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Does trifluoroethanol affect folding pathways and can it be used as a probe of structure in transition states?

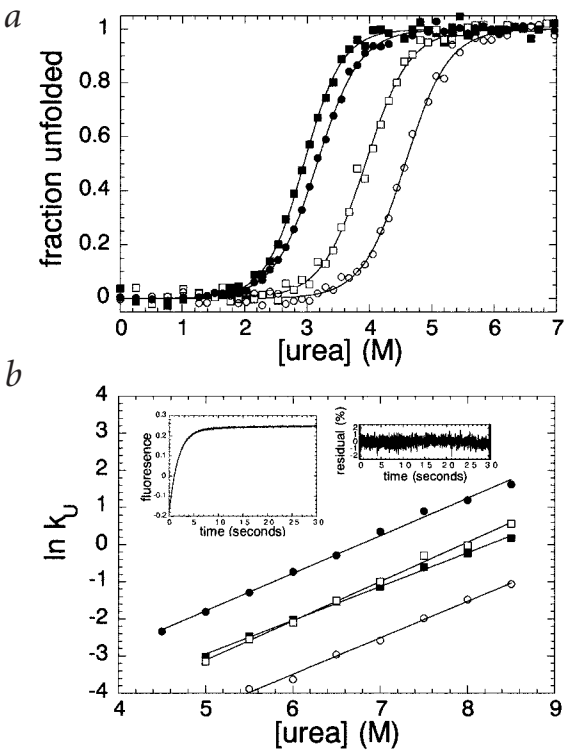
Ewan R.G. Main and Sophie E. Jackson

Cambridge University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK.

Nonaqueous co-solvents, particularly 2,2,2-trifluoroethanol (TFE), have been used as tools to study protein folding. By analyzing FKBP12, an α/β-protein that folds with two-state kinetics, we have been able to address three key questions concerning the use of TFE. First, does TFE perturb the folding pathway? Second, can the observed changes in the rate of folding and unfolding in TFE be attributed to a change in free energy of a single state? Finally, can TFE be used to infer information on secondary structure formation in the transition state? Protein engineering experiments on FKBP12, coupled with folding and unfolding experiments in 0% and 9.6% TFE, conclusively show that TFE does not perturb the folding pathway of this protein. Our results also suggest that the changes in folding and unfolding rates observed in 9.6% TFE are due to a global effect of TFE on the protein, rather than the stabilization of any elements of secondary structure in the transition state. Thus, studies with TFE and other co-solvents can be accurately interpreted only when combined with other techniques.

To thoroughly analyze the folding pathway of a protein, all species on that pathway must be characterized, both structurally and energetically. In the simplest case, where a protein folds in a two-state manner without populating intermediate states, this involves characterizing the unfolded state ensemble (U), transition state ensemble (‡) and native state (F). Although the structure of both native and unfolded states can be measured directly, through NMR and X-ray crystallography, the only method for characterizing the structure of transition states is indirectly by analyzing the kinetics of folding and unfolding. Two main experimental approaches have been used to study the structure of folding transition states. Protein engineering techniques (and Φ_F-value analysis)[1,2] give atomic level resolution, whereas more global information, such as the average solvent accessibility, heat capacity, enthalpy and entropy in the transition state, is gained by changing conditions such as temperature and denaturant concentration[3–5]. Recently, co-solvents, especially 2,2,2-trifluoroethanol (TFE), have been used in an attempt to characterize secondary structure in transition states of folding[6,7].

