Protein Folding Transition States: Elicitation of Hammond Effects by 2,2,2-Trifluoroethanol

C.-P. Benny Yiu,[a] Mauricio G. Mateu,[b] and Alan R. Fersht*[a]

Adaptation of the techniques of classical physical-organic chemistry to the study of protein folding has led to our current detailed understanding of the transition states. Here, we have applied a series of structure – activity relationships to analyse the effects on protein folding transition states of 2,2,2-trifluoroethanol (TFE), a reagent that is usually assumed to act by stabilising secondary structure. The folding and unfolding of the highly α-helical tetramerisation domain of p53 provides a useful paradigm for analysing its effects on kinetics: The first step of its folding consists of an association reaction with little, if any, formation of secondary structure in the transition state; and the final step of the folding reaction involves just the formation of bonds at subunit interfaces, with the α-helical structure being completely formed. We have systematically measured the effects of TFE on two sets of structure – activity relationships. The first is for Φ values, which measure the degree of non-covalent bond formation at nearly every position in the transition state. The second is for relative effects of the denaturant, guanidinium chloride, on kinetics and equilibria, which measure the gross position of the transition state on the reaction co-ordinate. We find that TFE modulated the kinetics by a variety of effects other than that on secondary structure. In particular, there were Hammond effects, movement of the position of the transition state along the reaction co-ordinate, which either significantly speeded up or slowed down protein unfolding, depending on the particular mutant examined. The gross effects of TFE on protein folding kinetics are thus not a reliable guide to the structures of transition states.

KEYWORDS:

Φ-value analysis · kinetics · protein folding · proteins · structure – activity relationships

Introduction

Transition states for protein folding differ considerably from those found for simple chemical reactions. A very large number of non-covalent bonds are in the process of being formed and broken in the diffuse transition state of the protein reaction in contrast to the small number of changes in covalent bonds in classical chemistry. Nevertheless, the introduction of protein engineering has allowed protein folding transition states to be analysed by techniques analogous to those of physical-organic chemistry (for review, see ref. [1]). Protein engineering is used to change the side chains at any position in the protein and so construct the equivalent of reagents with different substitutions. Kinetic and equilibrium measurements on the folding of the mutant proteins then allow the systematic construction of structure – activity relationships. In particular, Φ-value analysis, which parallels classical Brønsted β analysis, enables the degree of non-covalent bond formation to be ascertained for virtually every residue in the transition state. A Φ value of 1 for a side chain in a folding reaction (Φ_f) implies that the non-covalent bond is fully formed in the transition state, whereas a Φ value of 0 implies that the bond is as unformed as in the denatured state.

The protein transition state is also very susceptible to classic Hammond and anti-Hammond movements on mutation and change of solvent.[2] The protein studies have the advantage that the position of the transition state on the reaction co-ordinate can be measured indirectly by the effects of denaturants on kinetics and equilibria. Rate (k) and equilibrium (K) constants for folding are sensitive to the concentration of denaturants. The relative sensitivities are termed the "Tanford β value", with $\beta = (\Delta \log k/\Delta[\text{denaturant}])/(\Delta \log K/\Delta[\text{denaturant}])$. This β value is a measure of the surface area of the protein that is buried in the transition state compared with that in the native structure.

In contrast to the residue-by-residue precision of Φ-value analysis, the gross effects of solvents are often used to probe the structures of transition states. The cosolvent 2,2,2-trifluoroethanol (TFE) is used as a diagnostic tool in the studies of peptides and proteins because it promotes the formation of α-helical and β-sheet structures.[3] Although it induces the self-association of amphipathic helices, TFE weakens tertiary and quaternary interactions.[4] There is continuing controversy on the mode of action of TFE, whether it acts by specifically binding to different states of peptides[5] or whether it acts by a general solvent effect.[6] Jasanoff and Fersht showed that earlier experiments could be interpreted by either model.[7] Kentsis and Sosnick favoured the general nonspecific model, in which TFE destabilises the denatured state.[8] TFE also affects the kinetics of protein folding and unfolding[9] and so is used as a diagnostic tool for

[a] *A. R. Fersht, C.-P. B. Yiu*
Cambridge University Chemical Laboratory
and Centre for Protein Engineering, MRC Centre
Hills Road, Cambridge CB1 2QH (UK)
Fax: (+44) 1223-330445
E-mail: arf10@cam.ac.uk

