J. Mol. Biol. (1998) **279**, 49–57

JMB

Trifluoroethanol Stabilizes the pH 4 Folding Intermediate of Sperm Whale Apomyoglobin

Yongzhang Luo and Robert L. Baldwin*

Department of Biochemistry Stanford University School of Medicine, Stanford CA 94305-5307, USA

*Corresponding author

2,2,2-Trifluoroethanol (TFE) is known to stabilize peptide helices by strengthening hydrogen bonds. On the other hand, TFE destabilizes native proteins, as we confirm here, presumably by weakening the hydrophobic interaction. The stability of the pH 4 folding intermediate of apomyoglobin is known to depend both on the strength of the individual A, G, and H helices and on hydrophobic interactions between helices. We ask which effect of TFE dominates in this case: strengthening helices or weakening hydrophobic interactions between helices? Protein stability is measured by denaturant-induced unfolding curves, and two-state unfolding is tested by monitoring both far-UV CD and tryptophan fluorescence emission. Low concentrations of TFE strongly stabilize the pH 4 folding intermediate. Moreover, low concentrations of TFE compensate for helix-destabilizing mutations in the A and G helices. Consequently, enhancing helix propensity, rather than weakening the hydrophobic interaction, is the dominant effect of TFE on the folding intermediate. This result agrees with earlier mutational evidence that helix propensities are very important in determining the stability of the pH 4 intermediate. Although TFE destabilizes native holomyoglobin, as well as native lysozyme and ribonuclease A, nevertheless, TFE stabilizes native apomyoglobin.

© 1998 Academic Press Limited

Keywords: trifluoroethanol; folding intermediate; apomyoglobin; stability; helix propensity

Introduction

2,2,2-Trifluoroethanol (TFE), an organic solvent, is known to stabilize α-helical structure in peptides and unfolded proteins, in regions that have an intrinsic propensity to form the α-helix in aqueous solution (Dyson *et al.*, 1988; Nelson & Kallenbach, 1986; 1989; Segawa *et al.*, 1991; Barrow *et al.*, 1992; Buck *et al.*, 1995; Shiraki *et al.*, 1995). The average helix propensity of individual residues has been measured in repeating-sequence model peptides at various TFE concentrations. It increases regularly from 0 to 25% (v/v) TFE but levels off at higher TFE concentrations (Luo & Baldwin, 1997). Moreover, the strength of the hydrogen bond in a model compound, salicylic acid, increases in the same way with TFE concentration and also levels off above 25% TFE. These observations confirm earlier suggestions (Cammers-Goodwin *et al.*, 1996, and references therein) that TFE stabilizes peptide helices by strengthening peptide hydrogen bonds.

On the other hand, alcohols such as ethanol denature native proteins primarily by weakening the hydrophobic interaction (Brandts & Hunt, 1967). TFE, as an alcohol, can also denature proteins (Buck *et al.*, 1993; Alexandrescu *et al.*, 1994). For example, hen egg-white lysozyme in 50% TFE is denatured as indicated by reduced near-UV circular dichroism (CD) intensity and averaging of side-chain chemical shifts (Buck *et al.*, 1993). Similar observations were reported for α-lactalbumin (Alexandrescu *et al.*, 1994). Thus the dominant effect of TFE on native proteins is denaturation. The evidence that alcohols denature proteins by affecting the hydrophobic interaction (Brandts & Hunt, 1967) is based on similarities between the patterns of protein denaturation and the hydrophobic interaction studies with a simple model compound (argon).

E-mail: yluo@cmgm.stanford.edu

Abbreviations used: TFE, 2,2,2-trifluoroethanol; apoMb, apomyoglobin; Mb, myoglobin; holoMb, holomyoglobin; cyanoMb, cyanomyoglobin; I, apoMb pH 4 folding intermediate; CD, circular dichroism; FL, fluorescence; GdmCl, guanidine hydrochloride; WT, wild-type; C_m, the denaturant molarity at the midpoint of the unfolding transition.

0022–2836/98/210049–09 $25.00/0/mb981774

© 1998 Academic Press Limited

The pH 4 folding intermediate (I) of apomyoglobin (apoMb, myoglobin without heme) contains the A, G, and H helices of Mb (Hughson *et al.*, 1990) and it is an early transient intermediate in the folding kinetics of native apoMb at pH 6 (Jennings & Wright, 1993). I is known to be stabilized by non-polar side-chains that are buried in the AGH interface of holoMb (Kay & Baldwin, 1996) as well as by helix-stabilizing mutations (Kiefhaber & Baldwin, 1995; Luo *et al.*, 1997). The effects of mutations and other factors that affect the stability of I can be measured quantitatively because the urea-induced unfolding of I is a two-state reaction at pH 4.2, 4°C, 4 mM citrate (Kay & Baldwin, 1996). Note that 4 mM citrate provides a stabilizing anion, whose effects are comparable to those of 30 mM NaCl (see Luo *et al.*, 1997). Evidence for two-state unfolding is based on superposition of the unfolding curves measured by two very different probes (far-UV CD and fluorescence of partly buried tryptophan residues). Further evidence is provided by two-state kinetics of I (Jamin & Baldwin, 1996) and by single helix-destabilizing mutations, Gly or Pro, that affect the stability of the entire intermediate (Luo *et al.*, 1997). The cooperativity of the unfolding transition is affected by changes in the stability of I, caused either by stabilizing anions or by helix propensity mutations (Luo *et al.*, 1997).

There are two forms of I, I_a and I_b, in equilibrium at pH 4.2 (Jamin & Baldwin, 1998). I_b is converted to I_a at low urea concentrations, between 0 and 1 M, and the urea unfolding transition monitors the unfolding of I_a.

Low concentrations of TFE are used here with the aim of changing as little as possible the helical structure of the pH 4 intermediate. Unfolding curves are measured both by far-UV CD and by tryptophan fluorescence to determine if unfolding is close to a two-state reaction. The interpretation of these experiments becomes difficult when unfolding is not close to two-state. In order to study the effects of TFE in isolation, the only anion present is the weakly stabilizing anion 2 mM acetate. We also study whether the stabilizing effects of 5% TFE and a strongly stabilizing anion, 20 mM Na_2SO_4, are additive.

