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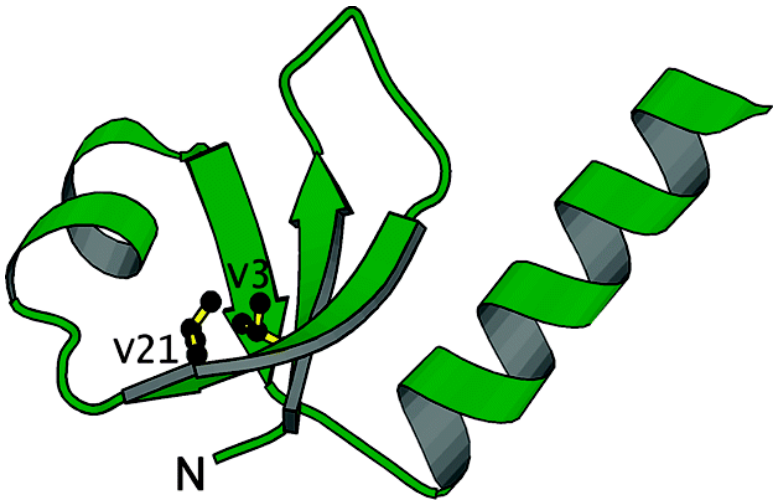

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Φ-Values beyond the Ribosomally Encoded Amino Acids: Kinetic and Thermodynamic Consequences of Incorporating Trifluoromethyl Amino Acids in a Globular Protein

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Much of our present knowledge of protein folding mechanisms has been derived from studies of the effects of point mutations upon folding kinetics and thermodynamics. Such experiments are usually interpreted using Φ-values, the ratio of the effect of the mutation on the stability of the transition state, $\Delta\Delta G^{\ddagger}$, to the effect on the stability of the protein $\Delta\Delta G°$.[1] Ideally, as conservative mutations as possible are chosen which cause a minimal structural perturbation. In some cases, it is obviously impossible to remove only one group. A Val to Ala substitution, for example, removes both γ-methyl groups. Such mutations might also lead to unanticipated effects because the core may repack to fill the "hole" created by mutation. Here, we report the use of trifluoromethyl-containing amino acids to extend Φ-value analysis. Unnatural amino acids offer the possibility of making conservative changes that could not be contemplated using only the 20 coded amino acids. Recent advances in peptide synthesis and expressed protein ligation have allowed the production of proteins containing single unnatural amino acids in sufficient quantities for biophysical experiments.[2] Amino acids containing trifluoromethyl groups are an attractive choice because they are reasonably isosteric to their nonfluorinated counterparts and are expected to cause a significant favorable change in $\Delta G°$.[3] This is an important point because conservative natural mutations can often lead to small changes in $\Delta G°$ of folding which prevent the accurate measurement of Φ-values. The vast majority of natural mutants decrease $\Delta G°$; thus substitutions which increase $\Delta G°$ also have the benefit of extending the range of Brønsted plots for distinguishing between parallel pathways and a single transition state.[4] In addition, a trifluoromethyl group is more hydrophobic than a methyl group, and this offers a novel way to modulate intraprotein interactions.[5] There is a long history of the use of fluoroamino acids as NMR and fluorescence probes, primarily involving aromatic amino acids.[6] Several studies of protein folding have made use of fluoroaromatic amino acids as site-specific spectroscopic probes, but fluoroamino acids have yet to be used in Φ-value analysis.[7] Trifluoromethyl-containing amino acids have recently been incorporated into coiled-coils by both synthetic and recombinant approaches, and an increase in global stability has been demonstrated.[3c,d,8] Somewhat surprisingly, there are no reports of the thermodynamic and kinetic consequences of the incorporation of trifluoromethyl-containing amino acids into globular proteins.

Two variants of NTL9 which contain single 4,4,4-trifluorovaline for valine substitutions were prepared by solid-phase peptide synthesis. NTL9 is a 56-residue globular α-β protein which has been shown to fold in a two-state fashion under a wide range of conditions.[9a] V3 and V21 were chosen as sites for substitution (Figure 1). These proteins are designated as tfV3 and tfV21. Both positions are largely buried in the wild-type protein. The trifluoromethyl valine residues have the *S* configuration at C_α but are a mixture of *S* and *R* at C_β (as in other published reports). Both variants are folded as judged by near and far UV CD with spectra very similar to that of the wild type. The NMR spectrum of NTL9 contains several ring current shifted methyl resonances as well as several C_α proton resonances downfield of water. These peaks are present in the spectra of the variants. The C_α proton chemical shifts of each variant are very close to the wild-type values (Supporting Information). For the V21 variant, all C_α proton shifts with the exception of V21 are within 0.1 ppm of the wild type. For the V3 variant, only three C_α proton resonances, in addition to the V3 C_α proton, differ from the wild type by more than ±0.1 ppm. Those three are within 0.3 ppm of the wild-type values. These protons are all close to one of the V3 methyls in the native state. In contrast, the C_α proton shifts for all three proteins are significantly different from random coil values. C_α proton chemical shifts are very sensitive to secondary structure, and the good agreement between the variants and wild type provides additional evidence that the substitutions have not significantly perturbed the native fold.