[b] *M. G. Mateu*
Universidad Autonoma de Madrid, Cantoblanco 28049, Madrid, (Spain)

transition state structure. However, Main and Jackson have questioned the use of TFE data in analysing kinetic pathways.[10] But recently, Hamada et al. have shown that there is a correlation between the effects of TFE and the number of local backbone hydrogen bonds in the native state.[11]

The tetramerisation domain of the tumour suppressor protein p53 (p53tet) has a folding pathway that is particularly suitable for analysing the effects of TFE on protein folding kinetics. The protein is a tetramer consisting of 4×30 residues in the structured region, with a "dimer of dimers" symmetry of mainly helical structures (Figure 1). It folds and assembles by a two-step mechanism [Eq. (1)]:[12]

$$4D \underset{}{\overset{k_f}{\rightleftharpoons}} 2I_2 \underset{k_u}{\rightleftharpoons} N_4 \quad (1)$$

In the first step, the denatured monomer (D), which has little α-helical structure, dimerises to form a native-like dimer (I_2). This then assembles rapidly into the tetramer (N_4). The transition states for the two steps have been analysed on the basis of Φ_f values, with every residue mutated to act as probes.[13] The transition state for the first step is highly unstructured. All Φ_f values are close to zero, indicating that no helical structure is present. The transition state for the second step is extremely structured: The Φ_f values are close to 1.0, so that the helices are fully formed. The only bonds not fully formed are those that span the dimer – dimer interface. We can thus examine the effects of TFE on the kinetics of formation at a step that has essentially no secondary structure present and one that has nearly 100% structure. In this study, we conduct a systematic analysis of the effects of TFE on the Φ values of the folding and also the Tanford β values. We find that TFE causes large Hammond effects that obscure its other effects.

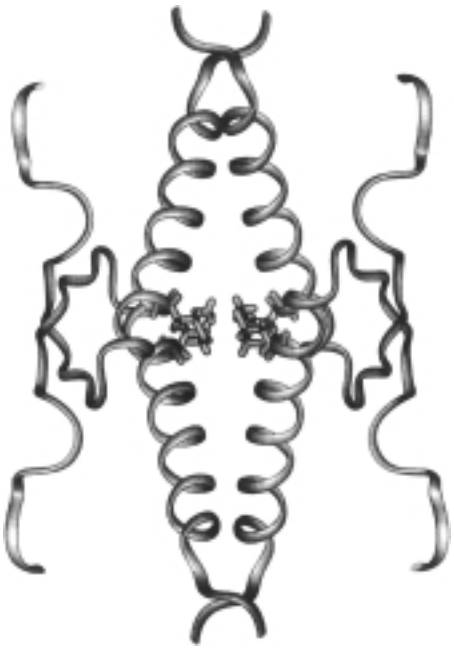

Figure 1. *Ribbon model of the three-dimensional structure of the p53 tetramerisation domain. The methionines found on the tetrameric interface at the residual position 340 of each monomer are shown as a stick representation.*

[*] *Members of the Editorial Advisory Board will be introduced to the readers with their first manuscript.*

Results

Dependence of refolding rate constants on TFE concentration ([TFE]): The refolding rate constants in 0, 2.3, 4.5, 6.8 and 9.1% TFE and their dependence on TFE concentrations are listed in Table 1. The wild-type protein (wt) refolded two times faster in 9.1% TFE, and about two-thirds of the mutants have similar values of $d(\log k_f)/d[TFE]$ to that of the wild-type protein (± 0.01). Many helical proteins fold much faster in TFE. For example, common-type acylphosphatase, muscle acylphosphatase[14] and FKBP-12[10] fold 20, 15 and 4 times faster, respectively, in solutions containing about 10% TFE, and stefan A,[15] lysozyme[16] and GCN-p1′[8] fold 45, 3.5 and 36 times faster in 11, 5.5 and 5% TFE, respectively.

In the range of [TFE] studied, a linear dependence was observed for wild-type protein and all mutants except M340A. The folding rate constants of M340A fit better to a nonlinear fit ($R = 0.99$) than a linear fit ($R = 0.93$) (Figure 2). Such a nonlinear

Table 1. *Rate constants for the first step (dimerisation) and their dependence on [TFE].*