Results

TFE destabilizes native holoMb, lysozyme and RNase A

The unfolding of holoMb in the presence of TFE was tested to determine whether TFE destabilizes Mb, as it does other native proteins (Buck *et al.*, 1993; Alexandrescu *et al.*, 1994; Shiraki *et al.*, 1995). We are able to obtain good reversibility (see below) but, as reported also by Hargrove & Olson (1996), long times are needed to reach equilibrium (see Materials and Methods). The GdmCl-induced unfolding of cyanoMb, monitored by far UV-CD at 222 nm (Figure 1(a)), confirms that native cyanoMb

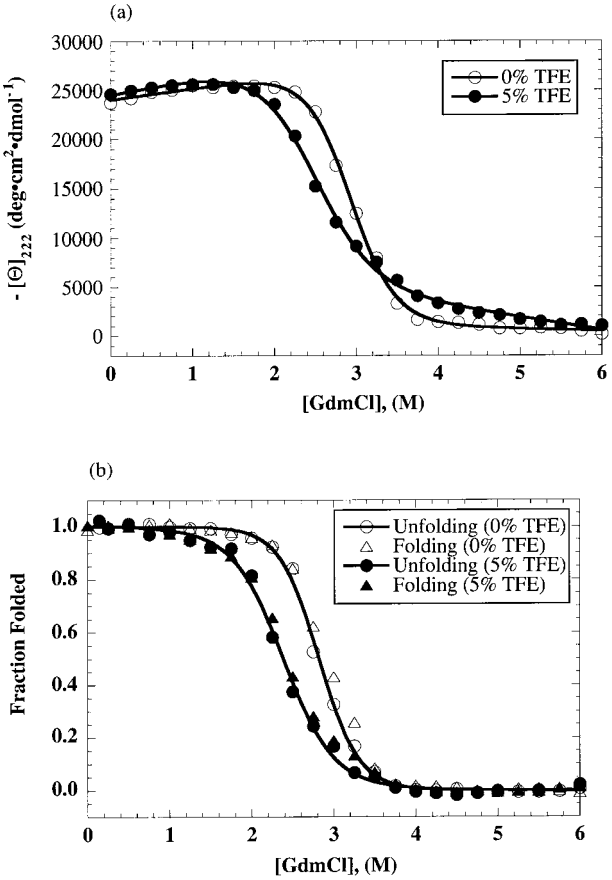

Figure 1. CyanoMb unfolding by GdmCl in the presence of TFE in 5 mM Tris-HCl (pH 7.8), 30°C, containing 2 μM hemin and 80 μM KCN. (a) Raw data; (b) normalized data showing both unfolding and refolding curves, with or without 5% TFE present.

is destabilized by low concentrations of TFE. The unfolding curves measured in the presence of either 0 or 5% TFE can be fitted by the two-state model (Figure 1(b)), which uses data inside as well as outside the transition region to fix the baselines (Santoro & Bolen, 1988). The unfolding of cyanoMb is reversible in these conditions, as tested by the superposition of the refolding curves (using predenatured cyanoMb in 6 M GdmCl) and the unfolding curves (using native cyanoMb) (Figure 1(b)). That TFE destabilizes other native proteins is confirmed by measuring the reversible denaturant-induced unfolding curves of hen egg-white lysozyme (Figure 2(a) and Table 1) and ribonuclease A (Figure 2(b) and Table 1). The fact that TFE destabilizes native holoMb, lysozyme, and RNase A is not surprising (Buck *et al.*, 1993; Alexandrescu *et al.*, 1994).

TFE stabilizes the pH 4 folding intermediate of apoMb

It is known that TFE stabilizes peptide helical structures by strengthening their H-bonds and by

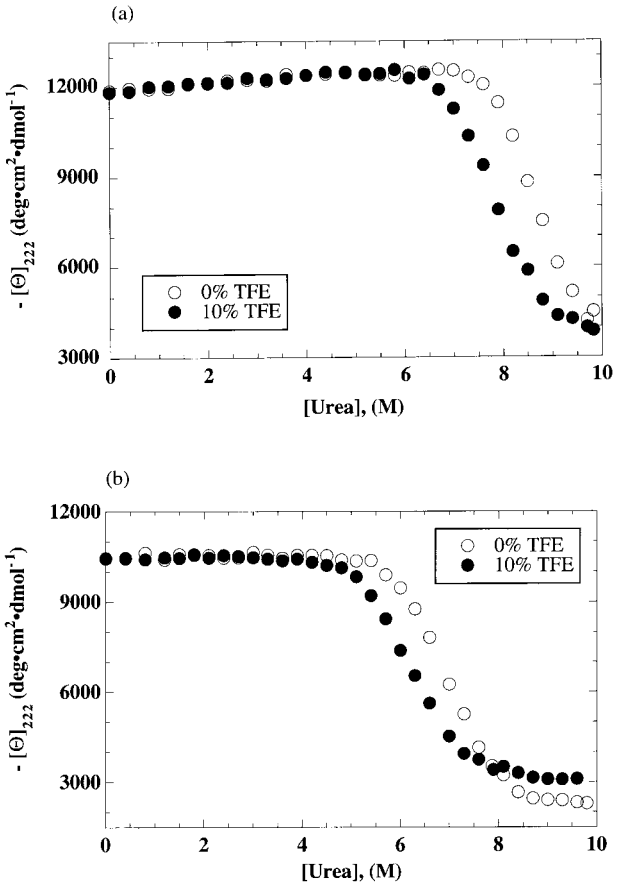

Figure 2. Urea-induced unfolding of native lysozyme and RNase A in the presence or absence of TFE monitored by CD at 222 nm. (a) Lysozyme in 5 mM NaAc, pH 4.0, 30°C; (b) RNase A in 5 mM Tris-HCl (pH 8.0), 30°C.

enhancing the helical propensities of the individual amino acids (Rohl *et al.*, 1996; Luo & Baldwin, 1997). We wanted to test whether low concentrations of TFE stabilize I. Low TFE concentrations are used with the aim of minimizing changes in the structure of I such as stabilizing other helices in I besides A, G, and H.

The urea-induced unfolding transition of I, monitored by both far-UV CD (Figure 3(a)) and FL (Figure 3(b)), at pH 4.2 is very broad in the pre-

Table 1. The stability of apomyoglobin, cyanomyoglobin, lysozyme and ribonuclease A at different TFE concentrations, measured by the C_m of denaturant unfolding

Native proteins	0 % TFE	5% TFE	10% TFE
apoMb-FL[a]	3.79	4.88	NM
apoMb-CD[a]	3.49	4.62	NM
cyanoMb-CD[b]	2.92	2.45	NM
Lysozyme-CD[a]	8.54	ND	7.76
RNase A-CD[a]	6.98	ND	6.14

C_m is the denaturant molarity at the midpoint of the unfolding transition. The conditions are: for apoMb, 5 mM Tris-HCl (pH 7.8), 4°C; for cyanoMb, the same buffer, 30°C; RNase A, 5 mM Tris-HCl (pH 8.0), 30°C; and for hen egg-white lysozyme, 5 mM NaAc, pH 4.0, 30°C. FL, tryptophan fluorescence emission at 320 mm (with excitation at 288 nm). CD, circular dichroism at 222 nm. NM, not measurable. ND, not determined.

