Experimental Investigation of the Frequency and Substitution Dependence of Negative ϕ-Values in Two-State Proteins†

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ABSTRACT: Negative ϕ-values, which arise, for example, when a mutation stabilizes the folding transition state while destabilizing the native state, have been the focus of significant theoretical interest. Here we survey the experimental folding kinetics literature to ascertain the frequency with which negative ϕ-values occur in two-state proteins and describe the detailed experimental characterization of a negative ϕ-value previously reported to be among the most statistically significant. We find that, while almost 9% of more than 500 reported ϕ-values (from a set of 16, well-characterized two-state proteins) fall below zero, many of these do not represent statistically significant observations. For example, only 6% of the ϕ-values for which estimates of precision are available fall even one reported "error bar" below zero, and only 4% are simultaneously negative, significant at this level and associated with free energy changes at or above 2.5 kJ/mol (below which ϕ-value analysis is widely considered unreliable). Moreover, given the asymmetric distribution of ϕ-values around zero and given that reported error bars may significantly underestimate true confidence intervals, the actual number of negative ϕ-values may be much smaller still. We have also performed detailed characterization of one of the most statistically significant negative ϕ-values reported in the literature to date, the V55F mutant of FynSH3. We find that substitution of the wild-type valine to other hydrophobic residues often increases folding rates without significantly altering folding free energy. This in turn leads to poorly defined ϕ-values, some of which are formally negative but only one or two of which fall statistically significantly below zero. In contrast, substitution to polar residues significantly destabilizes both the transition and native states, generally producing small but statistically significant positive ϕ-values of ~0.1. Thus, unlike other previously characterized ϕ-values, the negative ϕ-value associated with position 55 of the FynSH3 domain appears to be strongly dependent on the substitution employed to measure it, suggesting that subtlety will be required in order to develop a theoretical model of such behavior.

Protein engineering provides a high-resolution window into the folding transition state (*1*). The method, first applied in the late 1980s (*2*−*4*), was systematically refined by Fersht and co-workers (e.g., refs *5*−*8*), who termed it ϕ-value analysis. ϕ-value analysis measures the extent to which modification of a side chain alters the height of the folding barrier relative to the extent to which it alters the stability of the native state ($\phi = \Delta\Delta G^{\ddagger}/\Delta\Delta G_{eq}$). In the 15 years since ϕ-value analysis was first reported, the method has been applied with varying levels of completeness to more than two dozen proteins and has effectively become the standard benchmark by which theoretical and experimental folding studies are compared.

ϕ-values typically fall within a "canonical" range of 0−1. A ϕ-value of 0 is generally thought to imply a side chain interaction that is as poorly formed in the transition state as it is in the unfolded state. Conversely, a ϕ-value of 1 is generally taken to represent a side chain interaction that becomes nativelike in the folding transition state, and intermediate ϕ-values are generally taken to represent residues that adopt varying degrees of nativelike structure in the transition state (e.g., refs *5*−*8*). Extensive mutational analysis performed by Davidson (*9*, *10*) and Fersht (*11*) suggests that ϕ-values falling in this canonical range are largely context independent. That is, while they depend strongly on the position of the residue in question, they generally do not depend on the nature of the substitution employed to define them. This context independence holds even for highly deleterious mutations. For example, mutation of FynSH3's wild-type serine-41 to arginine, alanine, histidine, or valine produces ϕ-values that lie within experimental error of one another (*9*).

Not all ϕ-values, however, fall within the range of 0−1, and the origins of these noncanonical values have received considerable attention in both the theoretical and experimental literature (e.g., refs *9* and *12*−*21*). Perhaps the most widely held explanation for noncanonical ϕ-values is that that they arise due to the formation of non-native interactions in the folding transition state (see, e.g., ref *14*). We should note, however, that Sanchez and Kiefhaber have recently

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argued that noncanonical ϕ-values may represent experimental error arising from mutations that alter $\Delta\Delta G_{eq}$ insufficiently to ensure that ϕ is measured accurately; because ϕ is inversely related to the change in folding free energy induced by the probing mutation, it is particularly difficult to measure precisely when $\Delta\Delta G_{eq}$ is small (*15*).

Here we explore negative ϕ-values in detail. We report on the frequency and statistical significance of negative ϕ-values elucidated from a literature survey of a large set of well-characterized, single domain proteins (Table 1). We also report the results of an extensive experimental characterization of one of the most statistically significant negative ϕ-values reported to date, that assigned to the valine to phenylalanine substitution at residue 55 (V55F) of the FynSH3 domain (*22*). In an effort to better constrain the nature of this negative ϕ-value, we have characterized the kinetic consequences of a range of substitutions at this position.