Protein	k_f^{TFE} [10^{-5} s^{-1} M^{-1}]					Rate ratio	$\frac{\partial(\log k_f)}{\partial[TFE]}$
	[TFE] = 0%	2.3%	4.5%	6.8%	9.1%	(9.1 vs. 0% TFE)	[$\%^{-1}$]
wt (tet S)	2.89	3.31	4.64	5.38	6.18	2.1	0.038
β-strand mutants							
E326A	2.52	3.31	4.10	5.10	5.93	2.3	0.041
F328A	0.369	0.555	0.858	1.26	1.83	4.9	0.077
T329A	1.65	2.09	2.88	3.44	4.13	2.5	0.045
Q331A	2.75	3.58	4.12	4.87	5.55	2.0	0.033
R333A	1.86	2.31	3.04	3.44	4.37	2.4	0.040
α-helix mutants							
R335A	2.47	2.95	3.80	4.56	6.06	2.5	0.041
F338A	1.97	2.67	2.97	3.45	4.44	2.3	0.036
E339A	3.98	4.76	5.73	6.53	7.79	2.0	0.032
M340A	2.74	3.52	4.18	4.25	4.61	1.7	–[a]
R342A	1.85	2.06	2.52	2.41	2.99	1.6	0.021
E343A	6.65	6.56	12.3	–[b]	–[b]	–[b]	0.058
N345A	6.91	7.79	15.6	18.6	–[b]	–[b]	0.070
E346A	2.48	3.20	4.23	5.58	5.84	2.3	0.043
A347G	2.77	3.12	3.30	3.74	3.71	1.3	0.015
E349A	4.93	4.65	6.46	5.75	8.85	1.8	0.026
L350A	3.64	4.60	5.35	5.01	7.54	2.1	0.034
D352A	4.34	5.07	6.08	6.98	7.78	1.8	0.028

[a] Nonlinear behaviour. [b] Refolding rate too high to be accurately determined. wt = wild-type protein.

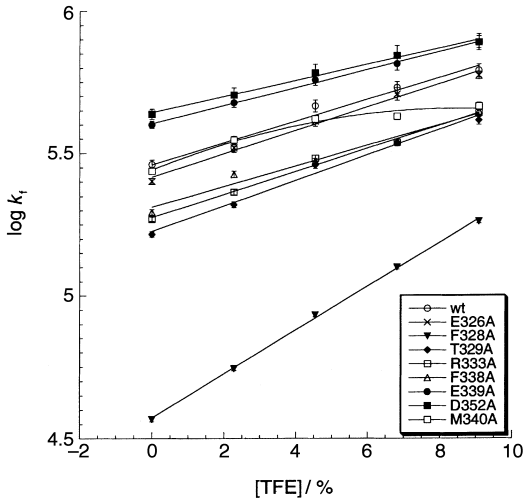

Figure 2. *Dependence of the logarithm of the refolding rate constants of wild-type p53tet and some mutants on TFE concentration (k_f in s^{-1}).*

behaviour is also observed for other proteins over a wide range of [TFE].[11]

The Tanford β value for folding: The Tanford β value for the first, dimerisation step of folding [the $4D \rightarrow 2I_2$ step in Eq. (1); see Eq. (6)] in water was found to be 0.20 (Table 2). This means that the transition state is fairly open and buries approximately 20% of the hydrophobic surface area relative to that in the native state. The value decreased to 0.11 in the presence of 6.8% TFE, indicating that the transition state has even less buried surface area and has become more similar to the denatured state.

Φ_f values for the first step: The Φ_f values for the dimerisation step in the folding process [Eq. (1)] in 0, 2.3, 4.5, 6.8 and 9.1% TFE were calculated for 17 point mutants (Table 3). Some mutations are helix-stabilising, for example R335A (N cap) and the hydrophobic mutations (hydrophobic side chains are less helix-breaking in TFE),[17] and A347G is helix-destabilising.[18] But for all the mutants (except for R342A), the Φ_f values were essentially zero at all concentrations of TFE studied, indicating that the transition state is as disordered as the denatured state. Main and Jackson also observed unchanged Φ_f values for the folding of urea-denatured FKBP-12 in 9.6% TFE, where also secondary structures are largely unformed in the transition state of this protein.[10]

