Stability Effects of Increasing the Hydrophobicity of Solvent-Exposed Side Chains in Staphylococcal Nuclease†

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ABSTRACT: A total of fifty single site surface phenylalanine substitution mutants have been made in the model protein staphylococcal nuclease. The fifty residues that were replaced with phenylalanine were chosen to give a broad sampling of solvent accessibility, secondary structure, and backbone conformations. The change in the stability of each mutant protein relative to wild type was measured by guanidine hydrochloride denaturation. These results were compared to previous results obtained when these same sites were substituted with an alanine and a glycine. By this means, changes in the stability due to the loss of interactions of the wild-type side chain can be separated from the effects of introducing the bulky, hydrophobic phenylalanine in these solvent-exposed positions. In general, our results agree with the conventional wisdom that placing a hydrophobic residue in a solvent-exposed position is destabilizing in most cases, but less destabilizing than most changes in the hydrophobic core of the protein. However, the degree to which a hydrophobic surface substitution destabilizes or stabilizes a globular protein is highly context-dependent, with some mutations being as destabilizing as those in the core. This indicates that steric and packing considerations are also important on the surface of a globular protein but generally are not as important as in the interior. No evidence for the widespread occurrence of the so-called reverse hydrophobic effect at solvent-exposed sites was found. In addition, this survey of numerous sites suggests that previous measurements of α-helix "propensities" often seriously underestimate the importance of the environment of the side chain.

In general, amino acids on the surface of soluble globular proteins are hydrophilic while those in the interior are hydrophobic. There are two possible explanations for the exclusion of hydrophobic residues from the surface. The first reason could be the need to prevent protein association and aggregation. A second reason could be that placing a hydrophobic amino acid on the surface of a globular protein is destabilizing. However, it has been generally accepted that surface amino acid substitutions in globular proteins usually have little effect on protein stability (*1*, *2*). In fact, the energetic consequences of placing nonpolar substitutions on the surface of a protein have not been as extensively examined as the effects of substitutions in the interior. Several studies have shown that the tolerance for substitution is clearly lower for buried residues than for surface residues (*3*−*5*). The most extensive tests have been in the T4 lysozyme (*6*) and in the Lac repressor (*7*) where the tolerance for 12 or 13 different side chains at 163 and 328 positions, respectively, was assessed. The most tolerant sites clearly were solvent-exposed residues. Still these studies did show effects at some solvent-exposed sites. Further, it should be noted that this work relied on qualitative assessments of protein stability through evidence of phenotypic activity. As a protein may still retain activity even if the structural stability has been significantly lowered, such studies leave open the question of the degree of destabilization caused by hydrophobic substitutions on the surface.

Indeed, it has been demonstrated in recent years that, in at least some instances, when a hydrophobic amino acid is substituted into a hyperexposed site on the surface of a protein, the protein can be destabilized, with the extent of the destabilization linked to the degree of hydrophobicity. This has been attributed to the "reverse hydrophobic effect" (*8*). The reverse hydrophobic effect is said to occur when a hydrophobic residue is more solvent-exposed in the native state than in the denatured state. It is argued that this burial of the hydrophobic surface in the denatured state stabilizes the denatured state relative to the native state. In these cases, a good correlation between increasing protein stability and decreasing side chain hydrophobicity of the substituted amino acid side chain is present. Although the initial observations of Pakula and Sauer (*8*) in the λ Cro repressor may be explained on the grounds of a three-state unfolding mechanism (*9*) rather than the reverse hydrophobic model, similar effects have been observed by other workers at hyperexposed sites in other proteins (*10*, *11*). Similar effects of hydrophobic substitution have also been observed at sites that are not hyperexposed to the solvent (*12*, *13*). Nevertheless, how common such reverse hydrophobic effects are is not clear.

In contrast to the destabilizing effects cited above, it has also been reported that a hydrophobic substitution on the

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surface of a globular protein can increase the stability of the protein. The thermal stability of the neutral protease found in *Bacillus stearothermophilus* was increased when a solvent-exposed surface residue was substituted with large hydrophobic amino acids (*14*). This effect was attributed to the hydrophobic contacts that the large hydrophobic amino acid was able to make with hydrophobic portions of the surrounding hydrophilic residues at the surface of the protein. It was suggested that this type of structural motif, termed a small exterior hydrophobic cluster (SEHC), occurs naturally in proteins such as plastocyanin. It has also been proposed that this motif could be important in helping to stabilize the tertiary structures of proteins (*15*). In fact, a SEHC motif was engineered in the neutral protease of *Bacillus subtilis* in imitation of the features found in the neutral protease of *B. stearothermophilus*. In this instance, the placement of a hydrophobic amino acid on the surface also stabilized the *B. subtilis* protein (*16*).

The examples above illustrate that the effects of placing a hydrophobic amino acid on the surface of a globular protein are not completely understood but may be important to the structure and stability of the protein in certain circumstances. Unfortunately, no large-scale systematic surveys of the quantitative thermodynamic effects of hydrophobic amino acid substitutions on the surface of proteins have been undertaken, so it is difficult to say how significant or frequent such effects are. Therefore, in this study, we made and examined 47 single-site phenylalanine substitutions on the surface of staphylococcal nuclease. We also include in our analysis 3 single-site surface phenylalanine substitutions prepared previously (*17*).

MATERIALS AND METHODS

Mutagenesis. The mutants were prepared by the method of Kunkel (*18, 19*) and screened as described previously (*17*).

Protein Expression and Purification. The mutated nuclease genes were transferred into an expression vector, expressed, and purified as described previously (*17*). The only exception was for the mutant K16F. When K16F is in urea and is dialyzed against a high-salt phosphate buffer to remove the urea, it precipitates. Therefore, this mutant was initially dialyzed against distilled water to prevent precipitation and then dialyzed against the native low-salt phosphate buffer. All other steps in the expression and purification of this mutant are identical to those previously reported.

Protein Stability Determinations. Guanidine hydrochloride denaturations were carried out as previously described (*20, 21*) on an Aviv model ATF-101 fluorometer except that the samples were heated to 55 °C for 5 to 10 min and allowed to cool to 20 °C before titrating. This was done to remove any metastable protein oligomers. Heated samples usually showed little stability difference from those not so treated. The K16F mutant, however, was not preheated because of the tendency of this protein to precipitate upon heating. To ensure that irreversible precipitation in the guanidine hydrochloride titration was not occurring, and to allow any metastable oligomers present the opportunity to unfold and disassociate, the titration of K16F was performed with both 5- and 25-min equilibration times between additions of guanidine hydrochloride (*21*). Titrations under both conditions were identical within experimental error.