[a] Urea-induced unfolding.

[b] GdmCl-induced unfolding.

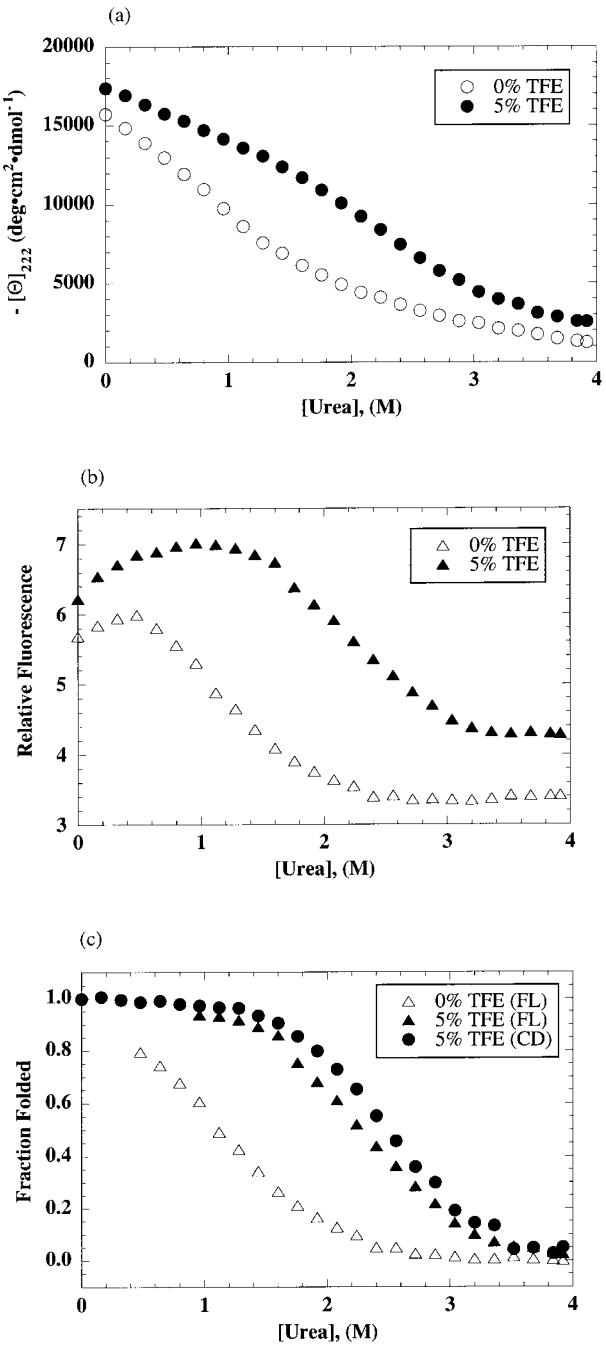

Figure 3. Urea-induced unfolding of the apoMb folding intermediate (WT) in the presence of TFE (0 and 5% TFE, v/v) in 2 mM NaAc, pH 4.2, 4°C: (a) raw data, CD-monitored; (b) raw data, FL-monitored; (c) normalized data for both CD and FL-monitored unfolding at 5% TFE. The data points below the highest fluorescence value at the beginning of unfolding are omitted in the two-state fittings. The normalized CD unfolding curve at 0% TFE is not shown in (c) because a reliable native baseline is not available (see (a)).

Table 2. The stability of apomyoglobin mutants at different TFE concentrations

apoMb	0 % TFE	C_m[a] 5% TFE	10% TFE
WT-FL	1.14	2.30	3.90
WT-CD	NM	2.53	4.24
Q8G-FL	NM	1.74	3.08
Q8G-CD	NM	2.06	3.76
E109G-FL	NM	1.65	3.09
E109G-CD	NM	1.84	3.33
E109P-FL	NM	1.44	NM
E109P-CD	NM	NM	NM

[a] C_m, denaturant molarity at the midpoint of the urea-unfolding transition. The conditions are 2 mM NaAc, pH 4.2, 4°C, with different TFE concentrations as indicated. FL, tryptophan fluorescence emission at 320 mm (with excitation at 288 nm). CD, circular dichroism at 222 nm. NM, not measurable.

sence only of a weak anion stabilizer such as acetate and the transition is not a two-state reaction in these conditions. Addition of TFE not only enhances the helical content of I but also dramatically stabilizes it, and unfolding monitored by CD at 222 nm is well resolved and can be fitted with the two-state equation (Table 2). In the case of FL-monitored unfolding, however, a deviation from two-state unfolding is observed at low urea concentrations from the rise in FL that occurs before urea-induced unfolding. This behavior is probably caused by the conversion of I_b to I_a at low urea concentrations, because this is known to be the explanation for a similar effect in other solvent conditions at pH 4.2, 4°C (Jamin & Baldwin, 1998). To separate the $I_b \rightleftharpoons I_a$ reaction from the urea-induced $I_a \rightleftharpoons U$ reaction, the FL data points below the highest value, near the beginning of urea-induced unfolding, are omitted in fixing the baselines. Examples of normalized unfolding curves, measured by both FL and CD, are given in Figure 3(c) (WT). The two unfolding curves are almost superimposable, showing that unfolding in 5% TFE is not far from being a two-state reaction (see Luo *et al.*, 1997). We use C_m values to measure relative stability here, because it is not possible to measure values of ΔG (unfolding) unless the reaction accurately follows two-state unfolding behavior. ΔG is proportional to C_m when unfolding does show two-state behavior and, provided the *m*-value remains constant in a series of mutants, the C_m values of the mutants provide the most precise estimation of their relative stabilities (Serrano *et al.*, 1992).

