1. We chose three of the alcohols most commonly used in protein studies, 2,2,2-trifluoroethanol (TFE), ethanol (EtOH), and methanol (MetOH), because their order of effectiveness in destabilizing tertiary structures and stabilizing helical secondary structures are known to be the same: TFE > EtOH > MetOH.[11] Changes in protein conformation were monitored by circular dichroism (CD) and NMR spectrocopies. We observe that small amounts of alcohols lead to the unusual effect of decreasing ΔC_p, a quantity otherwise mostly independent of temperature or environmental conditions and specific for a given protein.[8,9] This leads to flattening of the stability curve of the protein. However, a concomitant increase of ΔH_m leads to a decrease of T_S, thus shifting the whole stability curve to lower temperatures and widening the temperature range over which the protein is stable. The influence of alcohols on cold denaturation, as measured by the transition temperature, is much larger than that on the high temperature transition.

Experimental Section

Sample Preparation. *S. cerevisiae* Yfh1 and ^{15}N-labeled Yfh1 were expressed in *E. coli* as described by Adinolfi et al.[12] and by He et al.[13] The construct was expressed in *E. coli* as fusion protein with His-tagged glutathione-*S*-transferase (GST) either with tobacco etch virus (TEV) or PreScission protease cleavage sites. The soluble overexpressed proteins were first passed through a nickel column and eluted with imidazole (pH 8) and then further purified by gel filtration chromatography on a Superdex G75 16/60 column (Pharmacia). The purity of the recombinant proteins was checked by SDS-PAGE after each step of the purification and by mass spectroscopy on the final products. In all cases a single species was identified. ^{15}N-labeled samples were produced by growing the bacteria in minimal medium using ammonium sulfate as the sole source of nitrogen.

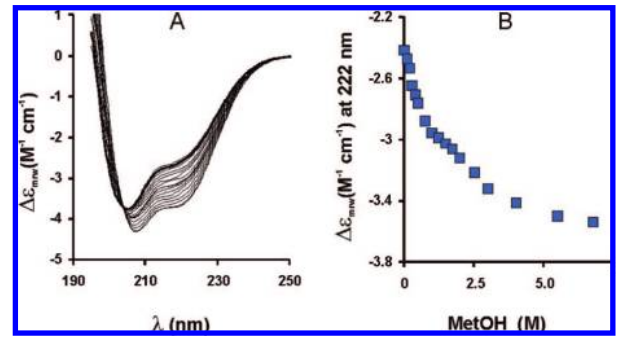

Figure 1. Influence of alcohols on the far-UV CD spectrum of Yfh1. (A) Spectra recorded in a 20 mM HEPES buffer at pH 7.0 and at 25 °C, with a protein concentration of 10 μM and increasing methanol content; (B) variation of the intensity at 222 nm as a function of alcohol concentration.

NMR Spectroscopy. NMR spectra were recorded on a Varian INOVA spectrometer and on a Bruker AVANCE both operating at 600 MHz ^{1}H frequency. Typically, measurements were carried out in a 20 mM HEPES buffer at pH 7.0 using a protein (unlabeled or ^{15}N uniformly labeled) concentration of 0.3−0.5 mM. Water suppression was achieved by the WATERGATE pulse-sequence,[14] heteronuclear single quantum correlated spectrum (HSQC) experiments were used as described by Bax et al.[15] The spectra were processed and zero-filled to the next power of two using the NMRPipe program.[16] Baseline correction was applied when necessary.

Far-UV CD Measurements. Far-UV CD spectra were recorded on a Jasco J-715 spectropolarimeter fitted with a cell holder thermostatted by PTC 348-WI Peltier unit. Measurements were carried out in a 20 mM HEPES buffer at pH 7.0 using a protein concentration of 10 μM and fused silica cuvettes of 1 mm path length (Hellma). Thermal unfolding was repeated at least twice on independent protein preparations to ensure reproducibility of the results. CD spectra were typically recorded with 0.2 nm resolution and were baseline corrected by subtraction of the appropriate buffer spectrum. Thermal unfolding curves were obtained by monitoring the ellipticity at 222 nm using 2 mm path length cells and a heating rate of 1 °C/min.

Results

We monitored the influence of alcohols on the far-UV CD spectra of Yfh1 dissolved in a 20 mM HEPES buffer at pH 7.0. This technique monitors directly the secondary structure of a protein. Addition of even very small amounts of any of the three alcohols used, that is, methanol, ethanol and TFE, leads to an increase in the population of the folded form. Figure 1A shows the far-UV CD spectrum of a buffered solution of Yfh1 recorded in different methanol−water mixtures. Increasing amounts of alcohol yield spectra consistent with there being a higher proportion of the folded conformation present. Figure 1B shows the variation of the intensity at 222 nm with alcohol concentration.