Data analysis was carried out as previously described (*20, 21*) except for 5 mutants that had noticeable downward sloping native baselines in their denaturation curves. These mutants were P47F, E52F, E57F, Q80F, and D143F. In addition to the usual titration at 50 μg/mL, these mutants were titrated at protein concentrations of 500 μg/mL. Next, P47F, E52F, E57F, and D143F were titrated in a high ionic strength initial buffer (600 mM NaCl, 25 mM sodium phosphate, pH 7.0). The values of ΔG_{H_2O}, C_m, and m_{GuHCl} for these mutants were calculated in all cases using a nonlinear least-squares analysis (*22*).

RESULTS

Phenylalanine substitutions were made at 47 sites, representing approximately half of all residues with even slight solvent exposure in the crystal structure of staphylococcal nuclease (*23, 24*). Lysine to phenylalanine mutations accounted for 17 of the 47 substitutions. Other wild-type residues mutated to phenylalanine were 8 glutamates, 4 aspartates, 4 glycines, 3 prolines, 3 glutamines, 3 alanines, 2 serines, 1 methionine, 1 arginine, and 1 histidine. There were also 3 solvent-exposed surface tyrosine residues that were mutated to phenylalanine previously whose stabilities were redetermined in this study (*17*). This brings the total number of phenylalanine substitutions to 50. The identities of these mutants and the parameters derived from the analysis of their guanidine hydrochloride denaturations are found in Table 1.

As described above, five mutants had unusual sloping native baselines. To explore the possibility that these sloping baselines might be due to protein association, these mutants were titrated at protein concentrations of 50 μg/mL and 500 μg/mL. The denaturation curves were superimposable within experimental error. The values calculated for the 50 μg/mL samples were the same as those calculated for the 500 μg/mL samples, within experimental error. In addition, P47F, E52F, E57F, and D143F were titrated in a high ionic strength initial buffer in order to determine if changes in ionic strength as guanidine hydrochloride is added play a role in the sloping native baseline. In all cases both low and high ionic strength titration curves appeared similar. For E57F and P47F calculated values of ΔG_{H_2O}, C_m, and m_{GuHCl} were identical within experimental error. For E52F the value of ΔG_{H_2O} was identical within experimental error, while C_m and m_{GuHCl} were slightly different. For D143F the value of ΔG_{H_2O} was 5.4 kcal/mol in a low ionic strength initial buffer versus 5.7 kcal/mol in a high ionic strength initial buffer with similar magnitude changes in C_m and m_{GuHCl}. While having greater than normal experimental error, these changes do not seem be of the degree expected if the sloping baseline were due to ionic strength changes. Analytical ultracentrifugation experiments also do not indicate major changes in the degree of reversible association (G. R. Schofield, W. E. Stites, unpublished observations). Thus, while protein oligomerization and ionic strength effects seem to be ruled out, the origin of this sloping baseline effect remains obscure.

The values for the tyrosine to phenylalanine mutations have previously been published (*17*). For two of these mutations the redeterminations were identical within experimental error. For one, Y85F, the redetermined free energy was 0.3 kcal/mol lower than that previously found. We have

Table 1: Solvent Accessiblity, Secondary Structure, Guanidine Hydrochloride Denaturation Parameters, and Relative Stabilities for Phenylalanine Substitution Mutants

mutant	SASA[a]	secondary structure[b]	m_{GuHCl}[c]	C_m[d]	ΔG_{H_2O}[e]	$\Delta\Delta G_{F\rightarrow WT}$[f]	$\Delta\Delta G_{F\rightarrow A}$[g]
K9F	0.39	E	0.93	0.70	4.3	1.2	−0.3
P11F	1.11	E	0.94	0.79	4.9	0.6	0.2
K16F	0.78	E	0.96	0.79	5.0	0.5	0.2
D19F	0.02	S	0.90	0.67	4.0	1.5	1.3
K24F	0.52	E	0.93	0.80	4.9	0.6	0.3
K28F	1.03	T	0.99	0.71	4.6	0.9	0.1
G29F	0.00	T	1.09	0.57	4.1	1.4	0.1
P47F	1.08	T	0.95	0.83	5.2	0.3	−0.3
K48F	1.28	T	0.91	0.83	5.0	0.5	0.5
K49F	0.88	T	0.94	0.89	5.5	0.0	−0.4
G50F	0.00	N	0.94	0.75	4.7	0.8	1.0
E52F	0.07	N	0.94	0.78	4.8	0.7	0.5
E57F	0.56	H	1.01	0.72	4.8	0.7	0.4
S59F	0.05	H	0.81	1.00	5.5	0.0	0.4
A60F	0.77	H	1.00	0.71	4.7	0.8	0.8
K63F	074	H	0.93	0.58	3.5	2.0	1.4
K64F	1.15	H	0.92	0.85	5.2	0.3	0.3
M65F	0.11	H	1.03	0.58	3.9	1.6	−0.4
E67F	0.36	H	0.97	0.64	4.1	1.4	0.3
K70F	1.06	H	0.96	0.83	5.3	0.2	0.0
K71F	0.77	N	1.00	0.84	5.5	0.0	−0.5
E73F	0.20	E	0.92	0.73	4.4	1.1	−0.4
Q80F	0.66	N	0.96	0.76	4.8	0.7	0.6
D83F	0.01	N	0.88	0.06	0.3	5.2	1.3
K84F	1.08	T	0.95	0.74	4.6	0.9	1.0
Y85F	0.92	T	0.98	0.82	5.3	0.2	−0.2
G86F	0.00	S	1.01	0.53	3.5	2.0	1.4
D95F	0.21	T	0.59	0.06	0.2	5.3	1.9
G96F	0.00	T	0.75	0.51	2.5	3.0	1.2
K97F	1.05	E	0.95	0.76	4.8	0.7	0.6
E101F	0.02	H	0.78	0.47	2.4	3.1	1.1
A102F	0.09	H	0.91	0.60	3.6	1.9	1.9
R105F	0.19	H	0.84	0.44	2.4	3.1	1.7
A112F	0.00	N	0.93	0.64	3.9	1.6	1.6
Y113F	0.99	N	0.98	0.84	5.5	0.0	0.0
Y115F	0.88	T	0.97	0.83	5.4	0.1	−0.2
K116F	0.98	T	0.91	0.91	5.4	0.1	0.2
P117F	0.74	T	0.92	0.96	5.8	−0.3	0.5
E122F	0.09	H	0.86	0.82	4.7	0.8	0.3
Q123F	0.82	H	0.94	0.78	4.8	0.7	0.3
H124F	0.64	H	0.93	0.94	5.8	−0.3	0.0
K127F	1.04	H	0.97	0.81	5.2	0.3	0.4
S128F	0.09	H	1.00	0.68	4.5	1.0	1.7
E129F	0.02	H	0.89	0.26	1.5	4.0	1.5
Q131F	0.39	H	0.95	0.77	4.8	0.7	0.5
K133F	0.55	H	0.93	0.66	4.1	1.4	−0.1
K134F	0.80	H	0.93	0.82	5.0	0.5	0.5
E135F	0.44	T	0.96	0.68	4.3	1.2	0.4
K136F	1.05	T	0.91	0.72	4.3	1.2	0.4
D143F	n/a[h]	n/a[h]	0.98	0.83	5.4	0.1	−0.1
WT[i]	n/a[h]	n/a[h]	1.00	0.83	5.5	0.0	n/a[h]