The co-solvent TFE, which has been used extensively to study the structure of short peptides, is known to stabilize secondary structure, for example, α-helices and β-hairpins[8,9]. It has also been used to study partially folded states and equilibrium intermediates[10,11]. More recently, several groups have observed changes in folding and unfolding rates on addition of TFE[6,7,12–14]. For lysozyme, an α/β-protein for which the majority of molecules fold through a slow pathway involving a populated intermediate state, TFE accelerates the folding rate even though the unfolding rate is unchanged. This has been attributed to a weakening of hydrophobic interactions and consequent destabilization of a misfolded species that acts as a kinetic trap on the folding pathway[13]. For acylphosphatase (AcP), an α/β-protein that folds with two-state kinetics, TFE increases both the folding and unfolding rates. In this case, the rate enhancements were attributed to TFE's ability to stabilize a transition state with a relatively hydrated and disorganized core but having a significant level of secondary, particularly α-helical, structure[6,7]. For stefin B, another α/β-protein, the addition of TFE increased the folding rate and caused an α-helical intermediate to become populated on the folding pathway[14]. However, when we studied the folding of FKBP12, an α/β-protein that folds with two-state kinetics, by protein engineering[15] and with TFE[16], we obtained conflicting results. Protein engineering experiments showed that, in the transition state, the α-helix is largely unstructured and no β-hairpins are fully formed[15]. Yet, 9.6% TFE (v/v) increased the folding rate by a factor of four (Fig. 1*c*) and decreased the unfolding rate by a factor of two, suggesting that the α-helix or a β-hairpin may have significant structure in the transition state[16]. These conflicting results imply that either addition of TFE has changed the folding pathway, or TFE does not act by stabilizing secondary structure in the transition state.



With the increased use of TFE as a tool for studying protein folding, it is essential to establish (i) whether TFE perturbs the folding pathway, and (ii) the exact cause of the observed changes in rate. To address these questions, we have analyzed the wild type as well as 15 mutants of the FKBP12 protein that probe different elements of secondary structure including the α-helix, the β-sheet and the hydrophobic core. The unfolding and refolding rates have been measured in water and in 9.6% TFE (v/v) and the results compared using both β_T-value and Φ_F-value analysis.

Equilibrium experiments

Urea-induced unfolding was used to measure the relative stabilities of wild type and mutant proteins in 9.6% TFE (v/v)

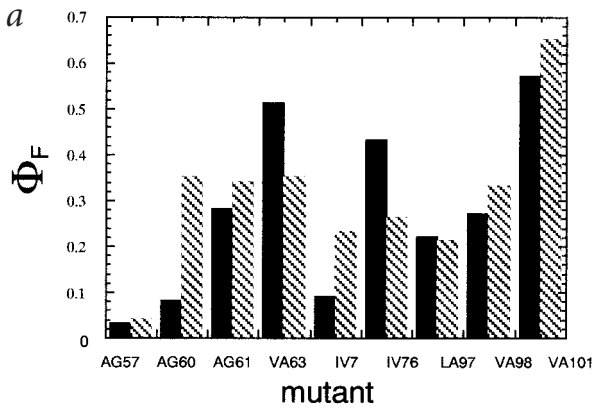


Fig. 2 ***a***, Comparison of Φ_F-values in 3.9 M urea for FKBP12 protein in 0% TFE (solid black bars) and 9.6% TFE (hatched bar). Φ_F-values were quoted at 3.9 M, rather than 0 M, to avoid large extrapolation errors. Typical errors are ±0.1; however, the mutants A60G and I76V have larger errors due to the small $\Delta\Delta G_{U\text{-}F}$. All values of Φ_F in 0% and 9.6% TFE are within experimental error. ***b***, Comparison of the transition state for folding of FKBP12 protein in 9.6% TFE (v/v) and in water. Φ_F-values between 0 and 0.1 are shown in green, 0.2–0.4 in yellow, 0.4–0.5 in orange and 0.5 and above in red.

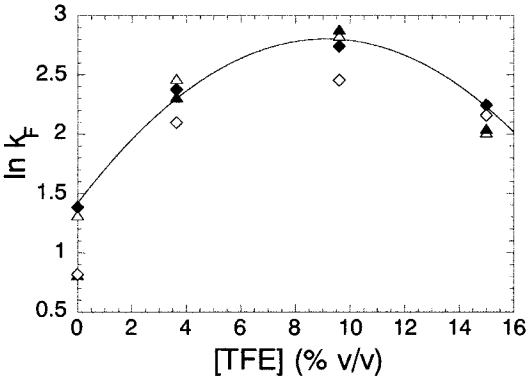


Fig.1 ***a***, Typical urea-induced denaturation curves of wild-type (○) and mutant FKBP12 protein (V101A ■; E61A, □; V98A, ●) in 9.6% TFE (v/v). ***b***, Typical urea-induced unfolding kinetics of wild-type (○) and mutant FKBP12 protein (V101A, ■; E61A, □; V98A, ●) in 9.6% TFE (v/v), with an example of a typical unfolding fluorescence trace and residual of wild type in 6 M urea. ***c***, Typical refolding kinetics of wild type (◆) and mutant FKBP12 protein (E61A, ◇ E61G, ▲; I76V, ◇) at final [TFE] of 3.64%, 9.6% and 15% (v/v). All solutions were in 50 mM Tris-HCl, 1 mM DTT, pH 7.5 at 25 °C.