2D and 1D NMR spectra of Yfh1 in different water-alcohol mixtures were recorded in the temperature range of 268−318

(8) Privalov, P. L. *Adv. Protein Chem.* **1979**, *33*, 167–241.
(9) Privalov, P. L. *Adv. Protein Chem.* **1982**, *35*, 1–104.
(10) Pastore, A.; Martin, S.; Politou, A.; Kondapalli, K. C.; Stemmler, T.; Temussi, P. A. *J. Am. Chem. Soc.* **2007**, *129*, 5374–5375.
(11) Bianchi, E.; Rampone, R.; Tealdi, A.; Ciferri, A. *J. Biol. Chem.* **1970**, *245*, 3341–3345.
(12) Adinolfi, S.; Trifuoggi, M.; Politou, A. S.; Martin, S.; Pastore, A. *Hum. Mol. Genet.* **2002**, *11*, 1865–1877.
(13) He, Y.; Alam, S. L.; Proteasa, S. V.; Zhang, Y.; Lesuisse, E.; Dancis, A.; Stemmler, T. L. *Biochemistry* **2004**, *43*, 16254–16262.
(14) Piotto, M.; Saudek, V.; Sklenar, V. *J. Biomol. NMR* **1992**, *2*, 661–666.
(15) Bax, A.; Ikura, M.; Kay, L. E.; Torchia, D. A.; Tschudin, R. *J. Magn. Reson.* **1990**, *86*, 304–318.
(16) Delaglio, F.; Grzesiek, S.; Vuister, G.; Zhu, G.; Pfeifer, J.; Bax, A. *J. Biomol. NMR* **1995**, *6*, 277–293.

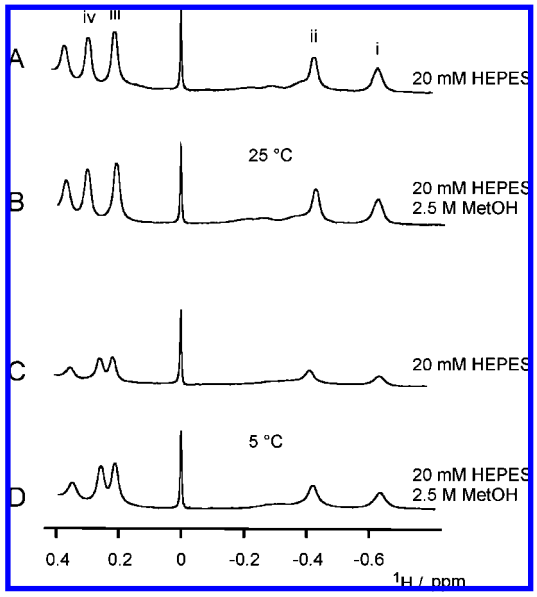

Figure 2. Recovery of the population of the folded conformer of Yfh1 upon addition of CD3OD. The four most high-field-shifted peaks in the NMR spectrum of Yfh1 are marked i to iv. The peak at 0 ppm corresponds to a fixed amount of TSP. (A) 1D spectrum in 20 mM HEPES at 25 °C; (B) 1D spectrum in 20 mM HEPES and 2.5 M CD_3OD at 25 °C; (C) 1D spectrum in 20 mM HEPES at 5 °C; (D) 1D spectrum in 20 mM HEPES at 5 °C and 2.5 M CD_3OD.

K, both to check the integrity of the tertiary structure and to complement the CD data with a technique that is sensitive both to the secondary and to the tertiary structures of proteins.

As can be seen from Figure 2, we observed no major effect on the conformation of the folded state upon the addition of alcohols, as judged from the position of high-field ring-current shifted resonances that occur at chemical shift values around 0 ppm or even at negative values. These peaks arise from aliphatic groups close to aromatic rings in the hydrophobic core of the protein and therefore monitor the stability of the tertiary structure of the protein. Since their chemical shifts are not influenced by the presence of alcohols, we can assume that the protein remains correctly folded, and retains the same 3D architecture. However, there is a profound influence of alcohols, even at very low concentrations, on the population of the folded conformer. Variations in the relative peak intensities can be directly linked to the population of the folded form of the protein. Figure 2 shows a comparison of the high field portions of the 1D NMR spectra of Yfh1 with and without methanol. It is easy to evaluate the intensities of the four high field peaks (i−iv) by comparison with the intensity of the reference peak at 0 ppm, corresponding to a fixed amount of 3-(trimethylsilyl) propionate sodium salt (TSP). At 25 °C addition of methanol (to make a 2.5 M solution, corresponding to 10% v:v) induces a modest increase of the population of the folded conformation. When the temperature of the HEPES buffered solution is decreased from 25 to 5 °C, that is, to a temperature just below the cold denaturation transition, the intensity of the four ring-current-shifted peaks (i−iv) drops to low values, as expected from the known behavior of Yfh1.[10] Careful inspection of Figure 2 shows that peaks iii and iv shift slightly when the temperature is lowered from 25 to 5 °C but all four peaks retain their positions (at 5 °C) when MetOH is added. At 5 °C, the progressive addition of methanol induces a recovery of the population of the folded conformation. The four peaks, in a 2.5 M solution of CD_3OD in HEPES buffer, although broadened as a consequence of the lower temperature, have recovered volumes closer to those at 25 °C in the HEPES solution. HSQC $^1H-^{15}N$ correlation spectra corresponding to all 1D spectra of Figure 2 are shown as Supporting Information.

We monitored the whole thermal denaturation curves of Yfh1 by CD spectroscopy, from low to high temperatures and back, in water alcohol mixtures at several different concentrations to quantify the effect of alcohols. Figure 3 shows the thermal denaturation curves of Yfh1 measured by monitoring the CD intensity at 222 nm as a function of temperature in the temperature range 273−333 K in the three water−alcohol mixtures. It is possible to observe that even very small percentages of alcohol have a profound effect on the form of the denaturation curve: the high temperature melting transitions are not much affected, whereas the low temperature melting transitions are shifted to much lower temperatures, thus widening the range of temperatures over which the protein is stable.