[a] Fraction of the solvent-accessible surface area of the wild-type side chain relative to the Ala-Xxx-Ala tripeptide in the extended conformation. [b] Secondary structure determination by the method of Kabsch and Saunders (*29*): E, β-sheet; H, α-helix; S, bend; T, hydrogen-bonded turn, and N, no regular secondary structure. [c] First derivative of ΔG_{H_2O} with respect to guanidine hydrochloride concentration. Expressed relative to the wild-type value of 6.60 kcal/(mol M). Error is estimated to be ±0.02 kcal/(mol M). [d] Concentration of guanidine hydrochloride at the midpoint of denaturation expressed in M. Error is estimated to be ±0.01 M. [e] The free-energy difference between the native and denatured states at 0 guanidine hydrochloride concentration expressed in kcal/mol. Error is estimated to be ±0.1 kcal/mol. [f] The difference in the stability of the wild-type protein ($\Delta G_{WT} = 5.5$ kcal/mol) and the stability of the respective phenylalanine mutants in kcal/mol where $\Delta\Delta G_{F\rightarrow WT} = \Delta G_{WT} - \Delta G_{Phe.}$ [g] The difference in the stability in kcal/mol between single-site alanine mutants and their corresponding single-site phenylalanine mutants where $\Delta\Delta G_{F\rightarrow A} = \Delta G_{Ala} - \Delta G_{Phe}$. ΔG_{Ala} values were taken from ref *25*−*27*. [h] n/a: not applicable. [i] WT: wild-type nuclease.

found that a number of samples show similar slight drops in stability after heating to remove metastable oligomers.

Of the 50 phenylalanine mutants, 15 had $\Delta\Delta G_{F\rightarrow WT}$ values ($\Delta\Delta G_{F\rightarrow WT} = \Delta G_{wild-type} - \Delta G_{Phe}$ where $\Delta G_{wild-type}$ is the stability of wild type, 5.5 kcal/mol, and ΔG_{Phe} is equal to the stability of the phenylalanine mutant, ΔG_{H_2O}, in Table 1) between 0 and 0.5 kcal/mol, 14 had $\Delta\Delta G$ values between 0.6 and 1.0 kcal/mol, 8 had $\Delta\Delta G_{F\rightarrow WT}$ values between 1.1 and 1.5 kcal/mol, and 5 had $\Delta\Delta G$ values between 1.6 and 2.0 kcal/mol. Only 2 mutants had $\Delta\Delta G_{F\rightarrow WT}$ values that were between −0.5 and −0.1 kcal/mol. In addition, there were only 6 mutants that had $\Delta\Delta G_{F\rightarrow WT}$ values that were between 2.1 and 5.5 kcal/mol (Figure 1A).

In previous studies, single-site alanine substitutions had been made at virtually every site in staphylococcal nuclease (*25*−*27*). Using these data, the difference in the stability of

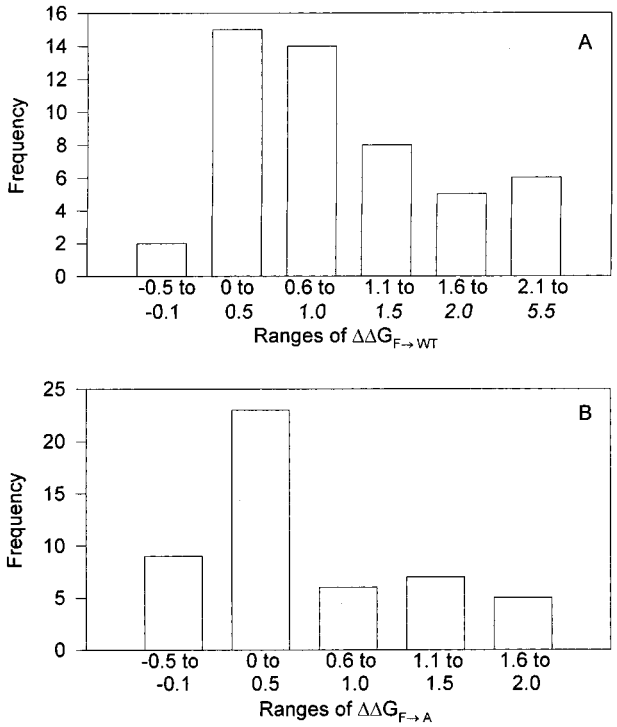

FIGURE 1: (A) A histogram showing the frequency of the occurrence of a given change in the stability relative to wild type for the 50 different mutants. (B) A histogram showing the frequency of the occurrence of a given change in the stability relative to an alanine substitution mutation at the same position.

the phenylalanine mutant and the corresponding alanine mutant was calculated ($\Delta\Delta G_{F\rightarrow A} = \Delta G_{Ala} - \Delta G_{Phe}$). Of the 50 phenylalanine mutants, 23 have a $\Delta\Delta G_{F\rightarrow A}$ of 0 to 0.5, 6 have a $\Delta\Delta G_{F\rightarrow A}$ of 0.6 to 1.0, 7 have a $\Delta\Delta G_{F\rightarrow A}$ value of 1.1−1.5, 5 have a $\Delta\Delta G_{F\rightarrow A}$ of 1.6 to 2.0, and 9 have a $\Delta\Delta G_{F\rightarrow A}$value of −0.1 to −0.5. These are summarized in histogram form in Figure 1B.