Table 3. *Φ_f Values for the first step of folding in TFE.*

Protein	Interactions of the deleted side chain	Φ_f [TFE] = 0%	Φ_f^{TFE} 2.3%	Φ_f^{TFE} 4.5%	Φ_f^{TFE} 6.8%	Φ_f^{TFE} 9.1%
β-strand mutants						
E326A	solvent-exposed	0.12	0.00	0.11	0.04	0.03
F328A	dimer core	0.21	0.18	0.17	0.15	0.12
T329A	solvent-exposed	0.15	0.12	0.13	0.11	0.10
Q331A	intermonomer H bond	0.04	−0.04	0.07	0.05	0.06
R333A	intermonomer H bond	0.12	0.09	0.12	0.12	0.09
α-helix mutants						
R335A	solvent-exposed	0.06	0.04	0.07	0.05	0.00
F338A	dimer core	0.05	0.03	0.06	0.06	0.04
E339A	solvent-exposed	−0.10	−0.12	−0.06	−0.07	−0.08
M340A	tetrameric interface	0.01	0.00	0.02	0.03	0.04
R342A	dimer core, solvent-exposed	0.35	0.35	0.47	0.60	0.54
E343A	interdimer H bond	−1.06	−0.90	−1.25	–[a]	–[a]
N345A	intermonomer H bond	−0.24	−0.25	−0.34	−0.23	–[a]
E346A	solvent-exposed	0.13	0.02	0.08	−0.02	0.04
A347G	tetrameric interface	0.02	0.01	0.09	0.09	0.12
E349A	intermonomer H bond	−0.10	−0.07	−0.06	−0.01	−0.07
L350A	tetrameric interface	−0.12	−0.20	−0.07	0.04	−0.12
D352A	intermonomer salt bridge	−0.16	−0.19	−0.11	−0.11	−0.10

[a] Refolding rate too high to be accurately determined.

Table 2. *The m values and Tanford β values of wild-type p53tet.*

[TFE] [%]	m_{D-N} [kcal mol^{-1} M^{-1}][a]	$m_{\neq-D}$ [kcal mol^{-1} M^{-1}][b]	β_{Tf}[c]	$m_{\neq-N}$ [kcal mol^{-1} M^{-1}][d]	β_{Tu}[e]
0	5.10 ± 0.20	−1.03 ± 0.01	0.20	0.44 ± 0.05	0.09
4.5	5.00 ± 0.12	−0.85 ± 0.04	0.17	0.40 ± 0.02	0.08
6.8	5.28 ± 0.31	−0.58 ± 0.04	0.11	0.26 ± 0.03	0.04

[a] $m_{D-N} = \partial(\Delta G_{D-N})/\partial$[GdmCl]. [b] See Eq. (4). [c] See Eq. (6). [d] See Eq. (5). [e] See Eq. (7).

Refolding amplitudes: The refolding amplitudes monitored by the CD signal at 222 nm, which is a measure of the extent of helix formation, were decreased only slightly in TFE for p53tet and some mutants (Figure 3). This would suggest little, if any, helix formation in the denatured state as well as in the transition state.

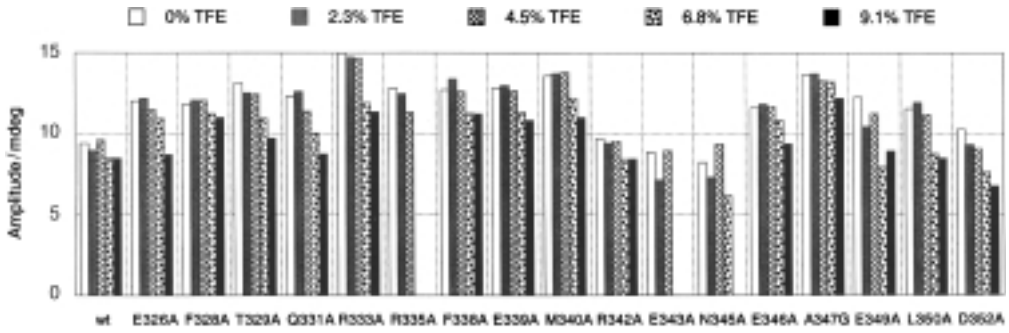

Figure 3. The amplitudes in the refolding of wild-type p53tet protein and 17 mutants at various concentrations of 2,2,2-trifluoroethanol.