MATERIALS AND METHODS

Literature Survey. A set of 512 ϕ-values was compiled from the literature describing 16 single-domain, two-state folding proteins (*9*, *19*, *20*, *22*−*37*). These 16 proteins represent the most exhaustively characterized proteins that fit to a two-state folding model. Error bars were adopted as reported; while few of these error bars are described in sufficient detail in the literature to identify whether they represent standard errors or specific confidence intervals, in the cases in which they are defined they are generally reported to represent estimated standard errors.

Mutagenesis and Protein Purification. The wild-type FynSH3 gene was subcloned into pET15b, which includes an amino-terminal six-His tag, which was not removed prior to these studies. Mutations were constructed by site-directed mutagenesis using three-step PCR methods. Primers were designed so that they were exactly complementary save for the desired change. PCR products were *Dpn*I digested to destroy contaminating traces of the original wild-type DNA template. *Escherichia coli* XL-1 cells were transformed with each gel-purified PCR product. Genetic constructs were then sequenced for validation of the desired construct. DNA was then purified using the Mini-Prep DNA purification kit (Stratagene, San Diego, CA) and used to transform *E. coli* BL21(DE3) pLysS cells for protein expression. Cells were grown in LB with Amp to an optical density of 0.4 and then induced with 1 mM IPTG. Cells were collected at an optical density of 1.0. Protein constructs were purified with Ni-NTA agarose resin (Qiagen, Inc., Valencia, CA) under native conditions, dialyzed exhaustively against 10 mM ammonium bicarbonate, lyophilized, and stored at −20 °C.

Kinetic Characterization. Chevron plots were fitted using standard methods (*39*). Folding and unfolding rates were observed by tryptophan fluorescence changes using an APP stopped-flow fluorometer (Applied Photophysics, Letatherhead, England). All experiments were performed in 50 mM potassium phosphate at a pH of 7.0 at 25 °C. Folding rates were measured by the rapid dilution of varying concentrations of guanidine hydrochloride (GuHCl).[1] Unfolding rates were measured by the rapid dilution of native conditions with the addition of varying concentrations of GuHCl. Data were fit to the equation:

$$\ln k_{obs} = \ln[\exp(\ln(k_f^\circ) + m_f[\mathrm{GuHCl}]/RT) + \exp(\ln(k_u^\circ) + m_u[\mathrm{GuHCl}]/\mathrm{RT})]$$

using Kalidagraph to estimate folding and unfolding rates extrapolated to 0 M GuHCl or to 1 and 5 M GuHCl, respectively. These kinetic values were also used to calculate ΔG_{eq} and $\Delta\Delta G_{eq}$. Confidence intervals for $\ln(k_f)$ and $\ln(k_u)$ were derived using standard errors estimated by Kalidagraph. Comparison of these values with the standard deviations of independent measurements made on the wild-type protein suggests that the estimated standard errors provide a reasonably accurate measurement of the true confidence intervals for $\ln(k_f)$ and $\ln(k_u)$ (data not shown). Estimated confidence intervals for ΔG_{eq}, $\Delta\Delta G_{eq}$, and $\Delta\Delta G^{\ddagger}$ were propagated from the standard errors of $\ln(k_f)$ and $\ln(k_u)$. Confidence bounds on ϕ were estimated using the maxima and minima of the ratios $(\Delta\Delta G^{\ddagger} \pm \epsilon_{TS})/(\Delta\Delta G_{eq} \pm \epsilon_u)$, where ϵ_{TS} and ϵ_u represent the 95% confidence intervals on $\Delta\Delta G^{\ddagger}$ and $\Delta\Delta G_{eq}$, respectively. The equilibrium folding free energies of representative mutants were also characterized using equilibrium chemical denaturation methods as described previously for the wild-type protein (*39*), with error bars representing estimated standard errors of the fits.

Table 1: Statistics Regarding the Data Set

protein	length	no. of reported ϕ	no. of negative ϕ	ref
ACBP	86	30	1	*36*
AcP	98	25	0	*35*
ADA2h	81	19	0	*37*
spectrin SH3	57	17	1	*25*
CI2	64	85	9	*19*
FKBP12	107	34	1	*33*
FNfn10	94	26	3	*32*
FynSH3	67	30	9	*9*
Im9	86	20	0	*31*
protein G	56	29	4	*30*
protein L	64	70	7	*29*
S6	97	19	1	*28*
src-SH3	56	16	1	*20*, *26*, *27*
Sso7d	63	24	6	*24*
TI127	89	26	1	*23*
TNfn3	90	42	2	*34*

RESULTS

Literature Survey. To access the frequency of negative ϕ-values, we have surveyed all of the reported mutations for the 16 most exhaustively characterized, two-state proteins described to date (Table 1) (*9*, *19*, *20*, *22*−*37*). Some 46 (∼9%) of the 512 ϕ-values reported for these proteins are negative (Figure 1). Many of these, however, may be associated with poor experimental precision. For example, only 25 (∼6%) of the 405 surveyed ϕ-values for which confidence estimates have been reported fall more than one "error bar" below zero (Figure 1). Similarly, ϕ precision is thought to be related to the magnitude of $\Delta\Delta G_{eq}$ (*15*), and the mean (absolute) reported $\Delta\Delta G_{eq}$ associated with the negative ϕ-values in our data set is, at 4.59 kJ/mol, far lower than the 7.03 kJ/mol observed across all of the mutations in our data set (Table 2). Consistent with this, only 27 negative ϕ-values (7%) are observed among the 396 mutations that

[1] Abbreviation: GuHCl, guanidine hydrochloride.