There are various ways to subdivide this set of 50 substitution mutants in order to look for trends. Several factors seem relevant. The degree of solvent exposure, the type of the side chain substituted with phenylalanine, the absence or presence of electrostatic interactions, and the type of secondary structure at the site of the substitution are all readily defined and appear to be likely to affect stability. Solvent accessibility was defined in relative rather than absolute terms. The fractional solvent-accessible surface area is a ratio of the solvent-accessible surface area of any given amino acid side chain in the protein crystal structure to the solvent-accessible surface area of that same amino acid in an extended Ala-Xxx-Ala reference tripeptide (*28*). The categories of solvent exposure we defined were hyperexposed sites (fractional solvent-accessible surface area was equal to or greater than 1), exposed sites (0.5 to 0.99), accessible sites (0.1 to 0.49), and marginally accessible sites (0.0 to 0.09). Helical, sheet, and turn residues were defined by the criteria of Kabsch and Sanders (*29*). The fractional solvent-accessible surface area and the secondary structure of each site substituted with phenylalanine are found in Table 1. Strained backbone conformations were defined as previously published (*30*) and we divide these into residues that are in left-handed helical backbone conformations and all other strained backbone conformations. Large enough numbers of lysine and glutamate residues were substituted with phenylalanine to make averages and ranges meaningful. Relatively small numbers of other residue types were substituted and thus are not as statistically significant. The impact of electrostatic interactions upon the substitution of wild-type lysine and glutamate side chains can also be examined. The effects of phenylalanine substitution upon each of these subdivisions are summarized in Table 2 and their meaning is considered in the discussion.

DISCUSSION

Why are hydrophobic residues seldom found on the surfaces of soluble proteins? There are two possibilities. Solvent-exposed hydrophobic residues may destabilize the folded state relative to the denatured state or they may lead to undesirable protein association or aggregation. It was primarily to determine the stability effects of substituting hydrophobic residues on the surface of a protein that this study was undertaken. Phenylalanine was the residue chosen to substitute into surface sites of staphylococcal nuclease because of its hydrophobicity and size. While other residues such as leucine and isoleucine are about as hydrophobic as phenyalanine (*31*), their side chains are not as large and bulky as phenylalanine and may not expose as much hydrophobic surface area to the solvent because of interactions with other surface residues.

The 47 sites that were mutated to phenylalanine in this study as well as the three mutated in a previous study (*17*) are surface sites. There are a variety of ways to define a residue as a surface residue (*32*). For this work, surface residues were chosen for mutation by inspection, with reference made to solvent accessibility data. Not every solvent-accessible site was chosen, but sites were chosen to give a broad, global distribution across the surface of the protein and to represent a range of solvent accessibility. Most of the side chains substituted with phenylalanine were hydrophilic. However a few solvent-exposed hydrophobic residues were substituted with phenylalanine as well.

Comparison to Alanine Mutants. In previous studies single-site alanine substitutions have been made at virtually every site in nuclease (*25*−*27*). Because of this, we are able to compare the stabilities of the single-site phenylalanine substitutions with the stabilities of the alanine mutants made at the same sites. Figure 2 shows that the stabilities of the phenylalanine mutants correlate well with the alanine mutants ($r = 0.8471$). In general, when an alanine mutant is destabilized with respect to wild type, the phenylalanine residue at the corresponding site is also destabilized to a roughly similar extent. The average loss in stability ($\Delta\Delta G_{F\rightarrow WT}$) for the phenylalanine mutants was 1.1 ± 1.2 kcal/mol, while the average loss in stability ($\Delta\Delta G_{A\rightarrow WT}$) for the alanine mutants at these same sites was 0.6 ± 0.9 kcal/mol. As this implies, the phenylalanine mutants tend to be less stable than the alanine mutants, with an average $\Delta\Delta G_{F\rightarrow A}$ equal to 0.5 kcal/mol. Therefore, it appears that as a general rule the phenylalanine substitutions on the surface of nuclease are almost twice as destabilizing as the alanine substitutions. By way of comparison, the mutation of eleven leucine side chains in staphylococcal nuclease, largely buried in the hydrophobic core, to alanine destabilizes the protein by an average 3.1 ± 1.5 kcal/mol and the nine valine to alanine mutants destabilize the protein by an average 2.4 ± 1.5 kcal/mol (*27*).

Table 2: Average $\Delta\Delta G$ Values for Various Groupings

group	no. in group[a]	avg $\Delta\Delta G_{F\rightarrow WT}$[b]	avg $\Delta\Delta G_{F\rightarrow A}$[c]
hyperexposed sites	10	0.6 ± 0.3	0.3 ± 0.3
exposed sites	17	0.5 ± 0.6	0.2 ± 0.5
accessible sites	8	2.0 ± 1.4	0.6 ± 1.8
marginally accessible sites	14	1.9 ± 1.3	1.1 ± 0.5
α-helical sites	20	1.2 ± 1.1	0.7 ± 0.7
β-sheet sites	6	0.8 ± 0.3	0.1 ± 0.3
turns	13	1.1 ± 1.5	0.4 ± 0.6
left-handed helix	7	2.0 ± 1.6	0.7 ± 0.7
strained backbone conformations	5	0.7 ± 0.5	0.5 ± 0.6
hydrophilic residues (D, E, K, H, Q, R, S)	36	1.2 ± 1.3	0.5 ± 0.6
hydrophobic residues (M, P, Y)	7	0.4 ± 0.6	-0.1 ± 0.3
lysines	17	0.7 ± 0.5	0.3 ± 0.5
glutamates	8	1.6 ± 1.2	0.5 ± 0.5
overall average	50	1.1 ± 1.2	0.5 ± 0.9

[a] The number of mutated sites in wild type that fit the criteria of the group under consideration. [b] The average $\Delta\Delta G_{F\rightarrow WT}$ was calculated from the differences in stability between wild type and the phenylalanine substitution (i.e., $\Delta\Delta G_{F\rightarrow WT} = \Delta G_{WT} - \Delta G_{Phe}$) at each site of interest. The standard deviations are also given. The stability of wild type (ΔG_{WT}) is 5.5 kcal/mol. Units are kcal/mol. [c] The average $\Delta\Delta G_{F\rightarrow A}$ was calculated from the difference in the stability between the alanine and phenylalanine mutants (i.e., $\Delta\Delta G_{F\rightarrow A} = \Delta G_{Ala} - \Delta G_{Phe}$) at each site of interest. The standard deviations are also given. Units are kcal/mol.