and water (Fig. 1*a*). The addition of 9.6% TFE increases the stability of the native state of wild type and mutant proteins, relative to the unfolded state, by an average of 1.61 ± 0.24 kcal mol^{-1} (Table 1). A similar stabilization energy (1.7 kcal mol^{-1}) has been observed for the dimeric α-helical coiled coil from GCN4 protein[12]. The difference in the change in free energy of unfolding between wild type and mutant proteins, $\Delta\Delta G_{U\text{-}F}$, is remarkably similar in water and in 9.6% TFE (Table 1). If 9.6% TFE stabilizes native-like helices in the unfolded

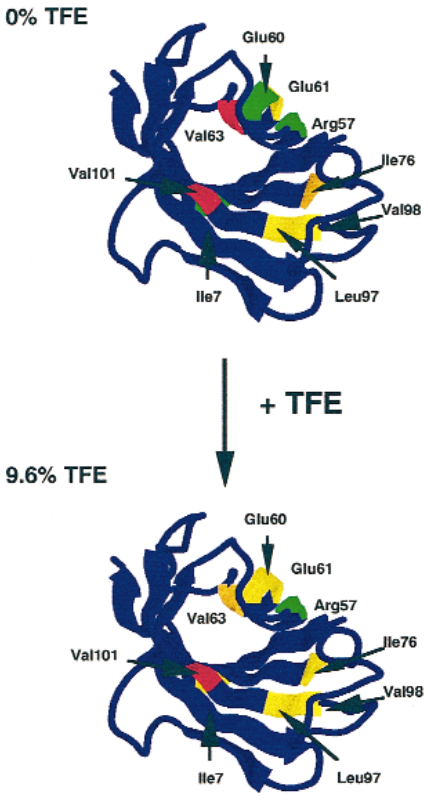


Table 1 Equilibrium and kinetic data for the unfolding and refolding of wild type and mutant FKBP12 in 0% and 9.6% (v/v) TFE