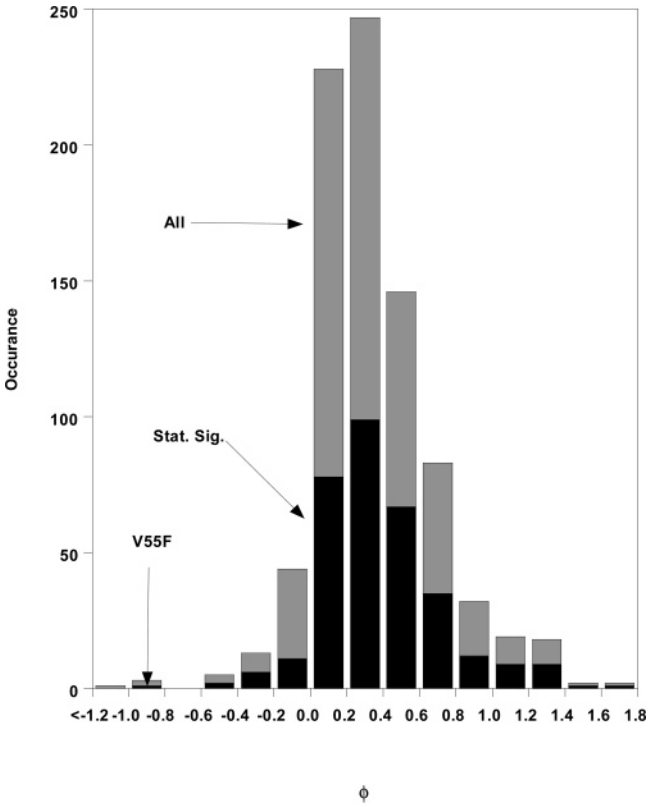

FIGURE 1: Negative ϕ-values are relatively uncommon. A survey of 512 ϕ-values derived from 16 two-state proteins is indicated, with those falling more than one reported error bar from zero indicated in black. Approximately 9% of all reported ϕ-values are negative, but less than two-thirds of these fall even one reported error bar away from zero (both positive and negative ϕ-values falling more than one reported error bar from zero are indicated in black). The FynSH3 substitution V55F, which we have characterized in detail in this work, was previously reported to be significantly negative: it is one of only four mutations previously reported to have a ϕ-value below -0.5 and appears to be by far the most statistically significant of these (*9*).

Table 2: ϕ Statistics

total no. of reported ϕ-values	512
total no. of ϕ-values below zero	46
no. of ϕ-values with reported confidence estimates	405
no. of these below zero	31
no. of these at least one error bar below zero	25
no. of these at least two error bars below zero	20
mean $\lvert\Delta\Delta G_{eq}\rvert$ of all mutants (kJ/mol)	7.03
mean $\lvert\Delta\Delta G_{eq}\rvert$ of those with ϕ below zero (kJ/mol)	4.59
no. of ϕ-values for which $\lvert\Delta\Delta G_{eq}\rvert \geq 2.5$ kJ/mol	396
no. of these below zero	27
no. of ϕ-values for which $\lvert\Delta\Delta G_{eq}\rvert \geq 2.5$ kJ/mol and for which confidence intervals have been reported	269
no. of these at least one error bar below zero	17
no. of ϕ-values for which $\lvert\Delta\Delta G_{eq}\rvert \geq 7$ kJ/mol	223
no. of these below zero	13
no. of ϕ-values for which $\lvert\Delta\Delta G_{eq}\rvert \geq 7$ kJ/mol and for which confidence intervals have been reported	143
no. of these at least one error bar below zero	6

change folding free energy by $\geq$2.5 kJ/mol (0.6 kcal/mol), a cutoff suggested by Fersht to be the limit below which ϕ determination becomes unreliable (*11*). Moreover, many of these ϕ-values lie within the range -0.01 to -0.05 and thus are of questionable statistical significance; only 4% of the ϕ-values in our data set for which confidence intervals have been estimated are simultaneously more than even a single error bar below zero and associated with free energy changes above this cutoff. Likewise, while 13 negative ϕ-values are observed (listed in Table 3) among the 223 mutations (5.8%) associated with the larger, 7 kJ/mol cutoff that Sanchez and Kiefhaber have claimed is required in order to achieve reasonable precision (*15*), only 6 of the 143 (4%) for which reliability has been estimated fall more than even a single error bar below zero.