TFE can rescue the effects of helix-destabilizing mutations in I

Because I becomes much less stable when single glycine or proline residues are introduced into either the A or G helices (Luo *et al.*, 1997) and, because TFE probably affects the stability of I by increasing helical propensity, it follows that TFE should restore the stability of mutants such as Q8G, E109G, and E109P, which contain helix-destabilizing mutations. To test this proposal, urea-induced unfolding was measured with these three mutants at different TFE concentrations. In the absence of TFE, at pH 4.2, 4°C, 2 mM NaAc, the apoMb intermediate is not very stable, only part of the CD unfolding curve can be observed, and the CD-monitored and FL-monitored unfolding curves are not superimposable, so that the C_m value is not measurable. In the presence of 5% TFE the unfolding reaction is nearly two-state, so that it is possible to measure C_m values of the mutants with TFE present (Table 2): see Figure 4(a), which shows results for Q8G. Similar results are obtained with E109G and E109P (Table 2). These results confirm that helical propensity contributes substantially to the stabilization of I. Figure 4(b) compares the helical contents of WT and the three mutants in the presence and absence of TFE.

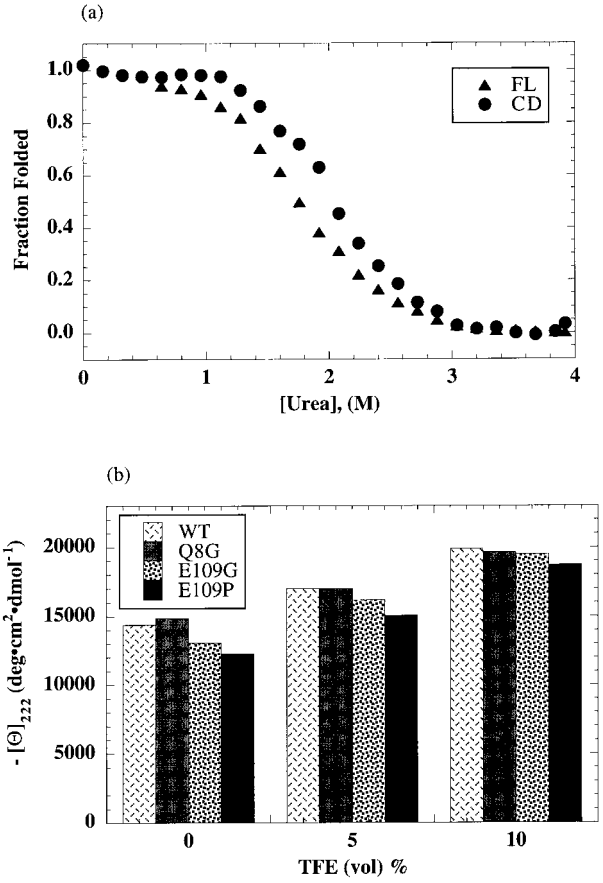

Figure 4. (a) Normalized CD and FL-monitored unfolding curves of Q8G in the presence of 5% TFE at pH 4.2. The normalized CD and FL-unfolding curves at 0% TFE are not shown because reliable native baselines are not available. See legend to Figure 3 and Materials and Methods for fitting the baselines of the FL-monitored unfolding curves. (b) Helical contents of WT and the three mutants in the presence or absence of TFE in 2 mM NaAc, pH 4.2, 4°C.

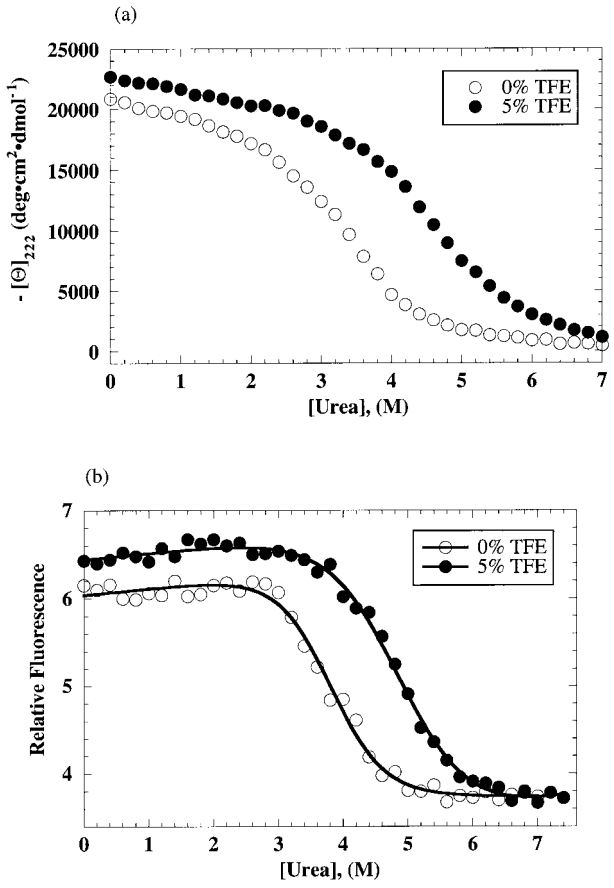

Figure 5. Urea-induced unfolding of native apoMb (WT) in 5 mM Tris-HCl (pH 7.8), 4°C in the presence or absence of 5% TFE. (a) CD-monitored; (b) FL-monitored.

TFE also stabilizes native apomyoglobin

Multi-dimensional heteronuclear NMR spectra of native apoMb show well-resolved lines in suitable conditions and the spectra closely resemble those of holoMb (Eliezer & Wright, 1996). Nevertheless, the F helix and part of the G helix are missing in NMR spectra of native apoMb, and we tested whether TFE stabilizes or destabilizes native apoMb. Surprisingly, the results show that 5% TFE stabilizes native apoMb at pH 7.8, as shown by urea-induced unfolding monitored by both far-UV CD (Figure 5(a) and Table 1) and tryptophan fluorescence emission (Figure 5(b) and Table 1). Similar results are also obtained when GdmCl is used to unfold apoMb (data not shown).

Discussion

Role of the hydrophobic interaction in the alcohol denaturation of proteins

The proposal that alcohols denature proteins probably by weakening the hydrophobic interaction was made by Hermans (1966), who showed that ethanol stabilizes peptide helices even though ethanol denatures proteins (see especially later work by Herskovits *et al.*, 1970). We show here that TFE behaves as a typical alcohol in denaturing RNase A, hen egg-white lysozyme and holoMb. Hermans suggested that the mechanism by which ethanol stabilizes helices is by strengthening peptide H-bonds: this suggestion has been supported by later work (see Cammers-Goodwin *et al.*, 1996, and references therein) and it was confirmed recently for TFE by Luo & Baldwin (1997), who compared the changes in helix propensity and H-bond strength at various TFE concentrations. Hermans (1966) pointed out that stronger peptide H-bonds should help to stabilize proteins and therefore that the denaturing action of alcohol must be attributed to weakening of the hydrophobic interaction, which must then be a major factor stabilizing proteins, as proposed by Kauzmann (1959) and Tanford (1962).