	Equilibrium data				Unfolding kinetic data		
0% TFE	m_{U-F} (kcal mol^{-1} M^{-1})	$[urea]_{50\%}$ (M)	ΔG_{U-F}[1] (kcal mol^{-1})	$\Delta\Delta G_{U-F}$[1,3] (kcal mol^{-1})	ln k_U ^{H2O}	$m_{\ddagger-F}$ (M^{-1})	ln k_U (3.9 M urea)[4]
Wild type	1.43 ± 0.05	3.87 ± 0.02	6.11 ± 0.08		-8.46 ± 0.16	0.89 ± 0.03	-4.97 ± 0.08
R57A	1.56 ± 0.07	3.36 ± 0.02	5.31 ± 0.07	0.81 ± 0.11	-7.66 ± 0.06	0.93 ± 0.02	-4.05 ± 0.02
R57G	1.66 ± 0.08	2.43 ± 0.02	3.84 ± 0.06	2.28 ± 0.10	-4.94 ± 0.16	0.84 ± 0.01	-1.65 ± 0.07
E60A	1.68 ± 0.10	2.53 ± 0.03	4.00 ± 0.07	2.12 ± 0.11	-5.32 ± 0.10	0.88 ± 0.03	-1.89 ± 0.04
E60G	1.50 ± 0.12	2.08 ± 0.05	3.29 ± 0.09	2.83 ± 0.12	-3.97 ± 0.19	0.82 ± 0.02	-0.79 ± 0.08
E61A	1.47 ± 0.05	3.34 ± 0.02	5.28 ± 0.07	0.84 ± 0.11	-7.17 ± 0.17	0.92 ± 0.03	-3.59 ± 0.07
E61G	1.57 ± 0.06	2.30 ± 0.02	3.63 ± 0.06	2.48 ± 0.10	-5.29 ± 0.21	0.95 ± 0.03	-1.59 ± 0.07
V63A	1.69 ± 0.10	2.00 ± 0.03	3.16 ± 0.06	2.96 ± 0.10	-5.88 ± 0.07	0.87 ± 0.01	-2.50 ± 0.03
I7V	1.41 ± 0.07	3.29 ± 0.02	5.20 ± 0.07	0.92 ± 0.11	-7.15 ± 0.14	0.92 ± 0.05	-3.55 ± 0.06
I76V	1.66 ± 0.10	3.39 ± 0.03	5.36 ± 0.08	0.76 ± 0.12	-7.71 ± 0.09	0.91 ± 0.01	-4.14 ± 0.04
L97A	1.89 ± 0.13	1.63 ± 0.04	2.58 ± 0.07	3.54 ± 0.11	-3.63 ± 0.21	0.82 ± 0.04	-0.42 ± 0.04
V98A	1.48 ± 0.17	2.51 ± 0.06	3.97 ± 0.11	2.15 ± 0.14	-5.95 ± 0.17	0.94 ± 0.02	-2.31 ± 0.08
V101A	1.63 ± 0.04	2.14 ± 0.02	3.38 ± 0.05	2.73 ± 0.10	-6.68 ± 0.07	0.94 ± 0.01	-3.00 ± 0.02
9.6% TFE							
Wild type	1.70 ± 0.09	4.58 ± 0.03	7.60 ± 0.05		-9.39 ± 0.24	0.98 ± 0.03	-5.57 ± 0.11
R57A	1.57 ± 0.06	4.28 ± 0.02	7.10 ± 0.04	0.50 ± 0.06	-8.87 ± 0.30	1.07± 0.04	-4.70 ± 0.14
R57G	1.55 ± 0.10	3.43 ± 0.03	5.69 ± 0.05	1.91 ± 0.07	-6.33 ± 0.21	1.01 ± 0.03	-2.40 ± 0.09
E60A	1.45 ± 0.09	3.62 ± 0.03	6.01 ± 0.05	1.59 ± 0.07	-6.38 ± 0.20	1.03 ± 0.03	-2.37 ± 0.09
E60G	1.65 ± 0.13	3.06 ± 0.04	5.08 ± 0.07	2.52 ± 0.09	-5.59 ± 0.14	1.09 ± 0.02	-1.36 ± 0.06
E61A	1.54 ± 0.10	4.17 ± 0.03	6.92 ± 0.05	0.68 ± 0.07	-8.41 ± 0.18	1.06 ± 0.03	-4.28 ± 0.08
E61G	1.60 ± 0.13	3.11 ± 0.04	5.16 ± 0.07	2.44 ± 0.09	-6.79 ± 0.22	1.15 ± 0.03	-2.30 ± 0.09
V63A[2]	1.82 ± 0.09	3.02 ± 0.02	5.01 ± 0.04	2.54 ± 0.06	-9.98 ± 1.20	0.87 ± 0.05[e]	-2.80 ± 0.20
I7V	1.80 ± 0.13	3.89 ± 0.03	6.46 ± 0.05	1.15 ± 0.07	-8.34 ± 0.20	1.10 ± 0.03	-4.06 ± 0.09
I76V	1.69 ± 0.07	4.23 ± 0.02	7.02 ± 0.04	0.58 ± 0.06	-9.10 ± 0.25	1.09 ± 0.04	-4.85 ± 0.11
L97A	1.68 ± 0.21	2.33 ± 0.08	3.87 ± 0.14	3.74 ± 0.14	-3.80 ± 0.21	0.82 ± 0.03	-0.62 ± 0.09
V98A	1.71 ± 0.06	3.16 ± 0.01	5.25 ± 0.02	2.36 ± 0.05	-6.84 ± 0.16	1.01 ± 0.02	-2.90 ± 0.07
V101A	1.93 ± 0.10	2.95 ± 0.02	4.90 ± 0.04	2.71 ± 0.06	-7.50 ± 0.11	0.91 ± 0.02	-3.95 ± 0.05
Wild type (3.6% TFE)	1.70 ± 0.09	4.55 ± 0.02	7.54 ± 0.04		-9.25 ± 0.1	0.91 ± 0.02	-5.71 ± 0.06
Wild type (17% TFE)	1.68 ± 0.071	3.65 ± 0.02	6.06 ± 0.04		-8.76 ± 0.2	1.15 ± 0.02	-4.28 ± 0.06