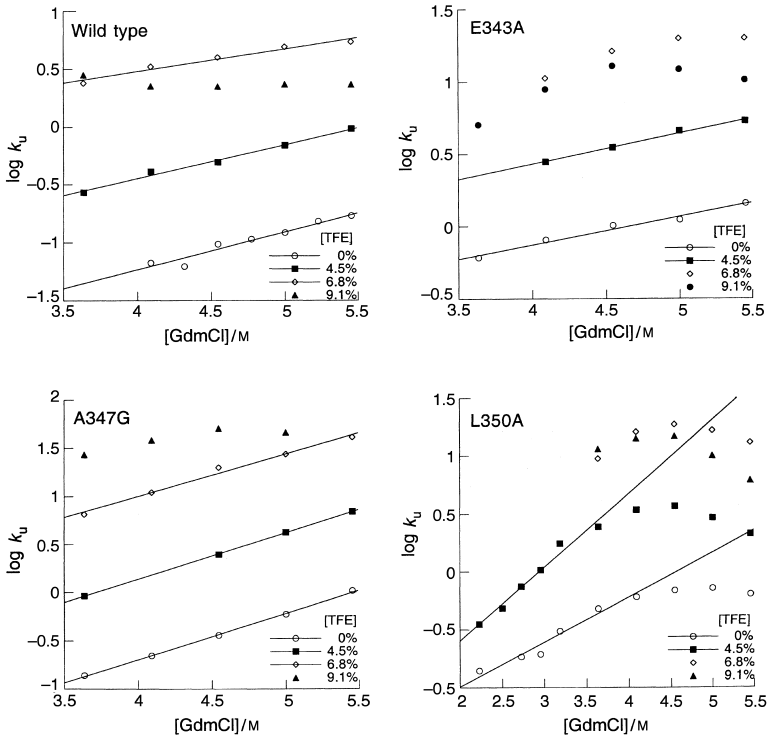

Figure 4. Dependence of the logarithm of the unfolding rate constants on [GdmCl] at various concentrations of TFE for p53tet and some mutants (k_U in s^{-1}). The linear fits were used for the determination of the unfolding rate constants in the absence of GdmCl and for determining the m *values.*

Dependence of unfolding rate constants on guanidinium hydrogen chloride concentration ([GdmCl]): p53tet unfolds in a single, first-order step to denatured monomers. At low concentrations of TFE (0, 4.5 and, for some mutants, 6.8%), wild-type protein and most mutants have a linear dependence of log k_u on [GdmCl] over the whole concentration range studied (Figure 4). But the dependence for F341L and L350A (two of the mutants on the tetrameric interface) was nonlinear at high [GdmCl]. At 9.1% TFE, wild-type p53tet and all but one mutant lost the linear dependence of log k_u on [GdmCl]. This suggests a compact transition state with a reduced sensitivity to [GdmCl].

[TFE] dependence of unfolding rate constants: At high [GdmCl], the unfolding of wild-type protein and all mutants was speeded up by TFE (Figure 4). Table 4 shows the TFE dependence of the unfolding rate constants in the absence of GdmCl. The unfolding rate constants of the wild type increased by ca. 8-fold and ca. 176-fold in the presence of 4.5 and 6.8% TFE, respectively. Conversely, proteins with a mutation in the tetrameric interface unfolded more slowly in 4.5% TFE (Table 4).

The Tanford β value for unfolding: The *m* values of unfolding as a function of [TFE] are shown in Table 4. Most of the proteins show a decrease in *m* value with increasing [TFE], except the mutants on the tetrameric interface.

The Tanford β value for unfolding [the $N_4 \rightarrow 2I_2$ step in Eq. (1); see Eq. (7)] decreased from 0.09 in water to 0.04 in 6.8% TFE (Table 2), indicating that the transition state of unfolding becomes more compact with more of the surface area buried in the presence of TFE.

Φ_f values for the second step: The Φ_f values for the second, tetramerisation step of folding [the $2I_2 \rightarrow N_4$ step in Eq. (1); see Eqs. (8) and (9)] were determined in 0, 4.5 and 6.8% TFE for a number of mutants (Table 4). In water, the values were found to be close to 1 for mutations at most residual positions and close to 0.5 for mutations at the tetramer interface, as found.[13] In 4.5% TFE, most mutants have the same Φ_f value of 1. Moreover, the tetrameric interface mutants F341L and L350A have Φ_f values increasing from 0.5 in water to approaching 1 with increasing [TFE] (calculated using the corresponding $\Delta\Delta G_{D-N}$ values in TFE). The Φ_f values of M340A increased similarly. Therefore, the transition state becomes more native-like in the presence of TFE.