Brandts & Hunt (1967) provided direct evidence that alcohols affect protein stability by changing the strength of the hydrophobic interaction. They compared the effects of ethanol–water mixtures on the stability of RNase A at a series of ethanol concentrations and temperatures (10°C, 30°C, and 50°C) with the corresponding effects on the solubility of argon, whose aqueous solutions resemble those of saturated hydrocarbons, both substances being relatively inert (see Gill *et al.*, 1985). Brandts & Hunt (1967) found that protein stability and argon solubility change with temperature and ethanol concentration in a strikingly similar manner. In most conditions ethanol lowers the stability of RNase A, but this behavior is reversed at low temperatures (5 to 10°C) and at ethanol concentrations around 10% (w/w); likewise ethanol increases the solubility of argon in most conditions, but this behavior is also reversed around 5°C at low ethanol concentrations. Dioxane–water mixtures affect the solubility of argon in the same manner as ethanol–water mixtures, which argues for the generality of the effect.

Stabilization of the apomyoglobin folding intermediate by TFE

The pH 4 folding intermediate (I) of apoMb is considered to be a typical molten globule intermediate. Its side-chains are mobile as evidenced by the lack of chemical shift dispersion in 1D ^{1}H-NMR spectra (see, for example, Loh *et al.*, 1995; also Eliezer & Wright, personal communication), while NMR H-exchange experiments show that the A, G, and H helices of holoMb are formed in I (Hughson *et al.*, 1990). An important property of I is that its unfolding is reversible and independent of protein concentration below 10 μM, so that its thermodynamic stability can be measured unambiguously. The fact that the A, G, and H helices are stable in I but not in isolated peptides with sequences corresponding to the individual A, G, and H helices is usually attributed to hydrophobic

interactions between helices (Barrick & Baldwin, 1993; Kay & Baldwin, 1996).

The contributions of some non-polar side-chains to the stability of I could be measured by mutagenesis after it was found that I shows two-state urea-induced unfolding at pH 4.2, 4 °C, 4 mM citrate (Kay & Baldwin, 1996). Mutagenesis was used to shorten non-polar side-chains that are buried in the AGH interface of holoMb, and values of $\Delta\Delta G$ for unfolding (wild type to mutant) were found to be one-half as large in I as in native apoMb (Kay & Baldwin, 1996). Similar results, and even larger values of $\Delta\Delta G$ for mutants, were found for the acid molten globule intermediate of cytochrome *c* (Marmorino & Pielak, 1995).

The stability of the apoMb folding intermediate depends strongly on the helix propensities of the individual helices of I. Its stability is sharply decreased by introducing single Gly or Pro residues into the A or G helices at solvent-exposed positions (Luo *et al*., 1997). Therefore, since TFE is known to stabilize peptide helices but to denature native proteins, it is interesting to determine which effect of TFE dominates its action on apoMb I: weakening the hydrophobic interaction or strengthening peptide H-bonds. We show here that I is stabilized by low TFE concentrations, which implies that increasing helix propensity is the dominant effect of TFE. Likewise, Cort & Andersen (1997) showed recently that 4% hexafluoroisopropanol, whose behavior is similar to that of TFE, induces the formation of a folding intermediate by acid-unfolded equine apoMb at pH 3.2.

Why should TFE also stabilize native apoMb? The native protein has a definite tertiary structure according to NMR spectra (Eliezer & Wright, 1996; Lecomte *et al*., 1996). Eliezer & Wright (1996) showed that resonances in the F helix and the beginning of the G helix are missing from the NMR spectra of N, probably because the lines are broadened from exchange between different conformations. Lin *et al*. (1994) suggested that native apoMb has some of the properties of a molten globule folding intermediate, because mutants often show low values of CD at 222 nm, suggesting that structural changes in N occur easily in mutants. The origin of the low CD values shown by apoMb mutants is still not understood, but the result found here, that TFE stabilizes the native conformation of apoMb, supports the suggestion that N has some properties resembling those of I, particularly since TFE denatures holoMb, just as it denatures other native proteins.

In determining whether TFE stabilizes or destabilizes the apoMb pH 4 intermediate, it is important to minimize structural changes and so we use a low TFE concentration (5%). It is not, however, possible to eliminate structural changes caused by TFE, because of the malleable character of the pH 4 intermediate: its structure changes in the presence of 5 to 20 mM trichloroacetate, which stabilizes the B helix (Loh *et al*., 1995). A recent NMR analysis of the structure of the pH 4 intermediate (Eliezer *et al*., 1998) shows that the ends of the A, G, and H helices are frayed, and detectable helix is present not only in the B region but also in the C and D/E regions. These observations suggest that the pH 4 intermediate has a dynamic, mobile structure, which can change in response to changing environmental conditions.

Relative importance of helix propensity and the hydrophobic interaction in stabilizing the pH 4 intermediate compared to native proteins

The balance between different factors contributing to stability can be quite different in a folding intermediate as compared to native proteins. This point is emphasized here by finding that low concentrations of TFE strongly increase the stability of the pH 4 intermediate, although they mildly decrease the stability of native proteins. A more quantitative comparison can be made by taking the ratio of the $\Delta\Delta G$ values for two types of mutation: one that changes helix propensity at a solvent-exposed position and another that changes the amount of buried non-polar surface area.

A survey of results for buried non-polar mutations in four different host proteins was made by Pace (1992). He gives $\Delta\Delta G$ for Met→Ala as 3.0(±1.0) kcal mol^{-1} and shows that this number, although based on a small number of mutants, is consistent with a larger set of results for mutations that shorten non-polar side-chains. Helix propensity mutations made in different host proteins also give self-consistent results, provided the mutation is made at a solvent-exposed position. If proline is excluded, the largest $\Delta\Delta G$ value is found for the mutation Ala→Gly for which $\Delta\Delta G$ is 0.9(±0.1) kcal mol^{-1}, according to a recent summary by Myers *et al*. (1997).