[1]Calculated in 0% TFE using an average m_{U-F} (1.59 ± 0.02) and in 9.6% TFE using an average m_{U-F} (1.63 ± 0.04).
[2]Obtained by fitting the unfolding data (ln k_U *versus* [urea]) to a second-order polynomial (for further details of this analysis see Fulton *et al.*[15]).
[3]Some small differences in $\Delta\Delta G_{U-F}$ are observed between water and 9.6% TFE for charged, solvent-exposed residues. In these cases, TFE affects the solvation energy in the unfolded and folded states, and also the strength of electrostatic interactions in the folded state. Calculation of the difference in $\Delta\Delta G_{U-F}$ for Ala→Gly mutations in the α-helix in water and in 9.6% TFE yields values of 0.06 ± 0.17, 0.22 ± 0.19 and 0.11 ± 0.18 kcal mol^{-1}, for positions 57, 60 and 61, respectively. These values are the same, within experimental error, indicating that 9.6% TFE does not significantly affect the stability of the α-helix in the unfolded state.
[4]Values calculated at 3.9 M are more accurate than those extrapolated to water.

state, it is expected that mutations in the α-helix would be less destabilizing in TFE than in water. This is not observed (Table 1).

Kinetic experiments

The rate constants for unfolding (k_U) were determined as a function of urea concentration in 0% and 9.6% TFE. The linear fit of plots of ln k_U *versus* urea concentration was used to extrapolate ln k_U at 0 M and 3.9 M urea (Fig. 1*b*). The unfolding of all proteins was slower in 9.6% TFE as expected (Table 1). The slope of the plot of ln k_U *versus* urea concentration, $m_{\ddagger-F}$, was very similar for all proteins in 0% and 9.6% TFE, suggesting a similar unfolding pathway.

Refolding rate constants (k_F) were determined by pH-jump experiments (from pH 1.5 to pH 7.5) or by TFE-jump experiment: from 40% (v/v) to concentrations between 3.6% and 15% (v/v) (Fig. 1*c*). The folding rates increased on addition of TFE, reaching a maximum rate enhancement at 9.6% TFE.

β_T-value analysis and Φ_F-value analysis

β_T-value analysis indicates the degree of compactness of the transition state by measuring the fractional change in degree of exposure of residues between the denatured or native state, and the transition state. Values for wild type and for mutant proteins in 0% and in 9.6% TFE are the same within experimental error (Table 2). These results suggest that there is no significant change in the folding pathway of FKBP12 on addition of 9.6% TFE.

To estimate the extent to which interactions are formed in the transition state, Φ_F-values are calculated. The Φ_F-value quantifies the degree of structure formation around each mutated residue in the transition state[1]. A value of $\Phi_F = 1$ indicates that the region around the site of mutation is as structured in the transition state as in the folded state. Conversely, a value of $\Phi_F = 0$ indicates that the region around the site of mutation is as unstructured in the transition state as in the unfolded state. If Φ_F is fractional, we have shown for FKBP12 that this corresponds to partial structure formation at the site of mutation in the transition state[15]. Furthermore, when the mutation does not involve a significant change in solvation energy (for example, a nonpolar to nonpolar substitution), fractional Φ_F-values correspond directly to the extent of structure formation[15]. In this study we analyze only nonpolar to nonpolar substitutions.

Table 2: Values for β_T and Φ_F calculated in 0% TFE and 9.6% TFE

0% TFE	β_T-value[1]	Φ_F-value[2]	
		Water	3.9 M urea
Wild type	0.63	–	–
R57A[3]	0.65	–	–
R57G[3]	0.70	–	–
A57G	–	-0.10	0.03
E60A[3]	0.69	–	–
E60G[3]	0.68	–	–
A60G	–	-0.13	0.08
E61A[3]	0.63	–	–
E61G[3]	0.64	–	–
A61G	–	0.33	0.28
V63A	0.70	0.48	0.51
I7V	0.61	0.16	0.09
I76V	0.67	0.57	0.43
L97A	0.74	0.15	0.22
V98A	0.63	0.31	0.27
V101A	0.66	0.60	0.57
9.6% TFE			
Wild type	0.66	–	–
R57A[3]	0.60	–	–
R57G[3]	0.61	–	–
A57G	–	-0.07	0.04
E60A[3]	0.58	–	–
E60G[3]	0.61	–	–
A60G	–	0.50	0.35
E61A[3]	0.59	–	–
E61G[3]	0.57	–	–
A61G	–	0.45	0.34
V63A	0.72	0.86[4]	0.35
I7V	0.64	0.46	0.23
I76V	0.62	0.70[4]	0.26
L97A	0.71	0.11	0.21
V98A	0.65	0.36	0.33
V101A	0.72	0.59	0.65