Table 3: Negative ϕ-Values Associated with $\Delta\Delta G_{eq} \geq 7$ kJ/mol

protein	mutant	ϕ	$\lvert\Delta\Delta G_{eq}\rvert$ (kJ/mol)	ref
protein G	G41A	-0.02 ± 0.02	11.88	*30*
Sso7d	V15A	-0.03 ± 0.04	9.58	*24*
TI127	V13A	-0.04 ± 0.01	9.00	*23*
TNfn3	P25A	-0.15 ± 0.01	7.24	*34*
FKBP12	F36A	-0.08 ± 0.01	14.8	*33*
FynSH3	F26I	-0.02 ± 0.02	8.32	*9*
	F20V	-0.03 ± 0.02	7.87	*9*
	A39F	-0.06 ± 0.02	9.20	*9*
	A6V/F20L	-0.11 ± 0.01	11.55	*9*
protein L	L56A	-0.01[a]	8.70	*29*
	F62V	-0.02[a]	15.61	*29*
	D38G	-0.05[a]	10.29	*29*
	G45A	-0.10[a]	9.33	*29*

[a] Estimated confidence intervals not reported.

More detailed consideration suggests that the number of truly negative ϕ-values is probably less than the above naive tabulation of the reported data would suggest. One source of this overestimation arises due to the asymmetric distribution of ϕ-values around zero; because the frequency of positive ϕ-values is at least 10−25 times that of negative ϕ-values, the number of ϕ-values that, due to inevitable sources of experimental error, are incorrectly recorded as negative is likely much larger than the number of negative ϕ incorrectly reported to be positive. Second, in our experience the error bars reported in the literature may very significantly underestimate the true standard error (because, for example, they ignore potentially important sources of interexperiment and interlaboratory variability; data not shown). Indeed, because detailed descriptions of the methods employed to estimate ϕ confidence intervals are almost entirely lacking from the relevant literature, it is difficult to discount the possibility that the true standard errors are much larger than the error bars typically estimated in the literature and thus it is equally difficult to discount the argument that the frequency of statistically significantly negative ϕ-values may be far less than the ~4% we have estimated above.

Position V55 of FynSH3. Only 4 of the more than 500 entries in our data set are reported to fall below -0.5. The most negative of these, Y73F in the protein ACBP (which comes in at -10.9), falls within error of zero (*36*). Similarly, the third most negative ϕ-value in our data set, that reported for the mutation T25A in protein G (which comes in at -0.81), lacks estimated error bounds and is associated with a $\Delta\Delta G_{eq}$ of only ~1 kJ/mol (*30*). In contrast, the second most negative ϕ-value in our data set, the $\phi = -0.82 \pm 0.08$ reported for the V55F mutation in FynSH3, is described as both highly statistically significant and associated with a $\Delta\Delta G_{eq}$ of 3.1 ± 0.2 kJ/mol (*22*).