Discussion

There are two important observations from this study. The first is that TFE can have significant effects on protein folding kinetics by affecting mainly tertiary interactions. The second is that TFE can cause either large increases or large decreases in the rate constant for unfolding, depending on the particular mutant

Table 4. *Rate constants and* m *values for unfolding and* Φ_f *values for the second step of folding in TFE.*

Protein	Interactions of the deleted side chain	k_u^{TFE} [s^{-1}] [TFE] = 0%	4.5%	6.8%	$m_{\ddagger-N}$ [kcal mol^{-1} M^{-1}][a] 0%	4.5%	6.8%	m_{D-N} [kcal mol^{-1} M^{-1}][b]	Φ_f^{TFE} [TFE] = 0%	4.5%	6.8%
wt		0.0029	0.024	0.22	0.4	0.4	0.3	5.1			
β-strand mutants											
E326A	solvent-exposed	0.0026	0.023	n.d.[c]	0.5	0.4	n.d.	5.0	1.0	1.0	n.d.
α-helix mutants											
R335A	solvent-exposed	0.0041	0.042	n.d.	0.5	0.4	0.3	5.8	1.0	0.9	n.d.
F338A	dimer core	0.0051	0.039	n.d.	0.6	0.5	n.d.	6.0	1.0	1.0	n.d.
M340A	tetrameric interface	11.22	0.065	0.067	0.3	0.5	0.3	6.5	**0.4**	**0.9**	**1.1**
F341L	dimer core	0.18	0.10	n.d.	0.2	0.7	n.d.	5.4	**0.4**	**0.8**	n.d.
R342A	dimer core, solvent-exposed	0.0025	0.032	n.d.	0.5	0.4	n.d.	5.1	1.1	0.9	n.d.
E343A	interdimer H bond	0.12	0.38	n.d.	0.3	0.3	n.d.	5.0	−1.5	−0.8	n.d.
A347G	tetrameric interface	0.0024	0.016	0.18	0.7	0.7	0.6	6.1	1.0	1.2	1.0
L350A	tetrameric interface	0.018	0.0079	n.d.	0.5	0.1	n.d.	4.9	**0.5**	**1.2**	n.d.
D352A	intermonomer salt bridge	0.11	0.097	n.d.	0.1	0.1	n.d.	5.2	0.7	0.7	n.d.

[a] See Eq. (5); determined at various TFE concentrations. [b] See footnote [a] in Table 2; determined in water.[13] [c] n.d. = not determined, and the dependence of $\log k_u$ on [GdmCl] being nonlinear.

examined. As discussed below, the paradox of these mixed effects of TFE results from large Hammond effects caused by TFE that can dominate its overall effects on kinetics.

TFE affects tertiary interactions: In the absence of TFE, the transition state of the first, dimerisation step of folding [Eq. (1)] involves essentially no formation of secondary structure.[13] The Φ_f values of p53tet remained approximately 0 for essentially all the mutated positions in the presence of TFE. The Φ_f values report back either on the degree of secondary structure formation (for those in the helices or strand) or the direct side-chain – side-chain interactions for those at the subunit interfaces (listed in ref. [13]). Therefore, the first transition state lacks secondary structure in TFE.

The transition state for the tetramerisation step has fully formed secondary structure, and only a few hydrophobic interactions on the subunit interface are partly formed. Yet, the unfolding step was speeded up greatly by TFE for the wild-type protein and most mutants. In 9.1% TFE, for example, the refolding rate constant of wild type was doubled. But, for proteins with mutations at the subunit interface, unfolding was slowed down. The Φ_f values relating to secondary structure [Eq. (1)] remained approximately 1 in the absence and presence of TFE, so TFE did not significantly affect secondary structure.

One possibility for the mechanism of rate enhancement in the initial folding step is that proposed by Kentsis and Sosnick;[8] the kosmotropic effect of TFE destabilised the denatured state because of its exposed hydrophobic side chains. The Tanford β value for folding shows that the first, dimeric transition state is slightly compact, with 20% of the solvent-accessible surface area buried relative to the native state from the denatured state. In TFE, the transition state moves significantly toward the denatured state. According to the Hammond postulate, the denatured state must be destabilised with respect to the transition state, supporting the Kentsis and Sosnick view. On the other hand, it is difficult to interpret the faster unfolding of wild-type protein and most mutants in TFE by the general solvent model since the transition state is less compact than the native structure. TFE should destabilise the transition state more than the native state if it causes more exposed states to become less stable and so slow down folding. Perhaps there is specific binding of TFE to the transition state.

TFE elicits Hammond effects: In the presence of TFE, the structure of the first transition state moves along the reaction co-ordinate to be closer to that of the denatured state, as indicated by the Tanford β value for folding decreasing from 0.2 in water to 0.11 in 6.8% TFE (Table 2). The Tanford β value for unfolding (see **Results** and Table 2) also shows a Hammond effect, in which the transition state of the wild-type protein becomes significantly more compact relative to the denatured state from the native state in TFE. Consistent with this, the Φ_f values of p53tet that were fractional in water, especially those in the tetrameric interface, all tend to 1 in the presence of TFE. This indicates that the hydrophobic interactions become more fully formed in the transition state of unfolding, as expected for the proposed Hammond behaviour.