[1]Errors in β_T are typically ±5%. Compound errors are calculated from the fitting errors associated with $m_{\ddagger\text{-}F}$ and $m_{U\text{-}F}$.
[2]Errors are typically ±0.1. Compound errors are calculated from errors in $\Delta\Delta G_{\ddagger\text{-}F}$ (calculated from the fitting errors in $\ln k_U$) and fitting errors associated with $\Delta\Delta G_{U\text{-}F}$.
[3]Φ_F-values are not calculated because mutations change the solvation energy; Φ_F values for the composite Ala→Gly mutation are reported instead.
[4]The discrepancy between Φ_F calculated in water and in 3.9 M urea for these mutants is a result of slight nonlinearity in plots of $\ln k_U$ *versus* [urea]. In these cases, the value at 3.9 M urea is more accurate than at 0 M urea because of the shorter extrapolation.

Unfolding data were used to calculate Φ_F-values in 0 M and 3.9 M urea for mutants in 0% and 9.6% TFE (Fig. 2 and Table 2). For a two-state system, Φ_F-values can also be calculated from refolding data, and these are similar to those calculated from the unfolding data as expected (data not shown). Remarkably, there is no difference (within experimental error) in the Φ_F-values determined in 0% and 9.6% TFE (Fig. 2). These results conclusively show that TFE does not change the folding pathway of FKBP12. The transition state in the presence of 9.6% TFE still has a largely unstructured α-helix and no fully structured β-hairpins (Fig. 2*b*). Thus, the observed rate enhancement cannot be attributed to stabilization of the α-helix or β-hairpin, as has been reported elsewhere[6,7]. These results suggest that TFE has a global effect on the protein structure rather than specifically stabilizing any individual element of secondary structure. A recent paper by Kentsis *et al.*[12] reported that TFE increases the refolding rate of an

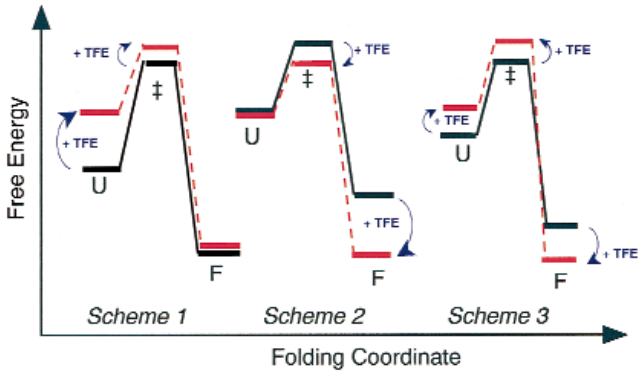


Fig. 3 Free energy diagrams for the folding of FKBP12 protein in 0% (black lines) and 9.6% (red lines) TFE. The unfolded, transition and native states are designated U, ‡ and F, respectively.

α-helical protein. Their analysis of two mutants suggested that TFE affects the unfolded ensemble, destabilizing it relative to both the transition and native states. We have now tested the generality of this conclusion in a structurally very different protein, FKBP12, which contains both α- and β-structural elements. By analyzing mutations throughout different regions of secondary and tertiary structure, we have shown that TFE does not affect any single element of structure in the transition state.

Which states on the folding pathway are affected by TFE?