To extract the relevant thermodynamic parameters for the unfolding processes it is necessary to make two assumptions: (i) that the folding transition is 2-state and (ii) that there is no temperature dependence of ΔC_p. Both assumptions proved consistent with the behavior of Yfh1 in several buffers (see ref 10 and references quoted therein) and we interpreted the absence of relevant conformational transitions due to alcohols as an indication that they still hold in water−alcohol mixtures at low alcohol concentrations. If it is assumed that the equilibrium between folded and unfolded forms of Yfh1 is a simple two-state equilibrium, the population of the folded form at any temperature, $f_F(T)$, is a function of $\Delta G°(T)$, the Gibbs free energy of unfolding. If we assume that the difference in heat capacity between the folded and unfolded forms, ΔC_p, is temperature independent, the Gibbs free energy is given by the Gibbs−Helmholtz equation modified so that the reference temperature is the midpoint of the high temperature transition (T_m, heat denaturation melting temperature).[1] A nonlinear least-squares fit to the observed CD signal[17] allows one to determine T_m, ΔH_m and ΔC_p. The corresponding values for low temperature unfolding (T_c and ΔH_c) can then be determined from a plot of $\Delta G°$ against temperature.[18] Values of the entropy change at T_c and T_m can be obtained from the Gibbs−Helmholtz's relationship $\Delta G = \Delta H - T\Delta S$. This analysis does, of course, make the assumption that the CD intensities of the heat- and cold-denatured forms are the same. The relevant thermodynamic parameters are summarized in Table 1.

Figure 4 summarizes the change of the thermodynamic parameters as a function of alcohol concentration. The difference in heat content between the folded and denaturated states (ΔC_p) decreases significantly with increasing alcohol concentrations, in an approximately linear fashion (Figure 4A) and is assumed to be the same for both temperature transitions. T_m has a complex behavior (Figure 4B): there is a sharp but small increase at very low alcohol concentrations followed by a progressive, small decrease at higher alcohol concentrations.

It is difficult to assess, from such data alone, the extent of the stabilizing effect since the increases are fairly small, at least for methanol and ethanol. From ∼29 °C in the absence of methanol to maxima of ∼33 °C (methanol), ∼35 °C (ethanol), and ∼39 °C (TFE). The enthalpy difference between the unfolded and native states measured at T_m (ΔH_m) also shows a small sharp increase at very low alcohol concentrations and then

(17) Masino, L.; Martin, S. R.; Bayley, P. M. *Protein Sci.* **2000**, *9*, 1519–1529.
(18) Szyperski, T.; Mills, J. L.; Perl, D.; Balbach, J. *Eur. Biophys. J.* **2006**, *35*, 363–366.

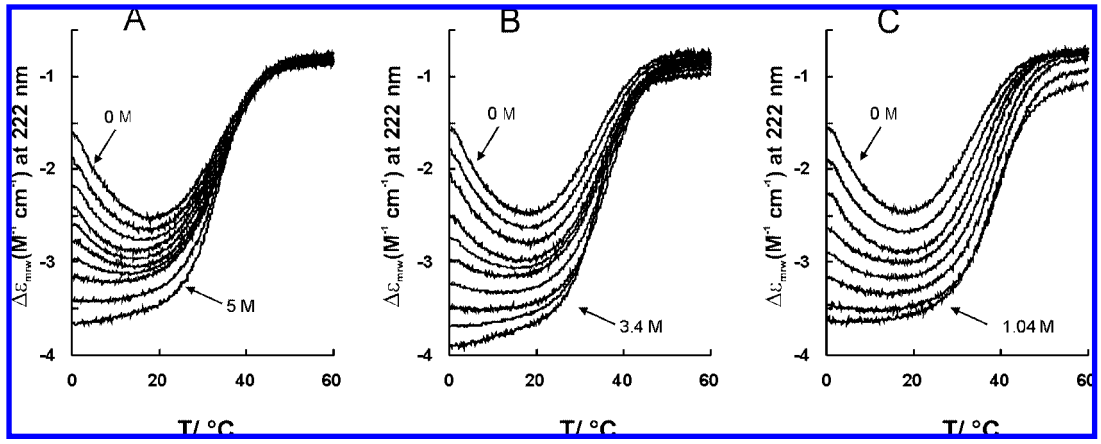

Figure 3. Thermal denaturation curves of Yfh1 in a 20 mM HEPES buffer at pH 7.0 with a protein concentration of 10 μM, measured by monitoring the CD intensity at 222 nm as a function of temperature in the temperature range 0−60 °C in the three buffer-alcohol mixtures. (A) denaturation curves in methanol−water (spectra comprised between the two extremes have the following molar concentrations: 0.25/0.37/0.49/0.74/0.99/1.5/2.0/3.2); (B) denaturation curves in ethanol−water (spectra comprised between the two extremes have the following molar concentrations: 0.086/0.17/0.34/0.51/0.68/1.03/1.37/2.04); (C) denaturation curves in trifluoroethanol−water (spectra comprised between the two extremes have the following molar concentrations: 0.035/0.07/0.14/0.21/0.28/0.56).