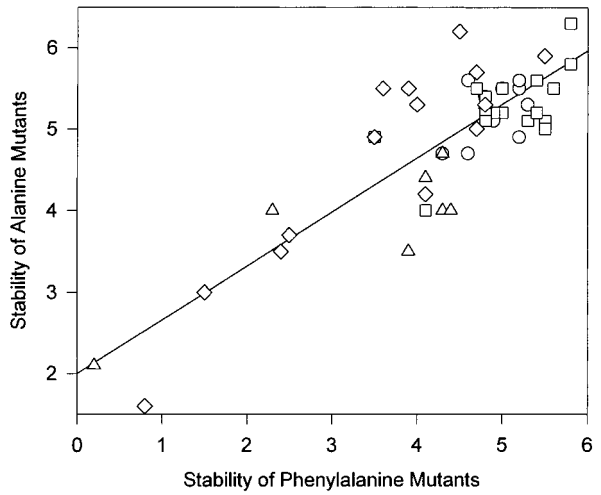

FIGURE 2: The stability of the alanine substitution at a given position versus the stability of phenylalanine substitution at the same site. Energies are in units of kcal/mol. Circles are hyperexposed sites, squares are exposed sites, triangles are accessible sites, and diamonds are marginally accessible sites. The stability of F and A substitutions at residue 143 is not included since solvent accessibility information is not available due to the lack of electron density at this site in the crystal structure.

This provides at least part of the answer to the main question that we wished to address: is the substitution of a large hydrophobic side chain on the surface of proteins usually destabilizing? Yes, it is and significantly more so than the substitution of a small hydrophobic residue. While not as profound in its effects as the truncation of a hydrophobic residue in the core of the protein, putting a hydrophobic residue on the surface does have an energetic cost that is a respectable percentage of the effects of altering a single residue in the core.

While in general a solvent-exposed phenylalanine is destabilizing, such generalizations can be dangerous as there are many exceptions to the rule. When the stabilities of the phenylalanine and alanine mutations are plotted against each other (Figure 2), there are numerous mutants both above and below the regression line. Therefore, there is a strong context dependence involved in determining whether a large hydrophobic side chain is energetically favorable or unfavorable compared to a smaller hydrophobic side chain at a specific surface site in nuclease. This raises two new questions. Why is the substitution of a phenylalanine usually destabilizing? And what factors influence whether a phenylalanine is more or less destabilizing than an alanine mutation? We consider first the influence of different degrees of residue solvent exposure and residue packing.

Solvent Accessibility. The method we have chosen to quantify this property is based on the fractional solvent-accessible surface area of the wild-type residue at each site. The fractional solvent-accessible surface area is a ratio of the solvent-accessible surface area of the amino acid side chain in the protein of interest to the solvent-accessible surface area of that same amino acid in the Ala-Xxx-Ala reference tripeptide (*28*). For example, as shown in Table 1, the solvent-accessible surface area at site K48 in nuclease is 1.28. This means that the lysine residue at site 48 is 28% more solvent-exposed in native nuclease than it is in the Ala-Lys-Ala model tripeptide.

With this in mind, we defined four different categories of surface sites on the basis of their fractional solvent-accessible surface area. These categories were hyperexposed sites, exposed sites, accessible sites, and marginally accessible sites. The hyperexposed sites were defined as those sites whose fractional solvent-accessible surface area was equal to or greater than 1. There were 10 of these sites chosen. Sites with a fractional solvent-accessible surface area of 0.5 to 0.99 were defined as exposed sites. Seventeen of these sites were chosen. The accessible sites were those sites that have a fractional solvent-accessible surface area of 0.1 to 0.49. There were 8 of these sites that were used in this study. Sites with a fractional solvent-accessible surface area of 0.0 to 0.09 were defined as marginally accessible sites. There were 14 of these sites chosen.

A possible pitfall of defining solvent accessibility in this manner is illustrated by the marginally accessible sites. These residues are typically those with small side chains that can be protected from the solvent by nearby residues with larger side chains. For example, G29 has little solvent exposure because of the lysine residue nearby at site 28. In addition, the C_α of G29 is approximately 4.1 Å away from the side chain oxygen of Y27. These residues shield the

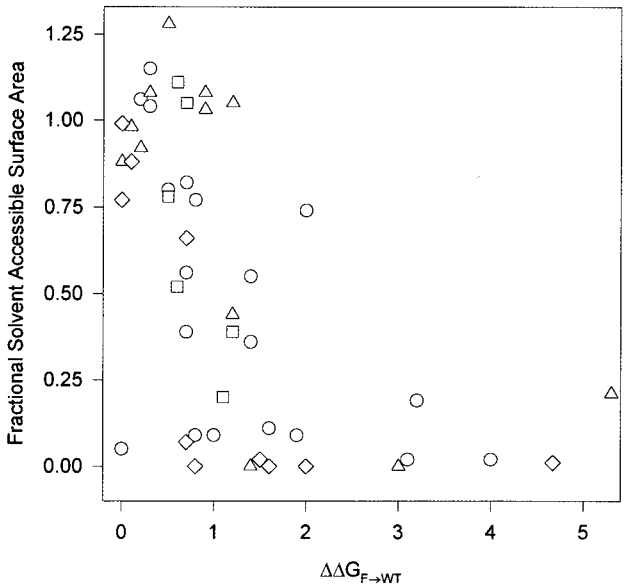

FIGURE 3: The fractional solvent-accessible surface area of the wild-type side chain at each site substituted with phenylalanine versus the difference in stability between the substituted protein and the wild-type protein ($\Delta\Delta G_{F\rightarrow WT} = \Delta G_{WT} - \Delta G_{Phe}$) at each site. Circles are sites in α helices, squares are sites in β sheets, triangles are sites in turns, and diamonds are sites that are not located in any type of secondary structure as defined by Kabsch and Saunders (*29*). The stability of the F substitution at site 143 is not included since solvent accessibility information is not available due to the lack of electron density at this site in the crystal structure.

glycine at site 29 from the solvent even though the glycine residue is located on the surface of the protein in a β turn, as readily apparent from the examination of the crystal structure (*23*, *24*). Any phenylalanine substituted in this site will be quite solvent exposed. Therefore what the fractional solvent accessibility value conveys is not necessarily how solvent-accessible a phenylalanine will be but rather some sense of how crowded the vicinity is and whether other residues will interact with the newly substituted side chain. Such interactions are, of course, potentially stabilizing or destabilizing.