Some results of this kind are also available for the pH 4 intermediate, although the number of mutants studied at present is quite small. The side-chain of Met131 is well buried in the AGH interface of holoMb and $\Delta\Delta G$ for Met131→Ala is 0.9 kcal mol^{-1} (Kay & Baldwin, 1996). Although this value is much smaller than the 3.0 kcal mol^{-1} found in native proteins, it should be noted that ΔG (unfolding) for wild-type (2.9 kcal mol^{-1}) is also substantially smaller for the pH 4 intermediate than for native proteins. Two glycine mutants have been made at solvent-exposed positions in the A or G helix: the $\Delta\Delta G$ values have been computed from the C_m values in 20 mM Na_2SO_4, where the mutants come close to showing two-state unfolding behavior (Luo *et al*, 1997). Using an average *m*-value for wild-type and the two mutants (1430(±225) cal mol^{-1} M^{-1}) gives $\Delta\Delta G$ as 0.33(±0.05) and 0.51(±0.08) kcal mol^{-1} for Q8G and E109G, respectively. When corrected for the increased helix propensity of Ala over Gln or Glu, the estimated values for Ala→Gly are 0.47 and 0.73 kcal mol^{-1}, giving an average $\Delta\Delta G$ of 0.60(±0.13) kcal mol^{-1}.

Thus, the striking result is that the ratio of $\Delta\Delta G$ values for these two classes of mutations is 0.6/0.9 or 0.7 for the pH 4 intermediate, whereas the ratio is 0.9/3.0 or 0.3 for native proteins. When compared in this way, helix propensities are twice as important relative to hydrophobic interactions in stabilizing the pH 4 intermediate *versus* native proteins. Thus, it is not surprising that low concentrations of TFE strongly stabilize the pH 4 intermediate although they mildly destabilize native proteins.

Additivity of the effects of TFE and a stabilizing anion

The experiments displayed in Figure 6(a) show that the effects of 5% TFE and the strongly stabilizing anion 20 mM Na_2SO_4 are roughly additive as regards C_m. The C_m in this solvent (5.0 M) is larger than the sum of the C_m values in 5% TFE (2.3 M) and in 20 mM Na_2SO_4 (2.1 M). Comparison of CD and FL-monitored unfolding curves (Figure 6(b)) shows that the unfolding behavior is close to two-state in each case. The unfolding curve is much more broad, however, with both 5% TFE and 20 mM Na_2SO_4 present and the *m*-value (740 cal mol^{-1} M^{-1}) is about one half of the value found when only one of the stabilizing additives is present, which indicates that ΔG for unfolding in water is nearly the same with both 5% TFE and 20 mM Na_2SO_4 present as when only one is present, even though the C_m is greatly increased with both present. The reason for this puzzling behavior is not known.

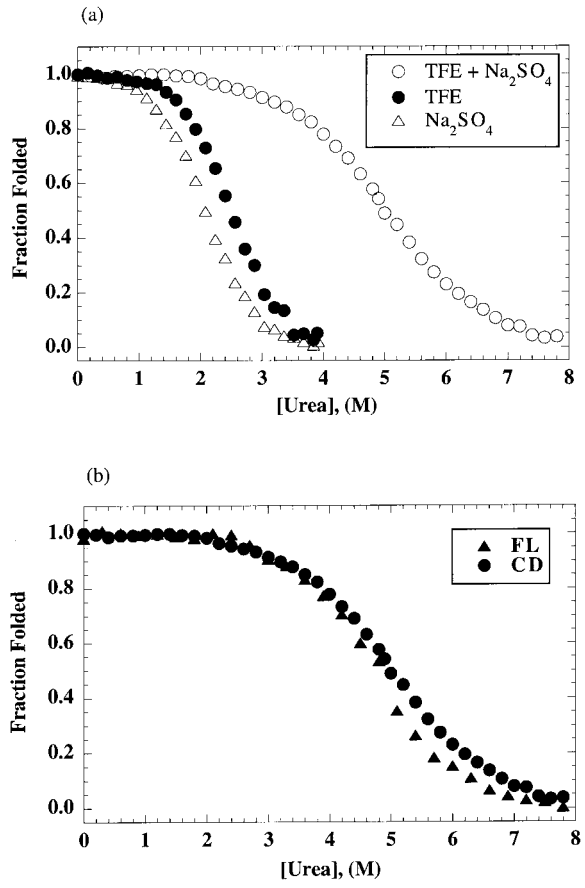

Figure 6. Urea-induced unfolding of I (WT) in the presence of either 5% TFE, 20 mM Na_2SO_4, or 5% TFE and 20 mM Na_2SO_4, in 2 mM NaAc, pH 4.2, 4°C. (a) Normalized data for CD-monitored unfolding; (b) normalized data for both CD and FL-monitored unfolding in the presence of 5% TFE and 20 mM Na_2SO_4.

Concluding remarks

There are two reasons for studying whether or not TFE stabilizes a folding intermediate. One is practical: it is common practice to add TFE when determining NMR structures of protein fragments and other marginally stable proteins (see, for example, Reymond *et al.*, 1997; Merutka *et al.*, 1995; and references therein). This is done because the NMR resonance lines are better resolved when TFE is added, and it is assumed implicitly that TFE improves the stability of the protein. Our results show that this assumption is correct not only for the pH 4 intermediate but also for native apoMb, which is not fully folded (see Eliezer & Wright, 1996) because the heme is missing. On the other hand, holoMb, which is fully folded, behaves as a typical native protein in losing stability when TFE is added. The second reason for studying the effect of TFE on the stability of I is to investigate the relative importance of helix propensities *versus* hydrophobic interactions in stabilizing I. Our results confirm the conclusion, based on mutational evidence (Luo *et al.*, 1997; Kay & Baldwin, 1996), that helix propensities are more important in stabilizing the pH 4 folding intermediate than native proteins.

Materials and Methods

Materials

2,2,2-Trifluoroethanol (TFE), hemin, and hen egg-white lysozyme were from Sigma. Ribonuclease A was from Sigma (type IIA). Ultrapure grade urea and guanidine hydrochloride were from Gibco BRL. Sodium acetate was from J. T. Baker. KCN was from Mallinckrodt. WT apomyoglobin and mutants Q8G, E109G, and E109P were prepared as described (Luo *et al.*, 1997). Apomyoglobin concentration was determined in 6 M guanidineHCl (pH 6.0), by UV absorbance at both 280 nm and 288 nm, assuming $\varepsilon_{280} = 15{,}200\ M^{-1}\ cm^{-1}$ and $\varepsilon_{288} = 10{,}800\ M^{-1}\ cm^{-1}$ (Edelhoch, 1967). Ribonuclease A concentration was determined by UV absorbance at both 278 nm and 287 nm, assuming $\varepsilon_{278} = 9{,}800\ M^{-1}\ cm^{-1}$ and $\varepsilon_{287} = 6{,}700\ M^{-1}\ cm^{-1}$ (Sela & Anfinsen, 1957). Hen egg-white lysozyme concentration was determined by UV absorbance at 280 nm, assuming $\varepsilon_{280} = 37{,}500\ M^{-1}\ cm^{-1}$.