Table 1. Thermodynamic Parameters of High and Low Temperature Unfolding of Yfh1 in Water/Alcohol Mixtures

alcohol (M)	ΔH_m (kcal mol^{-1})	T_m (°C)	ΔC_p (kcal K^{-1} mol^{-1})	ΔS_m (kcal K^{-1} mol^{-1})	T_c (°C)	ΔH_c (kcal mol^{-1})	ΔS_c (kcal K^{-1} mol^{-1})
			TFE				
0	20.5	29.2	1.89	67.8	8	−19.6	−69.6
0.035	29.1	31.6	2.04	95.5	3.9	−27.4	−99.0
0.070	34.7	33.5	2.04	113.2	0.7	−32.2	−117.7
0.14	38.1	35.4	1.87	123.5	−3.6	−34.8	−129.3
0.21	38.6	36.5	1.74	124.7	−5.8	−35.0	−131.0
0.28	39.9	37.5	1.65	128.5	−8.5	−36	−136.1
0.56	42.2	38.7	1.49	135.4	−14.6	−37.2	−144.0
1.11	46.8	38.1	1.27	150.4	−30	−39.7	−163.3
			EtOH				
0	20.5	29.4	1.89	67.8	8.3	−19.4	−68.9
0.086	26.3	30.5	2.01	86.6	5.1	−24.7	−89.0
0.17	30.1	32	1.89	98.7	1.3	−27.9	−101.8
0.34	34.4	33.5	1.91	112.2	−1.2	−32.0	−117.2
0.51	36	33.9	1.82	117.3	−4	−33.0	−122.6
0.68	37.2	34.3	1.7	121.1	−7.4	−33.7	−126.9
1.03	40.1	34.9	1.68	130.2	−10.4	−36.0	−137.1
1.37	43.1	35.4	1.54	139.7	−17.3	−38.1	−148.8
			MetOH				
0	19.4	28.3	1.99	64.4	9.2	−18.6	−65.9
0.25	26.8	30.6	1.93	88.3	3.7	−25.1	−90.8
0.37	27.2	30.9	1.89	89.5	3.1	−25.3	−91.8
0.49	27.1	31.1	1.91	89.1	3.5	−25.6	−92.6
0.74	30.2	31.7	1.72	99.1	−2	−27.8	−102.5
0.99	33.1	32.3	1.74	108.4	−4.2	−30.4	−113.1
1.48	34.8	32.5	1.66	113.9	−7.5	−31.6	−119.0
1.98	36.4	32.9	1.52	119.0	−12.5	−32.6	−125.2

a progressive, approximately linear, increase at higher alcohol concentrations (Figure 4D). Figure 4C and 4E show the corresponding curves for T_c (i.e., the low temperature unfolding midpoint), and for the difference in enthalpy between folded and denaturated states (ΔH_c), respectively. It is clear that the effect of the three alcohols on T_c is very much larger than that on T_m. For example, the T_m in the presence of 1 M ethanol is ∼5 °C higher than that in buffer whereas T_c is ∼19 °C lower than the corresponding value in the absence of ethanol. It is important to emphasize that the effect at any concentration of the three alcohols studied is to shift T_c to much lower values. Therefore, the main effect of the three alcohols is that of extending the temperature range over which the protein is stable.

The main results of the simultaneous analysis of the low and high temperature transitions in the three hydroalcoholic mixtures can be summarized as follows: (i) addition of even very small amounts of any of the alcohols used leads to an increase of the population of the folded form; (ii) the two unfolding temperatures, T_m and T_c, are affected in a different way; T_c moves monotonically to lower temperatures in a way roughly proportional to the amount of alcohol added whereas T_m has a small initial increase at low alcohol concentrations but then remains approximately constant at higher alcohol concentrations; (iii) the initial increase observed for T_m as a function of alcohol concentration follows the order TFE > EtOH > MetOH in terms of efficacy of the different alcohols; (iv) analysis of the unfolding curves shows that, within the range of concentrations examined, addition of alcohols causes a decrease of ΔC_p and a concomitant increase of ΔH_m and ΔS_m.