The average stability changes for each of the four solvent accessibility groups are summarized in Table 2 and illustrated more fully in Figure 3. The hyperexposed or exposed sites are roughly equivalent and are significantly less destabilized when substituted with phenylalanine than are the accessible and marginally accessible groups, which are also roughly equivalent to each other. This trend seems intuitively satisfying. Again, a low value of fractional solvent accessibility of the wild-type residue indicates that one or more other residues interact with it. Substitution with the bulky phenylalanine side chain could disrupt favorable and/or introduce unfavorable interactions. Thus, even though residues on the surface of proteins are more mobile than the hydrophobic core, as evidenced by the surface residues' generally higher temperature factors and greater degree of disorder in crystal structures, steric interactions and packing considerations on the surface of a protein are also important.

The Reverse Hydrophobic Effect. The reverse hydrophobic effect was proposed (*8*) as a possible explanation for the observation that substitutions at a hyperexposed site in λ Cro repressor showed decreasing stability as the hydrophobicity of the substituted residue was increased. It was suggested that such a site may be less solvent-exposed in the denatured state than in the native state. Therefore increasing the hydrophobicity of the side chain buries more hydrophobic surface in the denatured state and stabilizes it. While another explanation has been proposed for this particular observation in λ Cro repressor (*9*), others have reported possible evidence of the reverse hydrophobic effect at exposed sites in different proteins (*10*−*13*). If this is a common effect, it could be a major reason for the relative scarcity of solvent-exposed hydrophobic residues in proteins.

If the reverse hydrophobic effect were common in nuclease, we would expect to find phenylalanine substitution mutants at hyperexposed sites much less stable than alanine substitutions. Positions in nuclease where such evidence for the reverse hydrophobic effect is present are rare. In the case of hyperexposed residues, we find most phenylalanine mutations either had the same stability as the corresponding alanine mutant or were slightly less stable than the alanine mutant. As shown in Table 2, the average difference in stability between the alanine and phenylalanine mutants at hyperexposed sites was 0.3 ± 0.3 kcal/mol with the alanine mutants being more stable. The average difference in stability between the phenylalanine and alanine mutations for the exposed sites was only 0.2 ± 0.5 kcal/mol with the alanine mutants being more stable. Indeed, there were 6 exposed sites where the phenylalanine mutant was more stable than the alanine mutant. A phenylalanine side chain has one of the greatest hydrophobic-transfer free energies. For example, on the Fauchère−Pliška scale (*31*) the hydrophobic-transfer free energy of phenylalanine is −2.40 kcal/mol and of alanine is −0.42 kcal/mol. Thus, assuming complete solvent exposure in the native state and complete burial in the denatured state, a rough estimate of the maximal destabilizing reverse hydrophobic effect of a phenylalanine relative to an alanine is 2 kcal/mol. We believe that, for most of these sites, the minimal difference in stability between the relatively hydrophobic phenylalanine and the less hydrophobic alanine argues that the reverse hydrophobic effect is either very slight or nonexistent.

One site where the reverse hydrophobic effect may be acting was K84 since the K84F mutation was 1 kcal/mol less stable than the alanine mutant (Table 1). This difference in stability is much larger than the average difference between phenylalanine and alanine mutants at hyperexposed sites in general (*8*, *31*). There is also one exposed site that could be a possible candidate for evidence of the reverse hydrophobic effect. This was the K63 site where the phenylalanine mutant is 1.4 kcal/mol less stable than the alanine mutant at this site. But these are the exception to the rule. As Table 2 indicates, most cases where the phenylalanine is much less stable than the alanine involve positions with lower solvent accessibilities. A site with a lower accessibility seems more likely to be destabilized by unfavorable packing interactions in the native state.

Phenylalanine substitutions are in general somewhat less stable than alanine substitutions or the usually hydrophilic wild-type side chains and this may indeed be due to a reverse hydrophobic effect, but we estimate on the basis of the generally low value of $\Delta\Delta G_{F\rightarrow A}$ at hyperexposed and exposed positions that it can be no more than a few tenths of a kcal/mol. A reverse hydrophobic effect of a large energetic magnitude, if it occurs at all, occurs only at a small subset

of hyperexposed and exposed sites in staphylococcal nuclease.

Evidence For Hydrophobic Interactions Between Surface Residues. There also appears to be no evidence for the formation of "small exterior hydrophobic clusters" (SEHCs) (*15*) in staphylococcal nuclease upon hydrophobic substitution. We made substitutions at marginally solvent-accessible sites, in part to test for the formation of SEHCs. We thought it possible that the hydrophobic portions of the large neighboring hydrophilic side chains such as lysine and arginine could form favorable hydrophobic interactions with the substituted phenylalanine at these sites and perhaps stabilize the protein, as has been described previously in other proteins (*14*, *16*). As noted above, the relative hydrophobic-transfer free energies (*31*) mean that a phenylalanine could potentially stabilize a native state by about 2 kcal/mol relative to an alanine. However, it is also possible that large neighboring hydrophilic side chains could interact with the substituted phenylalanine unfavorably. For example, steric crowding, side chain repulsion, or unfavorable torsion angles all could destabilize the protein. Frigerio et al. (*16*) mention that these types of problems occurred in one of their designs when they engineered an SEHC into the neutral protease of *B. subtilis*.