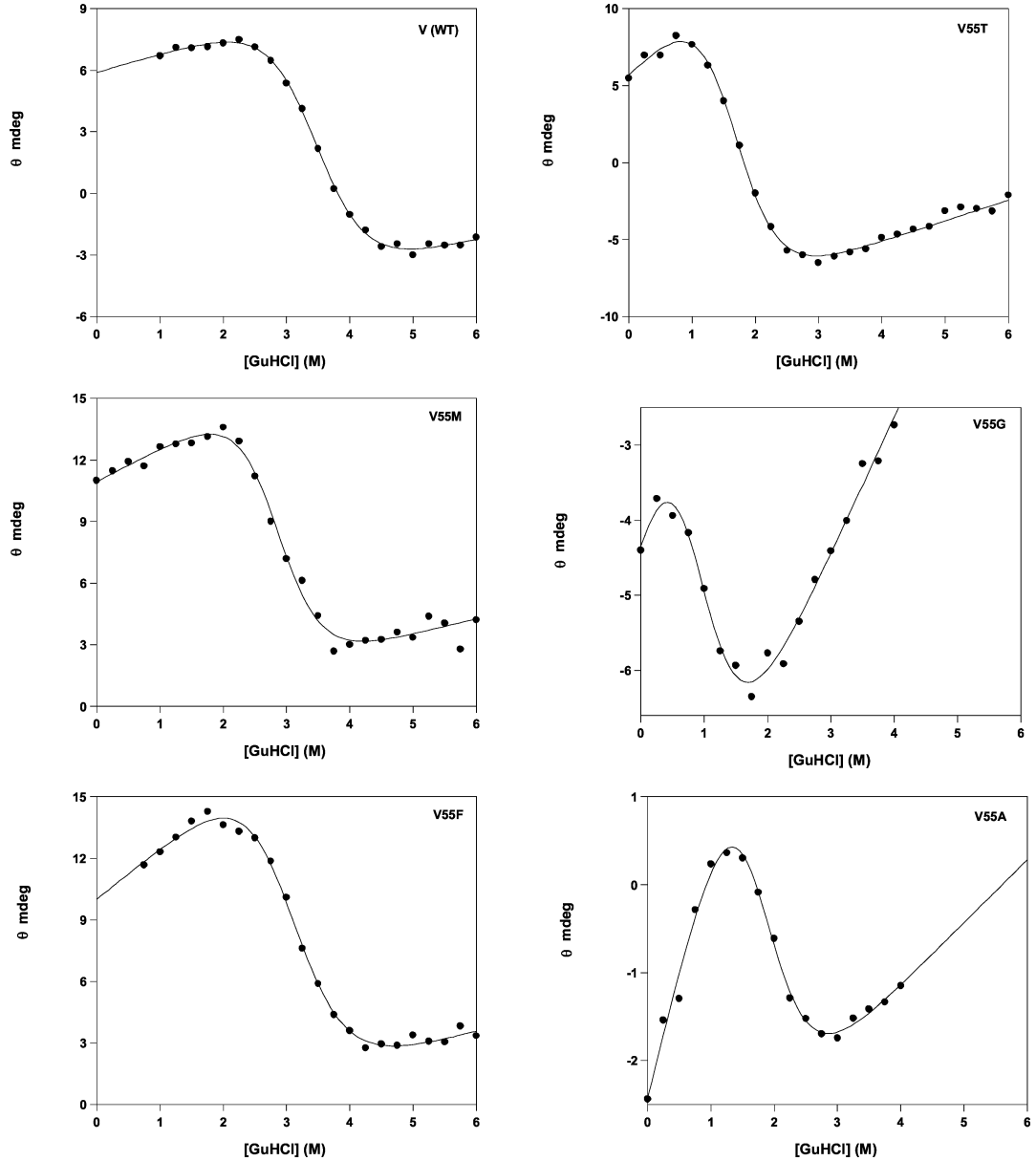

FIGURE 2: Equilibrium chemical denaturations of a representative subset of the mutants characterized here produce folding free energies within error of those observed via chevron analysis (Table 4). This suggests that mutations at position 55 of FynSH3 maintain the two-state folding of the wild-type protein.

To improve our understanding of the significance of negative ϕ-values, we have characterized numerous additional ϕ-values associated with mutations at position V55 of the FynSH3 domain. In total, we generated 15 mutations (representing all residues except P, Y, N, and D), 9 of which were sufficiently stable and well expressed for further characterization. In contrast, mutations that substitute the wild-type valine to a charged residue do not express and were therefore not subjected to analysis. We have performed equilibrium denaturation experiments on representative members of this set of proteins (Figure 2) and find that all unfold in a cooperative, apparently two-state manner.

We have estimated the folding free energies of the mutant proteins using chevron analysis (Figure 3). The free energy so obtained for the wild-type protein is 22.5 ± 0.4 kJ/mol, and the free energies of the characterized mutants range from 5.1 ± 0.5 to 22.7 ± 1.2 kJ/mol. Not surprisingly, substitutions to polar, uncharged amino acids typically produced significant reductions in native state stability. In sharp contrast, substitutions that replace valine with larger hydrophobic residues had little if any significant effect on native state stability. We note that, for each of the mutations for which we have obtained both, the equilibrium and kinetically derived folding free energies are effectively indistinguishable (Table 4). This suggests that these mutations do not disrupt the previously reported (*9*, *10*) two-state folding kinetics of this small protein.

We have characterized the kinetic consequences of substitutions from valine to the hydrophobic residues, phenylalanine, methionine, leucine, tryptophan, and alanine (Figure 3). Four of these five hydrophobic substitutions accelerate folding, the sole exception being tryptophan, which very slightly slows folding. Three of the five mutations also appear to destabilize the protein; only substitutions to leucine or tryptophan formally increase the estimated folding free energy (albeit not with any statistical significance). Conse-