Ideally, we would like to be able to assign the differences in rates observed in TFE to changes in the energy of a particular state on the folding pathway. Unfortunately, this is not possible because TFE affects several structural and physical properties of the system, as recently pointed out by Ionescu and Matthews[17]. For example, TFE may weaken hydrophobic interactions, strengthen intramolecular hydrogen bonds, change the dielectric constant of the solvent and act as an osmolyte. It is not possible to distinguish how these variables affect the energy of the unfolded, transition and native states (Fig. 3). One interpretation of our data is that TFE acts mainly as an osmolyte, disrupting the water structure surrounding the protein. In this case, we expect the unfolded state to be most destabilized, the transition state to a lesser extent and the native state the least (scheme 1, Fig. 3). This agrees well with recent work on the mechanism of helix induction by TFE, where it is proposed that, at a TFE concentration less than 20%, TFE stabilizes helical structure by disordering the local hydration shell around the polypeptide in a chaotropic manner[18]. Furthermore, a growing body of evidence supports the conclusion that low concentrations of TFE affect the solvation of the protein[12,19].

Efficacy of TFE in elucidating transition states and folding pathways

Although TFE has been used successfully to stabilize protein segments in order to study structural preferences for β-hairpins[8] and α-helices[9,20], its use in the elucidation of the structure of folding transition states is questionable, as shown by this and other studies[12]. We have shown that TFE does not significantly change the folding pathway of FKBP12 protein. However, although TFE affects the folding and unfolding rate of FKBP12, we have established that this effect, rather than being

caused by the stabilization of a single element of structure, appears to be global in nature, affecting all regions of the protein structure equally. There is evidence that other co-solvents may exert their effects by similar mechanisms[21]. Thus, an accurate interpretation of studies using TFE and other co-solvents can be achieved only by combining them with other techniques such as protein engineering.

Methods

All reagents were purchased from Sigma except the high-purity urea, which was purchased from Rose Chemicals Ltd.

Production of wild type and mutant proteins. Wild type and mutant proteins were expressed and purified as described[22].

Chemical denaturation. Equilibrium unfolding was monitored by changes in fluorescence and is described in detail elsewhere[22]. Final conditions were 50 mM Tris-HCl pH 7.5, 1 mM dithiothreitol (DTT) at 25 °C, with and without 9.6% (v/v) TFE.

Kinetics. Folding was monitored by fluorescence using stopped-flow techniques to initiate refolding and unfolding. Experimental details are described elsewhere[16]. Final conditions were 50 mM Tris-HCl pH 7.5, 1 mM DTT at 25 °C, with or without 3.6%, 9.6% or 15% (v/v) TFE.

Φ-value analysis. This value is described by equation (1).

$$\Phi_F = 1 - \frac{\Delta\Delta G_{\ddagger\text{-}F}}{\Delta\Delta G_{U\text{-}F}} \quad (1)$$

where $\Delta\Delta G_{\ddagger\text{-}F}$ is the difference in free energy change between transition and folded states, between wild type and mutant proteins, and $\Delta\Delta G_{U\text{-}F}$ is the difference in free energy between folded and unfolded states between wild type and mutant proteins. Assuming two-state folding, Φ_F can also be described by equation (2).

$$\Phi_F = \frac{\Delta\Delta G_{\ddagger\text{-}U}}{\Delta\Delta G_{U\text{-}F}} \quad (2)$$

where $\Delta\Delta G_{\ddagger\text{-}U}$ is the difference in free energy change between transition and unfolded states between wild type and mutant proteins. $\Delta\Delta G_{\ddagger\text{-}U}$ is obtained from refolding kinetics, $\Delta\Delta G_{\ddagger\text{-}F}$ is obtained from unfolding kinetics and $\Delta\Delta G_{U\text{-}F}$ is obtained from equilibrium unfolding experiments. A more detailed description of the analysis can be found elsewhere[1].

β_T-values. The value of $m_{\ddagger\text{-}F}$ can be related to the average fractional change in degree of exposure of residues between the native and transition states using a β_T-value[4,5]. This can be defined as:

$$\beta_T = 1 - \frac{m_{\ddagger\text{-}F}}{m_{U\text{-}F}} \quad (3)$$

where $m_{\ddagger\text{-}F}$ is the slope of the plot of ln k_U *versus* urea concentration and $m_{U\text{-}F}$ is the slope of the transition region of an equilibrium unfolding experiment. β_T is a measure of the fractional change in degree of exposure of residues between the unfolded state and the transition state.

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Correspondence should be addressed to S.E.J. *email: sej13@cam.ac.uk*

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