The average loss in stability ($\Delta\Delta G_{F\rightarrow WT}$) at the marginally accessible sites was significantly higher than the average stability losses at the hyperexposed and exposed sites. Interestingly, the average stability loss at the marginally accessible sites was about the same as the average stability loss at the accessible sites. Since this is the case, it seems highly unlikely that a SEHC exists at any of the accessible or marginally accessible sites. In fact, not one of the phenylalanine mutants at the marginally accessible sites was more stable than wild type and only one, S59F, had the same stability as wild type. However, it is not surprising that S59F had at least the same stability as wild type because this site is in an α helix and serine is known to be a nonhelix-stabilizing residue (*33*, *34*). In fact, from this perspective it is somewhat surprising that the S59F mutant is not more stable than wild type since phenylalanine is a helix-stabilizing residue (*33*, *34*). Moreover, the less hydrophobic alanine mutation at position 59 is more stable than the hydrophobic phenylalanine. All of this indicates that it is highly unlikely that this site is an example of a new SEHC.

The lack of newly formed SEHCs says nothing about whether stabilizing hydrophobic interactions might already be present on the surface of the wild-type protein. This can be addressed by examining the effects of mutating the seven surface residues that are reasonably large hydrophobes to begin with (P11, P47, M65, Y85, Y113, Y115, and P117). The average loss in stability ($\Delta\Delta G_{F\rightarrow WT}$) for these sites is 0.4 ± 0.6 kcal/mol. This is less destabilizing than the overall average of phenylalanine substitutions. Is this because the phenylalanines can partake in some stabilizing hydrophobic interaction? Probably not, since the average difference in stability between the alanine and the phenylalanine mutants ($\Delta\Delta G_{F\rightarrow A}$) at these seven sites was very small, only -0.1 ± 0.3 kcal/mol, and an alanine does not provide much scope for the hydrophobic interaction. Overall, this indicates that while substituting a surface hydrophobic site with another hydrophobic residue is not as destabilizing as substituting a surface hydrophilic residue with a hydrophobic residue, the effect is more likely due to greater tolerance of the phenylalanine's size rather than the presence of some stabilizing hydrophobic interaction(s) in a solvent-exposed hydrophobic cluster.

Helical Propensities. It is well-known that some amino acids are frequently part of α helices (e.g., alanine) while others are very seldom found in α helices (e.g., glycine or proline). This has led to the idea of helical propensities where the attempt is made to assess in some energetically quantitative manner the preference for a given type of residue to a reside in a helix (*33*−*40*). Typically these studies place each of the twenty amino acids in a given solvent-exposed position of a protein or peptide system and measure the differences in stability. These differences are usually attributed to the relative propensity of the substituted amino acid to adopt helical conformations. Many of the phenylalanine substitutions made for this work were in helical sites as determined by the method of Kabsch and Saunders (*29*). Since these same helical sites were mutated previously to alanine and glycine (*25*−*27*), it was possible to consider that the stability differences between these three side chains at helical sites could be due in whole or part to differences in the helical propensity. In contrast to earlier studies, although we consider only the relative stability of three residues, we do so at a large number of sites.

Table 3 shows the average differences in stability between phenylalanine, alanine, and glycine mutations at 20 solvent-exposed helical positions in the nuclease. While our calculated helical propensities are in the same rank order as most of those described previously (*33*−*39*), our values are usually larger. In addition, the standard deviations for these twenty sites are nearly as large as the "helical propensity" values that we find. This clearly indicates that there are other energetically significant interactions specific to each site and the changes in stability at these sites cannot be attributed solely to the differences in the helical propensities of the amino acids. This conclusion is reinforced if we also consider the effects of solvent exposure.

We examined the average differences in stability between the three residues for each helical position using the four classifications of solvent exposure previously described. Very similar averages were observed for helical hyperexposed and exposed positions, so these were grouped together. The accessible and marginally accessible helical positions also showed very similar averages, but distinct from the hyperexposed and exposed positions, so accessible and marginally accessible positions were grouped together. As Table 3 shows, the average difference between alanine and glycine at a hyperexposed or exposed site is less than the difference at an accessible or marginally accessible site. The range of effects, as illustrated by the standard deviations, is also less for the more exposed positions. Similarly, the average difference in free energy between a phenylalanine and a glycine residue in a helical position is less at the hyperexposed and the exposed sites than at the accessible and marginally accessible sites and the standard deviation of these differences is greater for the less solvent-accessible positions. This is precisely what one expects. The less exposed residues, that is, those in more crowded environments, show a wider range of effects. But even exposed residues show a wide range of stability effects.

Table 3: Helical Propensities

	$\Delta\Delta G_{G\rightarrow A}$	$\Delta\Delta G_{G\rightarrow F}$	$\Delta\Delta G_{F\rightarrow A}$
all helical residues[a]	1.2 ± 0.7	0.6 ± 0.9	0.7 ± 0.7
hyperexposed and exposed residues[b]	0.9 ± 0.5	0.5 ± 0.7	0.4 ± 0.4
accessible and marginally accessible residues[c]	1.5 ± 0.8	0.6 ± 1.2	0.9 ± 0.8
O'Neil and DeGrado[d]	0.77	0.41	0.36
Horowitz et al.[e]	0.91	0.22	0.69
Blaber et al.[f]	0.96	0.59	0.37
Charkrabartty et al.[g]	1.88	0.95	0.93
Yang et al.[h]	1.05	−0.06	1.11
Myers et al.[i]	0.90	0.34	0.56
Myers et al.[j]	0.98	0.37	0.61

[a] The average difference in free energy between two different side chains (e.g., $\Delta\Delta G_{G\rightarrow A} = \Delta G_{Ala} - \Delta G_{Gly}$) at each helical site mutated in staphylococcal nuclease. Units are kcal/mol. Stability of alanine and glycine mutants at helical positions in staphylococcal nuclease is from refs *25*−*27*. [b] The average difference in free energy between two different side chains (e.g., $\Delta\Delta G_{F\rightarrow A} = \Delta G_{Ala} - \Delta G_{Phe}$) at each hyperexposed and exposed helical site mutated in staphylococcal nuclease. Units are kcal/mol. The stability of alanine and glycine mutants at helical positions in staphylococcal nuclease is from refs *25*−*27*. [c] The average difference in free energy between two different side chains (e.g., $\Delta\Delta G_{G\rightarrow F} = \Delta G_{Phe} - \Delta G_{Gly}$) at each accessible and marginally accessible helical site mutated in staphylococcal nuclease. Units are kcal/mol. Stability of alanine and glycine mutants at helical positions in staphylococcal nuclease is from refs *25*−*27*. [d] Helical propensities calculated by placing the residues into a guest position in a noncovalent homodimer of α helices. Units are kcal/mol. The sign convention of the energy change has been reversed from the original work (*33*). [e] Helical propensities calculated by the substitution of different amino acids into the solvent-exposed site Ala32 in the second α helix of barnase. Units are kcal/mol. The sign convention of the energy change has been reversed from the original work (*35*). [f] Helical propensities calculated by the substitution of different amino acids into two sites of an α helix in T4 lysozyme (*33*). Units are kcal/mol. [g] Helical propensities measured by placing amino acid residues into an alanine-based peptide in the absence of helix-stabilizing side chain interactions. The sign convention of the energy change has been reversed from the original work. Units are kcal/mol (*36*). [h] Helical propensities measured by placing amino acid residues into the peptide succinyl-YSEEEEKAKKAXAEEAEKKKK−NH_2, where X is the position where the amino acid residue is placed into the peptide. The sign convention of the energy change has been reversed from the original work. Units are kcal/mol (*38*). [i] Helical propensities measured at site 21 in the single α helix of RNase T_1. The values listed are the free-energy values relative to Gly (*37*). [j] Helical propensities measured in a peptide with the same sequence as the single α helix of RNase T_1. The values listed are free-energy values relative to Gly (*37*).