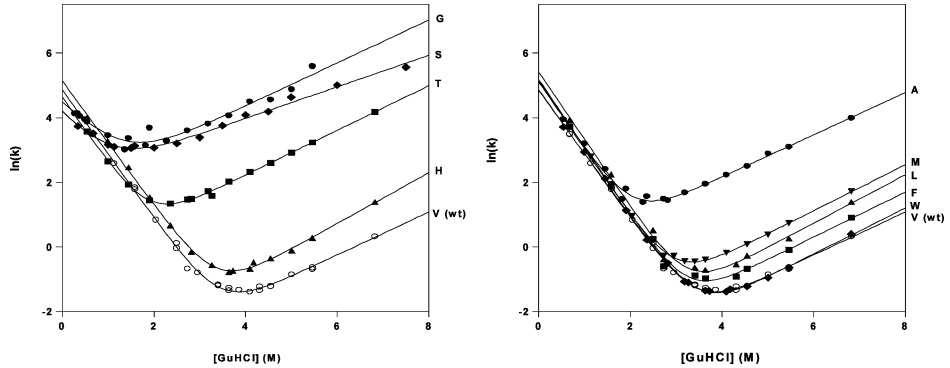

FIGURE 3: Chevron analysis of the folding of both hydrophilic (left) and hydrophobic (right) substitutions of valine-55 of FynSH3 (denoted as wt). In general, substitution of the wild-type valine to other hydrophilic residues tends to increase both folding and unfolding rates slightly, which results in statistically insignificant changes in stability. Substitution to hydrophobic amino acids, in contrast, typically accelerates unfolding significantly while only modestly decelerating folding.

Table 4: Kinetic and Thermodynamic Properties of Mutants at Position 44 of FynSH3

			ΔG_{eq} (kJ/mol)[a]				
protein	$\ln(k_f)$[a]	$\ln(k_u)$[a]	equilibrium	kinetic	$\Delta\Delta G^{\ddagger}$ [a] (kJ/mol)	$\Delta\Delta G_{eq}$[a] (kJ/mol)	ϕ
WT	4.85 ± 0.07	−4.24 ± 0.14	−22.0 ± 1.4	−22.5 ± 0.4			
V55F	5.17 ± 0.20	−3.86 ± 0.37	−18.5 ± 1.7	−22.4 ± 1.0	−0.80 ± 0.52	0.15 ± 1.11	−5.33[b,c]
V55L	5.40 ± 0.22	−3.76 ± 0.41	nd	−22.7 ± 1.2	−1.36 ± 0.57	−0.17 ± 1.22	8.00[c]
V55W	4.83 ± 0.05	−4.48 ± 0.13	nd	−23.1 ± 0.3	0.05 ± 0.22	−0.55 ± 0.52	−0.09[b,c]
V55M	5.10 ± 0.06	−3.13 ± 0.10	−20.8 ± 3.1	−20.4 ± 0.3	−0.62 ± 0.23	2.13 ± 0.48	−0.29[d]
V55A	5.14 ± 0.16	−0.45 ± 0.15	−15.2 ± 2.2	−13.9 ± 0.5	−0.72 ± 0.43	8.68 ± 0.67	−0.08[b]
V55H	5.12 ± 0.07	−4.00 ± 0.16	nd	−22.6 ± 0.4	−0.67 ± 0.45	−0.07 ± 0.58	9.57[c]
V55T	4.62 ± 0.08	−0.55 ± 0.06	−12.5 ± 1.1	−12.8 ± 0.2	0.57 ± 0.26	9.72 ± 0.46	0.06[e]
V55G	4.41 ± 0.15	1.69 ± 0.32	−6.5 ± 2.4	−6.7 ± 0.9	1.09 ± 0.41	15.79 ± 0.96	0.07[e]
V55S	4.07 ± 0.17	2.01 ± 0.11	nd	−5.1 ± 0.5	1.93 ± 0.46	17.43 ± 0.63	0.11[e]

[a] Error bounds represent standard errors. [b] Sign on ϕ not statistically significant due to poorly constrained $\Delta\Delta G^{\ddagger}$. [c] Sign on ϕ not statistically significant due to poorly constrained $\Delta\Delta G_{eq}$. [d] 95% confidence intervals +0.18/−0.12. [e] 95% confidence intervals +0.02/−0.03, +0.07/−0.05, and +0.07/−0.06, respectively.

quently, four of the five hydrophobic mutations are formally associated with negative ϕ-values (Table 2). However, because the free energy changes produced by the phenylalanine, leucine, and tryptophan substitutions are quite small ($|\Delta\Delta G_{eq}| < 0.6$ kJ/mol), it is very difficult to determine these ϕ-values with any precision. Even the negative ϕ-value associated with the valine to phenylalanine substitution, which motivated our study (*22*), appears in our experience to be statistically insignificant; while the best-fit value we obtain, −5.3, is formally negative, the $\Delta\Delta G_{eq}$ we estimate for this substitution is only 0.15 ± 1.11 kJ/mol, and thus the 95% confidence intervals on our estimate of ϕ overlap very significantly with nonnegative values. In contrast, the equilibrium free energy changes associated with the mutations V55M and V55A are statistically significant. The acceleration produced by the alanine mutant, however, is not significant at the 95% confidence level, and thus only the mutation V55M appears to produce a statistically significant negative ϕ-value.

In contrast to hydrophobic substitutions, the substitution of valine-55 with polar residues generally reduces both folding rates and stability (Figure 3). Mutation to serine, threonine, and glycine produces statistically significant, positive ϕ-values ranging from 0.06 to 0.11 (Table 4), values relatively close to the "mean" ϕ-value we obtain by fitting a line to $RT \ln(k_f)$ versus ΔG_{eq} across the data set comprised of all 10 constructs (see below). Substitution to histidine, however, does not significantly alter the protein's stability and, thus, does not produce a well-defined ϕ-value.