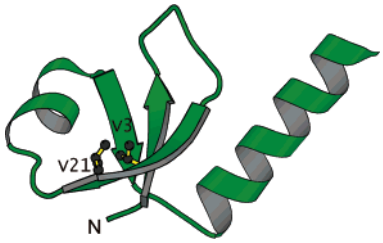

Figure 1. Ribbon diagram of NTL9 (PDB code: 1DIV) showing the position of V3 and V21. The N-terminus is labeled. In the wild type, the side chain of V3 is completely buried, while the V21 side chain has only 3.9 Å^2 of exposed surface area (calculated using a 1.4 Å probe). The figure was prepared with the Molscript program.[12]

The unfolding of both variants is reversible, as judged by comparing the signal before and after melting experiments. Both variants are noticeably more stable than the wild type. $\Delta G°$ of folding determined by GuHCl denaturation is 4.17 kcal mol^{-1} for the wild type, 4.96 kcal mol^{-1} for tfV3, and 5.61 kcal mol^{-1} for tfV21. The increase in stability upon insertion of a single trifluoromethyl group is impressive and is significantly larger than has been observed in coiled-coils on a per trifluoromethyl group basis.[3c,d,8a,b] The *m*-values for the variants and the wild type are virtually identical, suggesting that a similar amount of buried surface area is exposed upon unfolding. The midpoint of the thermal unfolding of each variant is also higher than that of the wild type. Thermodynamic parameters are listed in Table 1, and the GuHCl unfolding curves are shown in Figure 2.

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Table 1. Thermodynamic Parameters for NTL9, tfV3, and tfV21 in pH 5.4, 100 mM NaCl, and 20 mM Sodium Acetate Buffer; GuHCl Denaturations Were Conducted at 25 °C[a]

protein	$\Delta G_U°(H_2O)$ (kcal mol^{-1})	m (kcal mol^{-1} M^{-1})	C_M (M)	T_m (°C)
NTL9	4.17 ± 0.07	1.35 ± 0.02	3.08 ± 0.01	79.8
tfV3	4.96 ± 0.05	1.31 ± 0.01	3.78 ± 0.01	81.9
tfV21	5.61 ± 0.11	1.35 ± 0.03	4.17 ± 0.01	83.7

[a] Uncertainties represent the standard error to the fit.

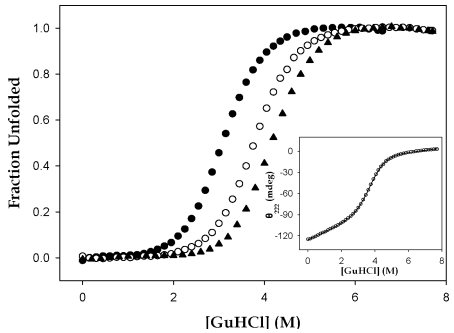

Figure 2. CD monitored equilibrium unfolding of NTL9 (●), tfV3 (○), and tfV21 (▲). Experiments were conducted at pH 5.4, 25 °C, 100 mM NaCl, and 20 mM sodium acetate. The inset shows the raw data for tfV3. Raw data for tfV21 and WT are shown in the Supporting Information.

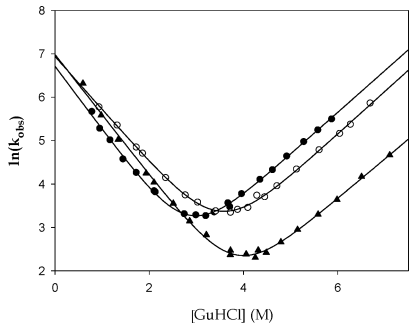

Figure 3. Plots of the natural log of the observed rate versus denaturant concentration, for NTL9 (●), tfV3 (○), and tfV21 (▲). Experiments were conducted at pH 5.4, 25 °C, 100 mM NaCl, and 20 mM sodium acetate.

The kinetics of folding and unfolding were measured via stopped-flow experiments. So-called cheveron plots, plots of ln $k_{observed}$ versus denaturant concentration, are shown in Figure 3. The variants and wild type show the classic V-shaped curve expected for two-state folding, and there is no hint of any roll over.[1c] Both $\Delta G°$ and the m-value calculated from the kinetic parameters are in excellent agreement with the equilibrium experiments (Table 2). The ratio of m_f to the equilibrium m-value, denoted θ_m, is related to the relative burial of surface area in the transition for folding and is traditionally used as a measure of the position of the transition state.[10] The θ_m values for NTL9 and the variants are similar, ranging from 0.55 to 0.60. Both variants unfold slower than the wild type, indicating that the trifluoromethyl substitution stabilizes the native state relative to the transition state. The decrease in unfolding rate suggests that introduction of the trifluoromethyl group does not cause significant strain in the native state.[11] Both variants fold faster than the wild type, although the effect on k_f is proportionally smaller than the effects on k_u. The faster folding rates suggest stabilization

Table 2. Kinetic Parameters for NTL9, tfV3, and tfV21 in pH 5.4, 100 mM NaCl, and 20 mM Sodium Acetate Buffer, 25 °C

protein	$k_f(H_2O)$ (s^{-1})	$k_u(H_2O)$ (s^{-1})	m_f	m_u	θ_m[a]
NTL9	824 ± 42	0.89 ± 0.08	−0.86 ± 0.02	0.57 ± 0.02	0.60 ± 0.02
tfV3	1031 ± 52	0.55 ± 0.08	−0.77 ± 0.02	0.57 ± 0.02	0.55 ± 0.02
tfV21	1082 ± 65	0.17 ± 0.03	−0.82 ± 0.02	0.54 ± 0.02	0.60 ± 0.02

[a] θ_m is defined as $m_f/(m_f - m_u)$.

of the transition state by hydrophobic interactions. The Φ-values are small but not zero, 0.16 for tfV3 and 0.11 for tfV21. The Φ-values for Ala substitutions are somewhat larger but are still small, 0.36 and 0.21 for V3 and V21, respectively.[9b] The results demonstrate the applicability of fluoroamino acids to Φ-value analysis and show that significant effects upon $\Delta G°$ can be expected for globular proteins.

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Supporting Information Available: Plots of C_α proton chemical shifts, raw data for the GuHCl denaturation, mass spectra, and 1D-^{19}F NMR spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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