Unfolding and refolding conditions of holomyoglobin

For reversible unfolding and refolding of holomyoglobin, it is necessary to use the cyanoMb form of holoMb and to use GdmCl instead of urea to unfold and refold

cyanoMb. Hargrove & Olson (1996) systematically studied the stabilities of different forms of holoMb and measured the rates and equilibrium constants for hemin dissociation from holoMb. For cyanoMb unfolding we used here 2 μM holomyoglobin (oxidized form) in the unfolding/refolding buffer (2 μM hemin, 80 μM KCN, 5 mM Tris-HCl, pH 7.8) at 30°C, with different amount of GdmCl present. The unfolding reaction was incubated for about 60 minutes (not less than 50 minutes) to reach equilibrium before taking CD measurements. Equilibrium is reached faster in the presence of 5% TFE than in the absence of TFE. For refolding holoMb was first unfolded in 6 M GdmCl at 80 μM holoMb, 80 μM hemin, 3.2 mM KCN, 5 mM Tris-HCl (pH 7.8), 30°C, and then diluted to 2 μM Mb in 5 mM Tris-HCl (pH 7.8), at different final GdmCl concentrations, 30°C. The cyanoMb concentration was calculated on the basis of the concentration of apoMb, taking into account the change in the volume upon reconstitution of holoMb by adding hemin and KCN. The Soret absorption of cyanoMb cannot be used to calculate the concentration of cyanoMb because of the interference from excess hemin.

Unfolding conditions used for I, apoMb, ribonuclease A, and hen egg-white lysozyme

The conditions used for measuring unfolding are as follows: for I, 2 mM sodium acetate, pH 4.2, 4°C; for native apoMb, 5 mM Tris-HCl (pH 7.8), 4°C; for ribonuclease A, 5 mM Tris-HCl (pH 8.0), 30°C; and for hen egg-white lysozyme, 5 mM sodium acetate, pH 4.0, 30°C.

CD and fluorescence measurements

Unfolding of apomyoglobin monitored by CD and FL has been described (Luo *et al.*, 1997). Briefly, CD data are collected at 222 nm with a 1 cm cuvette, using an Aviv 62A DS circular dichroism spectropolarimeter. Fluorescence measurements are made using a SLM AB2 spectrofluorimeter with a 1 cm light path. For urea-induced unfolding of I at pH 4.2, with or without TFE present, excitation was at 280 nm (with a bandpass of 4 nm) and emission was monitored at 330 nm (with a bandpass of 16 nm). For urea- or GdmCl-induced unfolding of native apoMb in 5 mM Tris-HCl (pH 7.8), with or without TFE present, excitation was at 288 nm (with a bandpass of 4 nm) and emission was monitored at 320 nm (with a bandpass of 16 nm). Protein concentrations of 1 μM and 2 μM of apoMb were used for fluorescence and CD measurements, respectively. 5 μM RNase A or lysozyme was used for CD measurements.

Data analyses

Urea-induced unfolding curves were analyzed by a two-state equation with linear baselines, according to the procedure of Santoro & Bolen (1988), which uses data inside as well as outside the transition zone to fix the baselines. The native baselines below 1 M urea for FL-monitored unfolding curves of I are strongly curved, and the extent of curvature depends in part on the stability of I. This effect results from a urea-dependent equilibrium between two forms of I that have different fluorescence spectra (see text, and Jamin & Baldwin, 1998). To avoid this problem, the fluorescence data below the highest value point were not used in fixing the baselines.

Acknowledgments

We thank Michael Kay for his preliminary data concerning the effects of TFE on the acid unfolding curves of apoMb, and Peizhi Luo and Michael Kay for discussions. Y. Luo is a fellow of the National Institutes of Health. This work was supported by a National Institutes of Health grant (GM 19988) to R. L. Baldwin.

References

Alexandrescu, A. T., Ng, Y. L. & Dobson, C. M. (1994). Characterization of a trifluoroethanol-induced partially folded state of alpha-lactalbumin. *J. Mol. Biol.* **235,** 587–599.

Barrick, D. & Baldwin, R. L. (1993). The molten globule intermediate of apomyoglobin and the process of protein folding. *Protein Sci.* **2,** 869–876.

Barrow, C. J., Yasuda, A., Kenny, P. T. & Zagorski, M. G. (1992). Solution conformations and aggregational properties of synthetic amyloid beta-peptides of Alzheimer's disease. Analysis of circular dichroism spectra. *J. Mol. Biol.* **225,** 1075–1093.

Brandts, J. F. & Hunt, L. (1967). The Thermodynamics of protein denaturation. III. The denaturation of ribonuclease in water and in aqueous urea and aqueous ethanol mixtures. *J. Am. Chem. Soc.* **89,** 4826–4838.

Buck, M., Radford, S. E. & Dobson, C. M. (1993). A partially folded state of hen egg white lysozyme in trifluoroethanol: structural characterization and implications for protein folding. *Biochemistry,* **32,** 669–678.

Buck, M., Schwalbe, H. & Dobson, C. M. (1995). Characterization of conformational preferences in a partly folded protein by heteronuclear NMR spectroscopy: assignment and secondary structure analysis of hen egg-white lysozyme in trifluoroethanol. *Biochemistry,* **34,** 13219–13232.

Cammers-Goodwin, A., Allen, T. J., Oslick, S. L., McClure, K. F., Lee, J. H. & Kemp, D. S. (1996). Mechanism of stabilization of helical conformations of polypeptides by water containing trifluoroethanol. *J. Am. Chem. Soc.* **118,** 3082–3090.

Cort, J. R. & Andersen, N. H. (1997). Formation of a molten-globule-like state of myoglobin in aqueous hexafluoroisopropanol. *Biochem. Biophy. Res. Commun.* **233,** 687–691.

Dyson, H. J., Rance, M., Houghten, R. A., Wright, P. E. & Lerner, R. A. (1988). Folding of immunogenic peptide fragments of proteins in water solution. II. The nascent helix. *J. Mol. Biol.* **201,** 201–217.

Edelhoch, H. (1967). Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry,* **6,** 1948–1954.

Eliezer, D. & Wright, P. E. (1996). Is apomyoglobin a molten globule? Structural characterization by NMR. *J. Mol. Biol.* **263,** 531–538.

Eliezer, D., Yao, J., Dyson, H. J. & Wright, P. E. (1998). Structural and dynamic characterization of partially folded states of apomyoglobin and implications for protein folding. *Nature Struct. Biol.* **5,** 148–155.