Fersht and Sato have argued that ϕ-values are relatively precise when they are calculated using folding and unfolding rates estimated at nonzero denaturant concentrations (thus lessening the errors induced by the long extrapolations required to estimate rates in the absence of denaturant) and limited to mutations that change ΔG_{eq} by at least 2.5 kJ/mol (*11*). Using these criteria we find that, when we estimate $\ln(k_f)$ and $\ln(k_u)$ at 1 and 5 M GuHCl, respectively, only five of our mutations lie confidently above the recommended free energy cutoff (data not shown). The ϕ-values thus obtained are within error of those obtained using rates extrapolated to no-denaturant conditions. The statistical significance of the signs on two of the five, however, is altered by the new analysis. Specifically, when calculated from rates estimated at 1 and 5 M GuHCl, the ϕ-value of V55G moves to within error of zero ($\phi = -0.04$ +0.08/−0.09), those of V55A (−0.17 ± 0.06) and V55M (−0.14 +0.07/−0.08) are statistically significantly negative, and those of V55T (0.07 ± 0.02) and V55S (0.06 ± 0.05) remain statistically significantly positive (error bounds represent 95% confidence intervals). Thus, once again we observe that the sign on the ϕ-value associated with position 55 of the FynSH3 domain is dependent on the substitution employed to measure it.

Davidson and co-workers have previously characterized four mutations at position 55 of FynSH3: V55F, V55I, V55L, and V55A (*22*). The observed change in free energy associated with two of these, V55L and V55I, was so small, however, that the authors declined to report ϕ-values. Nevertheless, using the kinetic data they report, we have

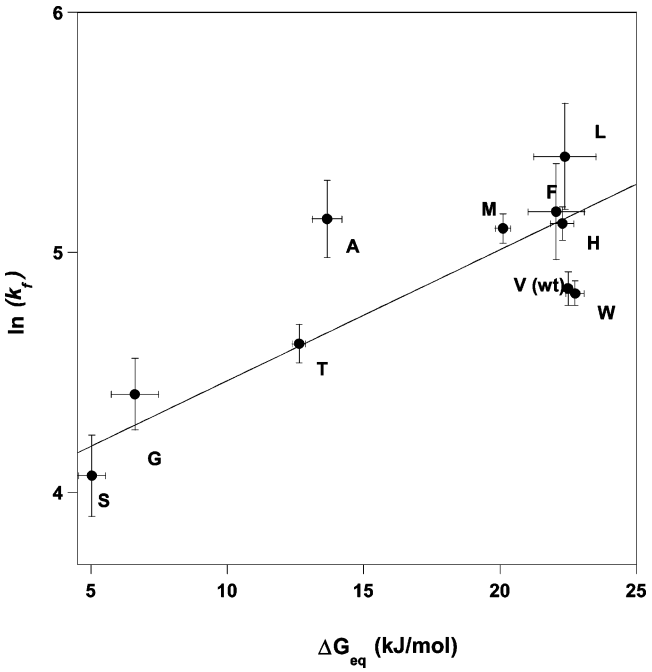

FIGURE 4: A plot of ln(k_f) versus folding free energy suggests that mutations at position 55 of FynSH3 may produce two classes of mechanistic effects. The pairwise slopes connecting hydrophilic substitutions to the wild-type sequence are positive and, although small, generally statistically significant (shown is a linear fit of the hydrophilic mutations, which produces a slope equivalent to $\phi = 0.14 \pm 0.02$ and a Patterson correlation coefficient, r^2, of 0.94). In contrast, many of the pairwise slopes connecting the hydrophobic substitutions to the wild-type valine are formally negative. Only for the V55M mutation, however, does this represent a statistically significant observation. It thus appears that negative ϕ-values at position valine-55 are, at best, context dependent and that subtlety will be required in order to develop a theoretical model of such behavior.

calculated ϕ-values for these mutations and find that, while V55L and V55I are formally negative and positive, respectively, neither of these results is statistically significant (data not shown). In contrast, however, Davidson and co-workers report much smaller confidence intervals than we are able to achieve for the mutant V55F, and thus the ϕ they report for this mutation is statistically significantly negative. Given the much larger error bars we observe, we cannot claim (and are not claiming) that this prior work is incorrect; we simply cannot reproduce this observation with sufficient precision to independently verify that this substitution at position 55 in FynSH3 produces an authentically negative ϕ. We can authoritatively state, however, that hydrophilic substitutions at this position produce small, but statistically significant, *positive* ϕ-values. Thus, again, the negative ϕ-value previously reported for position 55 of FynSH3 appears to be, at best, dependent on the substitution employed to measure it.