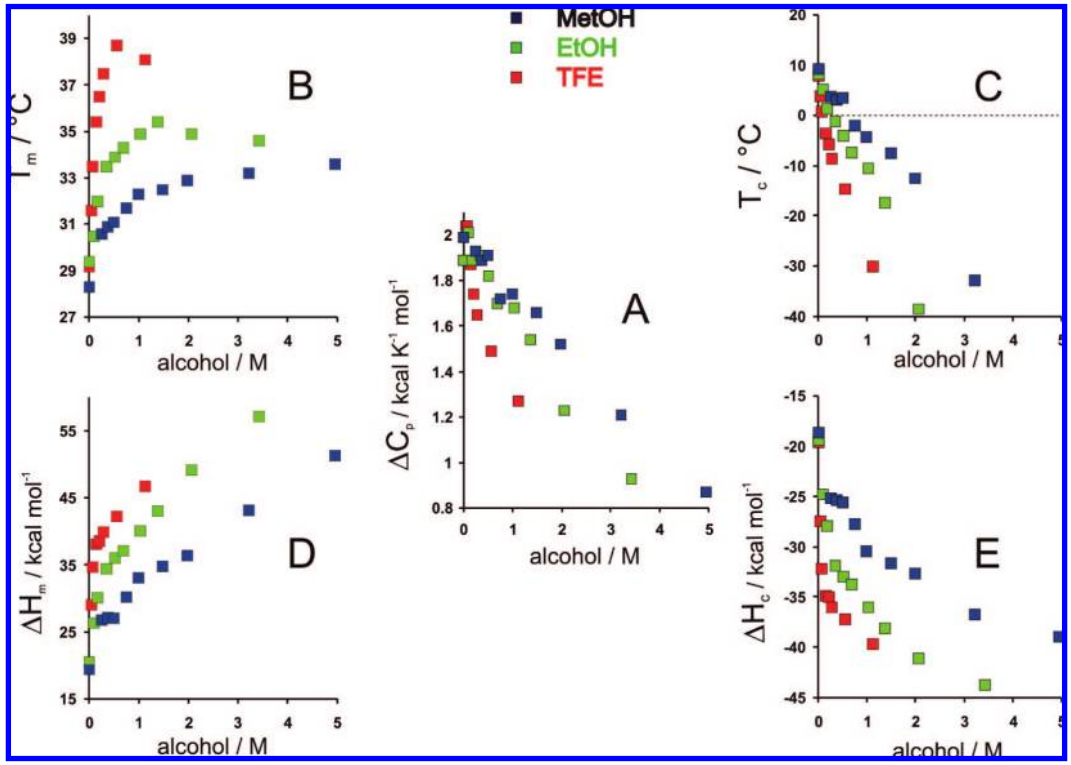

Figure 4. Change of thermodynamic parameters of Yfh1 for the high temperature transition as a function of alcohol concentration in the three water−alcohol mixtures: (A) the difference in heat content between folded and denaturated states (ΔC_p) plotted as a function of alcohol concentration; (B) plot of T_m, the high temperature unfolding midpoint, as a function of alcohol concentration; (C) plot of T_c, the low temperature unfolding midpoint, as a function of alcohol concentration; (D) plot of the difference in enthalpy between folded and denaturated states (ΔH), measured at T_m, as a function of alcohol concentration; (E) plot of the difference in enthalpy between folded and denaturated states (ΔH), measured at T_c, as a function of alcohol concentration.

The complex influence on the stability of Yfh1 is best illustrated using the stability curves introduced by Becktel and Schellman.[19] These authors showed that the difference in free energy between the denatured state and the native state of a protein (ΔG) varies with temperature in a way described by a modified Gibbs−Helmholtz equation:

$$\Delta G = \Delta H_m\left[1 - \frac{T}{T_m}\right] + \Delta C_p\left\{(T - T_m) - T\ln\left[\frac{T}{T_m}\right]\right\} \quad (1)$$

Stability curves can be calculated from this equation using the parameters of Table 1. Figure 5A shows these curves for increasing concentrations of ethanol. Figure 5B shows stability curves at similar concentrations (ca. 0.5 M) of the three alcohols. In all cases addition of alcohols makes the curves wider, raises the maxima, and shifts them to lower temperatures.

The increase of ΔH_m at low alcohol concentrations hints at a direct solvation of the folded conformation by alcohol molecules, and this view is supported by NMR data. We have previously observed that peaks arising from residues buried in the hydrophobic core show that alcohols increase the population of the folded form without any perturbation of the architecture of the fold, as shown by the lack of any chemical shift perturbation (see Figure 2).

However, it is possible to observe chemical shift perturbations of most resonances corresponding to exposed residues that, albeit consistent with the retaining of the fold architecture, hint at changes in solvation. Figure 6A shows the comparison of partial HSQC spectra of Yfh1 in TRIS and in a 2.5 M MetOH HEPES solution, restricted to a small low field region hosting four

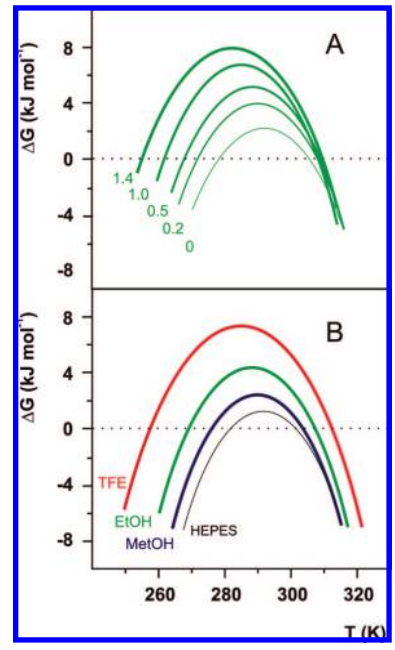

Figure 5. Variation of the stability curve of Yfh1 as a function of small alcohol concentrations: (A) Calculated stability curves after addition of increasing concentrations of ethanol, from 0 to 1.4 M; (B) calculated stability curves at similar concentrations of different alcohols. The black curve (thin inner line) corresponds to the Yfh1 solution in 20 mM HEPES at pH 7.0; the blue curve corresponds to 0.49 M MetOH concentration; the green curve corresponds to 0.51 M EtOH concentration; the red curve corresponds to 0.56 M TFE concentration.

interesting leucine resonances. As an example of the difference between isolated residues and residues belonging to exposed

(19) Becktel, W. J.; Schellman, J. A. *Biopolymers* **1987**, *26*, 1859–1877.