Gill, S. J., Dec, S. F., Olofsson, G. & Wadsö, I. (1985). Anomalous heat capacity of hydrophobic solvation. *J. Phys Chem.* **89,** 3758–3761.

Hargrove, M. S. & Olson, J. S. (1996). The stability of holomyoglobin is determined by heme affinity. *Biochemistry,* **35,** 11310–11318.

Hermans, J. (1966). The effect of protein denaturants on the stability of the alpha-helix. *J. Am. Chem. Soc.* **88,** 2418–2422.

Herskovits, T. T., Gadegbeku, B. & Jaillet, H. (1970). On the structural stability and solvent denaturation of proteins. I. Denaturation by the alcohols and glycols. *J. Biol. Chem.* **245,** 2588–2598.

Hughson, F. M., Wright, P. E. & Baldwin, R. L. (1990). Structural characterization of a partly folded apomyoglobin intermediate. *Science,* **249,** 1544–1548.

Jamin, M. & Baldwin, R. L. (1996). Refolding and unfolding kinetics of the equilibrium folding intermediate of apomyoglobin. *Nature Struct. Biol.* **3,** 613–618.

Jamin, M. & Baldwin, R. L. (1998). Two forms of the pH 4 folding intermediate of apomyoglobin. *J. Mol. Biol.* **276,** 491–504.

Jennings, P. A. & Wright, P. E. (1993). Formation of a molten globule intermediate early in the kinetic folding pathway of apomyoglobin. *Science,* **262,** 892–896.

Kauzmann, W. (1959). Factors in interpretation of protein denaturation. *Advan. Protein Chem.* **14,** 1–63.

Kay, M. S. & Baldwin, R. L. (1996). Packing interactions in the apomyoglobin folding intermediate. *Nature Struct. Biol.* **3,** 439–445.

Kiefhaber, T. & Baldwin, R. L. (1995). Intrinsic stability of individual alpha helices modulates structure and stability of the apomyoglobin molten globule form. *J. Mol. Biol.* **252,** 122–132.

Lecomte, J. T., Kao, Y. H. & Cocco, M. J. (1996). The native state of apomyoglobin described by proton NMR spectroscopy: the A-B-G-H interface of wild-type sperm whale apomyoglobin. *Proteins: Struct. Funct. Genet.* **25,** 267–285.

Lin, L., Pinker, R. J., Forde, K., Rose, G. D. & Kallenbach, N. R. (1994). Molten globular characteristics of the native state of apomyoglobin. *Nature Struct. Biol.* **1,** 447–452.

Loh, S. N., Kay, M. S. & Baldwin, R. L. (1995). Structure and stability of a second molten globule intermediate in the apomyoglobin folding pathway. *Proc. Natl Acad. Sci. USA,* **92,** 5446–5450.

Luo, P. & Baldwin, R. L. (1997). Mechanism of helix induction by trifluoroethanol: a framework for extrapolating the helix-forming properties of peptides from trifluoroethanol/water mixtures back to water. *Biochemistry,* **36,** 8413–8421.

Luo, Y., Kay, M. S. & Baldwin, R. L. (1997). Cooperativity of folding of the apomyoglobin pH 4 intermediate studied by single glycine and proline mutations. *Nature Struct. Biol.* **4,** 925–930.

Marmorino, J. L. & Pielak, G. J. (1995). A native tertiary interaction stabilizes the A state of cytochrome *c*. *Biochemistry,* **34,** 3140–3143.

Merutka, G., Dyson, H. J. & Wright, P. E. (1995). 'Random coil' ^{1}H chemical shifts obtained as a function of temperature and trifluoroethanol concentration for the peptide series GGXGG. *J. Biomol. NMR,* **5,** 14–24.

Myers, J. K., Pace, C. N. & Scholtz, J. M. (1997). A direct comparison of helix propensity in proteins and peptides. *Proc. Natl Acad. Sci. USA,* **94,** 2833–2837.

Nelson, J. W. & Kallenbach, N. R. (1986). Stabilization of the ribonuclease S-peptide alpha-helix by trifluoroethanol. *Proteins: Struct. Funct. Genet.* **1,** 211–217.

Nelson, J. W. & Kallenbach, N. R. (1989). Persistence of the alpha-helix stop signal in the S-peptide in trifluoroethanol solutions. *Biochemistry,* **28,** 5256–5261.

Pace, C. N. (1992). Contribution of the hydrophobic effect to globular protein stability. *J. Mol. Biol.* **226,** 29–35.

Reymond, M. T., Huo, S., Duggan, B., Wright, P. E. & Dyson, H. J. (1997). Contribution of increased length and intact capping sequences to the conformational preference for helix in a 31-residue peptide from the C terminus of myohemerythrin. *Biochemistry,* **36,** 5234–5244.

Rohl, C. A., Chakrabartty, A. & Baldwin, R. L. (1996). Helix propagation and N-cap propensities of the amino acids measured in alanine-based peptides in 40 volume percent trifluoroethanol. *Protein Sci.* **5,** 2623–2637.

Santoro, M. M. & Bolen, D. W. (1988). Unfolding free energy changes determined by the linear extrapolation method. 1. Unfolding of phenylmethanesulfonyl alpha-chymotrypsin using different denaturants. *Biochemistry,* **27,** 8063–8068.

Segawa, S., Fukuno, T., Fujiwara, K. & Noda, Y. (1991). Local structures in unfolded lysozyme and correlation with secondary structures in the native conformation: helix-forming or -breaking propensity of peptide segments. *Biopolymers,* **31,** 497–509.

Sela, M. & Anfinsen, C. B. (1957). Some spectrophotometric and polarimetric experiments with ribonuclease. *Biochim. Biophys. Acta,* **24,** 229–235.

Serrano, L., Kellis, J. T., Jr, Cann, P., Matouschek, A. & Fersht, A. R. (1992). The folding of an enzyme. II. Substructure of barnase and the contribution of different interactions to protein stability. *J. Mol. Biol.* **224,** 783–804.

Shiraki, K., Nishikawa, K. & Goto, Y. (1995). Trifluoroethanol-induced stabilization of the alpha-helical structure of beta-lactoglobulin: implication for non-hierarchical protein folding. *J. Mol. Biol.* **245,** 180–194.

Tanford, C. (1962). Contribution of hydrophobic interactions to the stability of the globular conformation of proteins. *J. Am. Chem. Soc.* **84,** 4240–4247.

Edited by P. E. Wright

(Received 21 January 1998; received in revised form 6 March 1998; accepted 6 March 1998)