The Brønsted plot is a method for measuring noncovalent interactions that occur in the protein-folding transition state. This analysis method, which plots ln k versus folding free energy for all of the characterized mutations at a given position, produces a slope that is proportional to the mean ϕ-value associated with the position (Figure 4). Whereas the use of Brønsted plots for the analysis of multiple mutations has been called into question on the grounds that it assumes that all of the mutations probe the same mechanistic aspect of folding (*38*), at a more sophisticated level it provides a useful means of discriminating between mutations sharing a common mechanistic effect on the mechanism (which will fall on a single line) and those that do not (*9*). That is, if a number of mutations fall on a single line in a Brønsted plot, the most parsimonious explanation is that the transition-state interactions disrupted (or created) by the various mutations are equally well formed in the transition state. We find that hydrophilic mutations at position V55 exhibit such a simple, linear relationship between $RT \ln(k_f)$ and folding free energy ($r^2 = 0.94$), with a slope equivalent to $\phi = 0.14 \pm 0.02$ (Figure 4). This relationship suggests that the free energy associated with the transition-state interactions altered by hydrophilic substitutions is relatively context-independent and relatively small compared to the free energy produced by the same interactions in the native state. The equivalent picture is less clear for hydrophobic substitutions. The ϕ-values associated with most of these are experimentally ill-defined. Several of these mutants, however, produce folding rates that fall far from the fitted line (Figure 4), suggesting that some of these substitutions may have significantly altered the folding mechanism.

DISCUSSION

It appears that statistically significant negative ϕ-values are, at best, rather rare: while 9% of reported ϕ-values are negative, only 4% are simultaneously negative, statistically significant (even at the "one error bar" level), and associated with the minimum free energy change thought necessary to ensure reasonably reliable ϕ determinations. Moreover, due to the asymmetric distribution of ϕ-values around zero and the potential for underestimated standard errors in the literature, a proposition that is difficult to discount given the almost universal omission of details regarding the definition of a given error bar and how it was estimated, the fraction of statistically significant negative ϕ-values may be much smaller still. It thus appears that authentically negative ϕ-values may be very rare indeed among the well-characterized two-state proteins investigated to date.

Perhaps consistent with the relative paucity of negative ϕ-values, we find that even one of the most experimentally robust negative ϕ-values reported to date is very sensitive to the nature of the mutation employed to probe it. Mutations of valine-55 in FynSH3 to hydrophobic residues frequently accelerate folding without concomitantly producing significant changes in folding free energy. Because of this behavior, several of these mutations produce formally negative ϕ-values, but given the precision of our data, only one or two of these observations represent statistically significant evidence of ϕ-values falling below zero. In contrast, irrespective of the precise mutation employed, we observe that substitution of wild-type valine-55 with hydrophilic residues generally produces small, but statistically significant, positive ϕ-values of $\sim$0.1.

Irrespective of the precise ϕ-values associated with the substitution, the folding rate of FynSH3 increases when valine-55 is mutated to any of several other hydrophobic residues. We presume this acceleration is due to non-native hydrophobic interactions that stabilize the expanded transition state (perhaps by stabilizing nonspecific or otherwise non-native contacts) but, due to steric clashes, do not similarly stabilize the more compact native state (*13*, *21*). This said,

the lack of clearly negative ϕ-values at position 55 in FynSH3 is perhaps somewhat surprising given the observation of mutations at this site that accelerate folding. Mutations can stabilize the native state, destabilize the native state, or be energetically neutral. Yet the majority of the V55 mutations that *stabilize the folding transition state* fall into the last category. That is, the stabilizing effect these mutations have on the folding transition state is counteracted in the native state (i.e., the protein's unfolding and folding rates are both increased), leading to no net change in equilibrium free energy. This could, of course, occur if the residue at this position does not make any interactions in the native state, and thus mutations at this site can stabilize the transition state via non-native interactions without adversely affecting native state stability. Both inspection of the native state structure (PDB ID 1shf), which indicates that the valine-55 side chain is completely sequestered from the solvent, and the observation that polar mutations reduce native state stability suggest, however, that position 55 participates in significant native state interactions. Our observations thus beg the question: where are the mutations that stabilize the transition state but *overcompensate* in the native state, destabilizing it relative to the wild-type protein and thus producing robustly negative ϕ-values? We do not have an answer to this question.

Examination of both existing ϕ-value data and the experimental data presented here suggests that positions that exhibit negative ϕ-values are quite rare and that those that do exist may be mechanistically complex. This contrasts sharply with the situation for positive ϕ-values, which are both common and, for positions that have been studied at a similar level of detail in FynSH3 and other proteins, appear to be perhaps surprisingly independent of the mutation used to define them (*10*, *11*, *22*). More generally, while ϕ-value analysis is a powerful technique for defining specific transition-state interactions, the results presented here suggest that their mechanistic interpretations may require significant subtlety.

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