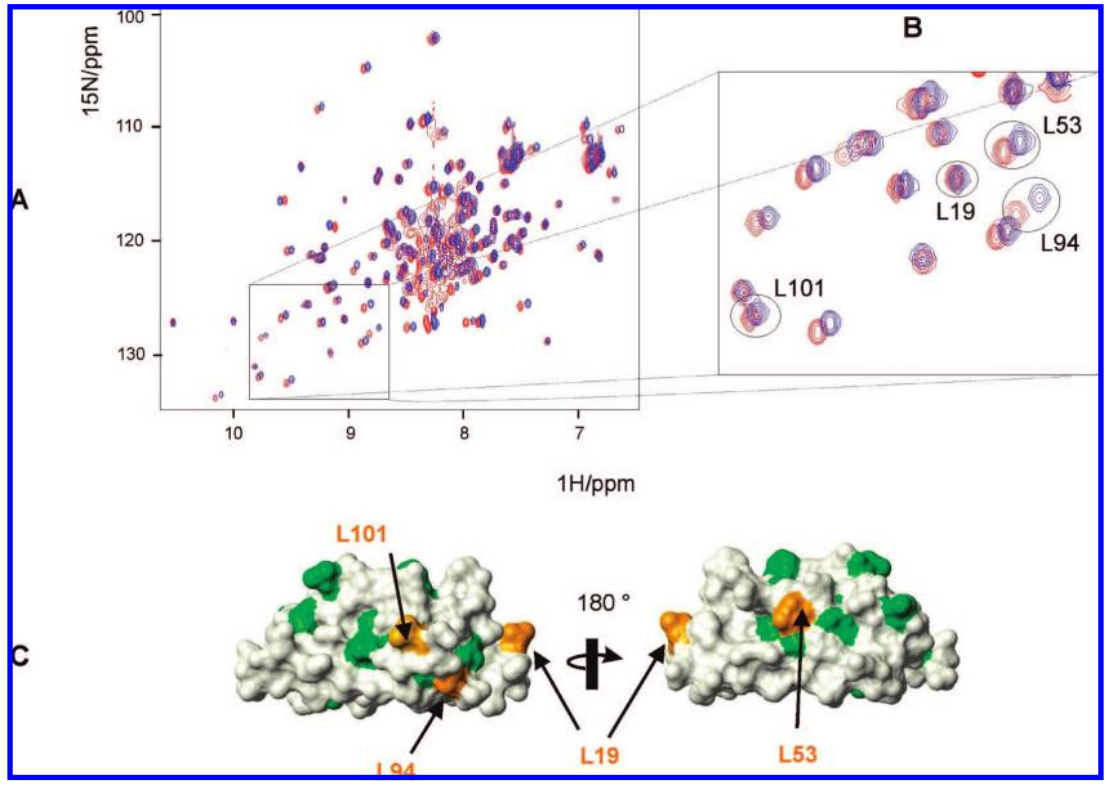

Figure 6. Preferential solvation of Yfh1. (A) Comparison of HSQC $^{1}H-^{15}N$ correlation spectra of Yfh1 in 20 mM HEPES at pH 7 and at 25 °C (red) and in the same HEPES solution containing MetOH (2.5 M, blue peaks). (B) Expanded region of spectrum A showing the shift of four leucines residues. Resonances of L19, L53, L94, and L101 (discussed in the text) are circled. (C) Two views of the contact surface of Yfh1 highlighting hydrophobic residues as green atoms. L19, L53, L94, and L101 are colored orange. The figure was generated by MOLMOL.[20]

hydrophobic patches, we focused on leucines 19, 53, 94, and 101 (circled in Figure 6B). The resonances of L19 and L101 do not shift appreciably (L19) or less (L101) than those of L53 and L94. As shown by Figure 6C, L19 and L101 are exposed to solvent but, at the same time, are completely isolated from other hydrophobic residues whereas L53 and L94 are at the center of hydrophobic patches. These NMR observations are consistent with current hypotheses regarding TFE action on protein structure that at low concentrations TFE interacts with hydrophobic groups on the surface of proteins.[21]

Discussion

In the present investigation, we have studied the effect of alcohols on protein stability monitoring both high and low temperature transitions of the model protein Yfh1 over a wide range of alcohol concentrations. The unique possibility, offered by Yfh1, to study both high and low temperature unfolding in an environment devoid of cosolvents (other than alcohols) has allowed us to shed light on the influence of alcohols on protein stability and, at the same time, to validate the current understanding of the mechanism of cold denaturation.

Alcohols have been reported to be potent inducers of secondary structure both in small peptides (e.g., see ref 22 and refs therein) and in proteins.[23–28] Generally, they are known to favor helical conformations but it has also been shown[28] that they can induce a natively α-helical protein to adopt non-native β-structures. Alcohols are also described as protein denaturants owing to their known ability to disrupt protein tertiary structure. Both disruption of tertiary structures and promotion of secondary conformations in proteins occur at relatively high concentrations, for example, greater than 15% (v/v) for TFE[23] (corresponding to ca. 2 M TFE) and even greater (30−40% (v:v)) for ordinary aliphatic alcohols[29–31] (corresponding to ca. 7−10 M MetOH). Owing to the paucity of studies, it remains unclear whether alcohols also affect the tertiary structure of proteins at low concentrations. The first explicit hint at a possible *stabilizing* effect of alcohols at low concentration can be found in a paper by Brandts and Hunt[32] in which it was shown that alcohols have different effects on the stability of ribonuclease, depending on *both* concentration and temperature: while above room temperature the main effect of ethanol is invariably to decrease the