Hammond effects on rate constants: The most striking conclusion from this study is that the Hammond effect can cause dramatic changes in the effects of TFE. In water, the rate-limiting step in unfolding involves the breaking of a few hydrophobic interactions, and so mutation of those hydrophobic residues resulted in a large increase in the unfolding rate. In TFE, since the Hammond effect reduces the extent of bond breaking at the subunit interface in the transition state of unfolding, the mutations no longer affect unfolding kinetics, and the rates tend to those of the wild type. Therefore, the movement of the transition state closer to the native structure in the presence of TFE actually reverses the large increase in rate in the unfolding of wild-type protein so that some mutants unfold more slowly in TFE. TFE thus has an indirect effect on kinetics via the Hammond effect. This leads to the effects of TFE on unfolding being idiosyncratic, depending on the precise mutant observed.

Conclusion

Transition states for protein folding are especially susceptible to changes in the surrounding medium. Probes, such as TFE, that perturb the whole structure and report back on gross properties are particularly unreliable as a guide to mechanism. They are too crude and can distort the transition state and cause artefacts in kinetics. Hammond movements, for example, can lead to rate changes that eclipse the other effects of TFE on kinetics. Φ values provide currently the most precise experimental data on the structure of transition states. But, as emphasised frequently, mutations should be made that just perturb the energetics slightly, but measurably, since all changes in structure can lead to movement of the transition state.[1] It is very satisfying that methods evolved in classical physical-organic chemistry for the study initially of acid/base catalysis and then covalent-bond changes in general can be so readily adapted to complex protein reactions involving a myriad of non-covalent-bond changes. Indeed, structure – activity and structure – reactivity relationships have been the major experimental tools responsible for our current understanding of protein folding transition states at near-atomic resolution.

Experimental Section

Protein expression and purification: The tetS version (residues 311 – 367) of p53tet[19] was expressed in *E. coli* C41(DE3) cells, a variant of the bacterial strain BL21(DE3). 1 mM isopropylthio-β-D-galactoside was used for induction when $A_{600} = 0.7$. The cells were allowed to grow overnight at 25 °C. Cells were harvested and the proteins were purified as previously described.[19] One litre of culture can yield > 20 mg of protein.

Chemical denaturation: p53tet (40 μM monomer in 25 mM sodium phosphate buffer, pH 7) was denatured at various concentrations of GdmCl and the equilibrium monitored by measuring the CD signal at 222 nm (pathlength 1 mm). The data were converted to the fraction denatured (f_D) as a function of denaturant concentration with the program CDMan (D. Veprintsev, MRC, Cambridge), and ΔG_{D-N}, the free energy change of unfolding, $[D]_{50\%}$, the midpoint of transition, and m_{D-N}, a constant of proportionality, were calculated by using Equations (2) and (3),[13] where P_t is the monomer concentration and [D] is the concentration of GdmCl.

$$\Delta G_{D-N} = -RT\ln[4P_t^3 f_D^4/(1-f_D)] \quad (2)$$

$$\Delta G_{D-N} = m_{D-N}([D]_{50\%} - [D]) - RT\ln(P_t^3/2) \quad (3)$$

Thermal denaturation: The melting of p53tet was followed, as previously described,[19] in the presence of 0, 4.5, 6.8 and 9.1 % TFE (v/v). For the calculation of $\Delta\Delta G_{D-N}$, the change in the free energy change of unfolding caused by mutation, a constant value of ΔC_p of 0.425 kcal mol^{-1} K^{-1} was used. Although ΔC_p is expected to decrease in the presence of a monohydric alcohol,[20] small changes in ΔC_p have only a small effect on $\Delta\Delta G_{D-N}$.[21] The calculation gives essentially the same $\Delta\Delta G_{D-N}$ using a range of ΔC_p values from 0 to 0.43 kcal mol^{-1} K^{-1}.

Stopped-flow CD measurements: Protein folding measurements were made using an Applied Photophysics SX18MV stopped-flow unit with a CD1 accessory. The wavelength was 222 nm and the bandpass 9 nm. A second monochromator with a bandpass of 23 nm was used to remove stray light. Between 3 and 10 traces were averaged for each kinetic measurement.