(20) Koradi, R.; Billeter, M.; Wüthrich, K. *J. Mol. Graph.* **1996**, *14*, 51–55.
(21) Roccatano, D.; Colombo, G.; Fioroni, M.; Mark, A. E. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 12179–12184.
(22) Tancredi, T.; Carra′, G.; Guerrini, R.; Arduini, M.; Calo′, G.; Regoli, D.; Salvadori, S.; Temussi, P. A. *Chem.—Eur. J.* **2005**, *11*, 2061–2070.
(23) Buck, M.; Radford, S. E.; Dobson, C. M. *Biochemistry* **1993**, *32*, 669–678.
(24) Buck, M.; Schwalbe, H.; Dobson, C. M. *Biochemistry* **1995**, *34*, 13219–13232.
(25) Buck, M. *Q. Rev. Biophys.* **1998**, *31*, 297–355.
(26) Bhattacharjya, S.; Balaram, P. *Proteins* **1997**, *29*, 492–507.
(27) Babu, K. R.; Douglas, D. J. *Biochemistry* **2000**, *39*, 14702–14710.
(28) Perham, M.; Liao, J.; Wittung-Stafshede, P. *Biochemistry* **2006**, *45*, 7740–7749.
(29) Hermans, J. *J. Am. Chem. Soc.* **1966**, *88*, 2418–2422.
(30) von Hippel, P. H.; Schleich, T. In *Structure and Stability of Biological Macromolecules*; Timasheff, S., Fasman, G., Eds.; Dekker: New York, 1969; Vol. 2, p417.
(31) Parodi, R. M.; Bianchi, E.; Ciferri, A. *J. Biol. Chem.* **1973**, *248*, 4047–4051.
(32) Brandts, J. F.; Hunt, L. *J. Am. Chem. Soc.* **1967**, *89*, 4826–4838.

free energy of denaturation, moderate amounts of alcohol at 10 °C stabilize the native fold.[32,33] Only very recently a thorough study of the effect of TFE on lysozyme structure and function[34] has suggested that TFE interacts and stabilizes the protein at low TFE concentrations, whereas this effect is reversed at concentrations of TFE above 10% (2.6 M) with denaturation of the protein.

Most other studies at low alcohol concentrations however, seem to be at variance with our conclusions. Two of these studies bear specific relevance to our work. Velicelebi and Sturtevant[7] and Fu and Freire[35] showed that addition of small amounts of alcohols to lysozyme and cytochrome c respectively, induces a decrease of T_m. However, both studies report that the decrease of T_m with increasing alcohol concentration is paralleled by an increase of ΔH_m and by a decrease of ΔC_p, two observations consistent with increased stability (vide infra). We suggest a way to rationalize and generalize these observations into a unifying model on the basis of simple thermodynamics considerations. In particular, it is possible to show that the moderate decreases of T_m reported in other studies[7,35] are not inconsistent with a large increase of the temperature range of protein stability. The increment of the area of stability for Yfh1 in the presence of alcohols, as shown by the curves of Figure 5, stems mainly from the increase of ΔH_m and the concomitant decrease of ΔC_p (Table 1). Both effects should lead to higher values of T_m and *symmetrically* lower values of T_c. In fact, a decrease of the value of ΔC_p leads to a broadened stability curve, because the curvature of the stability curve is given[19] by $\partial^2 \Delta G/\partial T^2 = -\Delta C_p/T$. However, an increase of the ratio $\Delta H_m/\Delta C_p$ leads to a more or less pronounced shift of the stability curve toward low temperatures, depending on the actual relative changes of ΔH_m and ΔC_p.

It is easy to show that:

$$T_S = T_m \exp\left[-\frac{\Delta S_m}{\Delta C_p}\right] = T_m \exp\left[-\frac{\Delta H_m}{T_m \Delta C_p}\right] \qquad (2)$$

If a decrease of ΔC_p is accompanied by a concomitant increase of ΔH_m, as observed with the addition of small amounts of alcohols (see Table 1 and refs 7, 35), the ratio $\Delta H_m/\Delta C_p$ will increase, thus causing T_S to decrease, and the whole stability curve to shift toward lower temperatures.

As a consequence of the increase of the ratio $\Delta H_m/\Delta C_p$ we may have a partial compensation of the increase of T_m, particularly for methanol (see the methanol curve of Figure 5B) and an enhancement of the decrease of T_c. The real nature of the effect is shown by the large decrease of T_c. As mentioned above, the great advantage offered by Yfh1 resides in the possibility to observe directly a large part of the cold denaturation curve. With a slightly different combination of values for ΔH_m and ΔC_p, the shift of the stability curve toward lower temperatures can be more pronounced, leading to a moderate *decrease* of T_m as observed by Velicelebi and Sturtevant[7] for lysozyme and by Fu and Freire[35] for cytochrome c. Thus, it is possible to have a concomitant widening of the temperature range of protein stability *and* a decrease of T_m. A graphic illustration of these possibilities, based on the scheme proposed by Nojima et al.[36] to rationalize the stability of proteins from thermophilic organisms is reported in the Supporting Information.

Can we extend our understanding from the thermodynamic interpretation to a description at the molecular level? It is difficult to explain the asymmetric effect on T_m and T_c by a direct solvation of the folded structure common to both transitions. By the same token, to explain the increase of T_m, it is not possible to invoke exclusion of the cosolvent from the native conformation as proposed by Gekko and Timasheff (1981) for glycerol and other polyhydric compounds,[37] because also in this case we should observe symmetric effects on cold and heat denaturations. Besides, our simple monohydric alcohols cannot be compared to polyhydric alcohols. It has been proposed that the low-temperature unfolded state is, in general, intrinsically different from the corresponding state at high temperature.[38] The HSQC spectrum of the low temperature unfolded Yfh1 looks different from the corresponding high temperature one.[10] It is impossible to attribute these differences to specific structural features without a full resonance assignment. We can, however, try to explore the possibility that alcohols can also interfere with solvation of the unfolded structure and that the observed asymmetric effects can be explained by the mechanisms of denaturation. Any direct interaction of the unfolded states with alcohols should lead to increased stabilization of these states and, as a consequence, to decreased protein stability, as observed for all typical denaturants.[39] We suggest that the much larger effect of alcohols on T_c than on T_m is due not only to the solvation of the folded conformation by the alcohol molecules but also, in part, to an indirect effect on the unfolded state, possibly mediated by water.

According to the currently accepted interpretation,[1] the phenomenon of cold denaturation is caused by the very specific and strongly temperature-dependent hydration of buried nonpolar groups of proteins. Hydration of these groups, in contrast to expectations, is characterized by a negative free energy of hydration and increases in magnitude as the temperature is decreased. Therefore, at a sufficiently low temperature, the tightly folded native structure unfolds and exposes otherwise buried nonpolar groups to water. In contrast, the high temperature transition is not critically dependent on hydration of apolar protein side chains.[1] Accordingly, a simple phenomenological interpretation of the effects described in this paper is that alcohols may compete with apolar groups of the protein for water solvation, effectively making the hydration process of the apolar side chains more difficult. Since the larger heat capacity of the denatured form with respect to the folded form of a protein is caused by different hydration of the folded and unfolded forms and, particularly, by different hydration of apolar groups, a competition by alcohols for the hydration of these groups, hindering exposure of buried apolar groups, will thus shift the low temperature transition toward lower temperatures.

The competition of alcohols with the apolar groups of the protein core for hydration is in good agreement with the prevailing view of water−alcohol interactions,[40] intertwined with the introduction of the hydrophobic force as one of the

(33) Brandts, J. F. In *Structure and Stability of Biological Macromolecules*; Timasheff, S. N., Fasman, G. D., Eds.;Dekker: New York, 1969; Vol. 2, p 213.
(34) Povey, J. P.; Smales, C. M.; Hassard, S. J.; Howard, M. J. *J. Struct. Biol.* **2007**, *157*, 329–338.
(35) Fu, L.; Freire, E. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 9335–9338.
(36) Nojima, H.; Ikai, A.; Oshima, T.; Noda, H. *J. Mol. Biol.* **1977**, *116*, 429–442.
(37) Gekko, K.; Timasheff, S. N. *Biochemistry* **1981**, *20*, 4677–4686.
(38) Whitten, S. T.; Kurtz, A. J.; Pometun, M. S.; Wand, A. J.; Hilser, V. J. *Biochemistry* **2006**, *45*, 10163–10174.
(39) Pfeil, W.; Privalov, P. L. *Biophys. Chem.* **1976**, *4*, 33–40.
(40) Frank, H. S.; Evans, M. W. *J. Chem. Phys.* **1945**, *13*, 507–532.

main forces contributing to protein stability.[41] Several studies on the dissolution of small nonpolar molecules in water had shown convincingly that the hydration of these molecules is associated with a significant entropy decrease of the system, which was interpreted as an increase of order in water.[40,42] In turn, at the molecular level, the entropy decrease was attributed to formation of iceberg-like or clathrate-like structures in the water next to the nonpolar molecule.[40] By similar reasoning, the well-known observation that the entropy of the system increases much less than expected for an ideal solution of randomly mixed molecules when an alcohol is mixed with water, was attributed by Frank and Evans[40] to the formation of iceberg-like features in the water surrounding the hydrophobic moieties of the alcohols. The very details of the interaction between water and alcohols have been recently questioned,[43–45] in favor of clustering[44] or of incomplete mixing at the molecular level.[43] Such models would nonetheless be consistent with our observations, because segregation of water and alcohols in different clusters would effectively hinder hydration of protein side chains by decreasing the activity of water.

We can conclude that alcohols, at low concentration and physiological pH, *stabilize* proteins by greatly widening the range of temperatures over which the protein is stable. A direct experimental observation of this stabilization requires an accurate determination of the whole stability curve. This was actually possible with our model system based on Yfh1, but it may be rather difficult in a general case, owing to the inaccessibility of cold denaturation temperatures. On a molecular level, our experimental findings may be interpreted as a direct interaction of the folded conformation with alcohols and as an indirect interference of alcohols with the hydration of the unfolded conformation at low temperature.

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Supporting Information Available: Graphic illustration of the scheme proposed by Nojima et al.; HSQC spectra.

This material is available free of charge via the Internet at http://pubs.acs.org.

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(41) Kauzmann, W. *Adv. Protein Chem.* **1959**, *14*, 1–63.
(42) Frank, H. S.; Wen, W.-Y. *Disc. Faraday Soc.* **1957**, *24*, 133–140.
(43) Soper, A. K.; Dougan, L.; Crain, J.; Finney, J. L. *J. Phys. Chem. B* **2006**, *110*, 3472–3476.
(44) Dixit, S.; Crain, J.; Poon, W. C.; Finney, J. L.; Soper, A. K. *Nature* **2002**, *416*, 829–832.
(45) Guo, J. H.; Luo, Y.; Augustsson, A.; Kashtanov, S.; Rubensson, J. E.; Shuh, D. K.; Agren, H.; Nordgren, J. *Phys. Rev. Lett.* **2003**, *91*, 157401–157404.