Unfolding experiments were done by mixing one volume of native protein (330 μM, in 25 mM sodium phosphate buffer, pH 7.0) with ten volumes of GdmCl solution (in 25 mM sodium phosphate, pH 7.0) in the presence of various concentrations of TFE. A time constant from 5 to 50 ms was used, depending on the rate of reaction. Refolding was achieved by mixing one volume of acid-denatured protein (198 μM, in 62 mM sodium phosphate, pH 2.2) with ten volumes of various concentrations of TFE (in 20 mM sodium phosphate), with the resulting buffer being 25 mM sodium phosphate (pH 7.0). A time constant of 3.7 ms was used.

Determination of rate constants and amplitudes of reactions: The folding reaction was fitted to a double-exponential equation. The amplitude was found by extrapolating the signals back to zero time, allowing for the dead time of the instrument, 7 ms, which was determined by the colorimetric reaction of 100 mM dichloroindophenol with various concentrations of ascorbate.[22] The unfolding reaction was fitted to a single-exponential equation. The logarithm of the unfolding rate constant ($\log k_u$) of p53tet and its mutants sometimes demonstrated a nonlinear dependence on GdmCl concentrations. In these cases, the unfolding rate constant in water or in the presence of TFE was either determined by extrapolating the linear portion (if present) of the curve to zero denaturant or not determined. The linear fits were used for the determination of the *m* values as defined below.

Tanford β values: $m_{\ddagger-D}$ and $m_{\ddagger-N}$ are constants of proportionality obtained by applying Equations (4) and (5) to Equation (1).

$$m_{\ddagger-D} = \partial(RT/2.303\log k_f)/\partial[\text{GdmCl}] \quad (4)$$

$$m_{\ddagger-N} = \partial(RT/2.303\log k_u)/\partial[\text{GdmCl}] \quad (5)$$

The β_T value for folding, β_{Tf}, is a measure of the average degree of exposure in the folding, dimeric transition state [for the $4D \rightarrow 2I_2$ step in Eq. (1)] relative to that of the native tetramer (N_4) from the denatured state (D) and is defined by Equation (6), in which, for the reversible chemical denaturation of p53tet, m_{N-D} equals $-m_{D-N}$.

$$\beta_{Tf} = m_{\ddagger-D}/m_{N-D} \quad (6)$$

β_{Tu} is the β_T value for unfolding, which is a measure of the average degree of exposure in the unfolding, tetrameric transition state [for the $N_4 \rightarrow 2I_2$ step in Eq. (1)] relative to that of the denatured state (D) from the native state (N_4) and is defined by Equation (7).

$$\beta_{Tu} = m_{\ddagger-N}/m_{D-N} \quad (7)$$

Φ_f-Value analysis: The Φ_f values for the first step of folding were obtained for the folding transition state ($4D \rightarrow 2I_2$) with Equations (8) and (9).

$$\Delta\Delta G_{D-\ddagger} = -RT\ln(k_{f(mut)}^{TFE}/k_{f(wt)}^{TFE}) \quad (8)$$

$$\Phi_f = 2(\Delta\Delta G_{D-\ddagger})/\Delta\Delta G_{D-N} \quad (9)$$

The Φ_f values for the second step of folding were obtained for the unfolding transition state ($N_4 \rightarrow 2I_2$) with Equations (10) and (11).

$$\Delta\Delta G_{\ddagger-N} = -RT\ln(k_{u(wt)}^{TFE}/k_{u(mut)}^{TFE}) \quad (10)$$

$$\Phi_f = 1 - (\Delta\Delta G_{\ddagger-N}/\Delta\Delta G_{D-N}) \quad (11)$$

$\Delta\Delta G_{D-\ddagger}$ is the free energy difference between the folding transition states of wild-type and mutant proteins (per dimer), relative to the

denatured states; $\Delta\Delta G_{\ddagger-N}$ is the free energy difference between the unfolding transition states of wild-type and mutant proteins (per tetramer), relative to the native states; $k_{f(wt)}^{TFE}$ and $k_{u(wt)}^{TFE}$ are, respectively, the refolding and unfolding rate constants in water or TFE for the wild-type protein, and $k_{f(mut)}^{TFE}$ and $k_{u(mut)}^{TFE}$ are the corresponding rate constants for the mutants. The $\Delta\Delta G_{D-\ddagger}$ and $\Delta\Delta G_{\ddagger-N}$ values in water or TFE are small (data not shown), and the $\Delta\Delta G_{D-N}$ values in water[13] were used for the calculation, unless otherwise stated.

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