The importance of the environment is demonstrated in another example. Six surface helical lysine residues (K63, K64, K70, K127, K133, and K134) and five surface helical glutamate residues (E57, E67, E101, E122, and E129) were mutated in this study. The stability of the wild-type residue, lysine or glutamic acid, can be used with the stability of the alanine, glycine, and phenylalanine mutations at these same sites to calculate the average helical propensity values relative to glycine. The rank order of our helical propensities calculated for the lysine sites was K > A > F > G and for the glutamate sites was E > A > F > G. These results are different from the rank order of helical propensities previously reported which is usually A > K > F > E > G (*33*−*39*). This demonstrates that the environment of the helical site is very important in determining the best helical residue for a particular site since in both cases the wild-type residue had the best helical propensity.

Consequently, we believe that the disagreement in helical propensities reported by other workers can be explained largely by the differences in the environment of the particular site where the substitutions were done. Further, we believe that our data clearly shows the danger of interpreting stability changes in any system as being solely due to any one factor.

Other Factors. We also examined the effects of several other factors, including the identity of the wild-type side chain, the presence or absence of side chain electrostatic interactions, and the effects upon residues in β sheets, turns, and strained backbone conformations. These effects are summarized in Table 2, and because of the generally lower numbers of residues involved and/or the lack of clear conclusions, only a few noteworthy points will be discussed briefly.

Residues that are in a left-handed helix or other strained backbone conformations are notably different from average in their behavior (Table 2). Much of this, though not all, is due to four wild-type glycine residues which as a subgroup are even more notably divergent from the overall averages. The average $\Delta\Delta G_{F\rightarrow WT}$ value of 1.8 ± 0.8 kcal/mol at these four wild-type glycine sites and especially the average $\Delta\Delta G_{G\rightarrow A}$ value of -0.9 ± 0.8 kcal/mol are significantly different from the overall averages of 1.1 ± 1.2 and 0.6 ± 0.9 kcal/mol, respectively. Thus, as previously noted (*30*), an alanine substitution at these positions is worse than an alanine substitution elsewhere and a phenylalanine substitution is more unfavorable than a phenylalanine substitution elsewhere. However, less predictably, the average $\Delta\Delta G_{F\rightarrow A}$ value of 0.9 ± 0.5 kcal/mol is much greater than the overall average of 0.5 ± 0.9 kcal/mol. This indicates that the phenylalanine substitution is more unfavorable than the alanine substitution seems to predict. It is not clear whether this is due to the strained conformation of the backbone in wild type, the low solvent accessibility of these positions, or some other factor(s).

Electrostatic interactions are a common occurrence between hydrophilic residues, and phenylalanine substitution obviously disrupts such interactions. (A table summarizing all electrostatic interactions that involve side chains substituted with phenylalanine is available as Supporting Information in the microfilm edition.) Only three of the lysine side chains substituted are involved in salt bridges or hydrogen bonds. Though of limited statistical significance, it is interesting to note that mutation to either an alanine or a phenylalanine at these three sites was generally more destabilizing than the average found for the 14 lysines not participating in a salt bridge or hydrogen bond. All but two of the glutamate side chains are involved in salt bridges or hydrogen bonds, opposite of the situation found for lysine side chains. Again, mutation to a phenylalanine or an alanine at the two sites not participating in a salt bridge or hydrogen bond was generally less destabilizing than for the six sites

that do have electrostatic interactions. Although this is suggestive, the range of values found does not allow clear conclusions about the energetic significance of electrostatic interactions.

Similar Sites, Similar Stabilities? There are many different factors to consider when trying to explain differences in stability at different sites in a protein. These factors include changes in hydrophobicity, electrostatic interactions, loss of hydrogen bonds, differences in solvent accessibility between sites, and differences in secondary structure. One could look at a single site and using these different factors rationalize the reasons for a decrease or an increase in the stability due to a mutation at that particular site. However, such ad hoc rationalizations have a strong possibility or even probability of being incorrect. In contrast, one can look at similar mutations at different sites that have at least some similar characteristics, such as solvent accessibility and secondary structure, and see if these are sufficient to give mutants similar stabilities.

Mutants K64F, K70F, and K127F provide one example. All had solvent exposure in the hyperexposed group as defined previously, all were in α helices, and none of them participated in any side chain hydrogen bonding according to the crystallographic data (*24*). Interestingly, their stability values are all the same within experimental error. Similarly, the two mutants K49F and K116F which are both in turns and are both in the exposed solvent-accessible group, also have stabilities that are the same within experimental error. These examples are still not entirely convincing since the stability changes upon alanine substitution are different and the loss of stability is quite small at these sites. Perhaps more convincing would be the mutants K16F and K24F, which also have the same stabilities within experimental error, but with a more significant stability drop. These mutants are both in β sheets and have solvent-accessible surface areas in the exposed range as well. The effects of alanine substitution at these sites are also identical within experimental error. Consequently it appears that if the environments of the two positions are indeed sufficiently similar, even by these admittedly crude measures of the environment, identical substitutions have very similar energetic effects.

CONCLUSIONS

In general, placing the large hydrophobic phenylalanine side chain on the surface of nuclease was destabilizing, more so than the small hydrophobic alanine. Although surface residues are widely regarded as tolerant of substitution, the energetic changes observed here are not negligible and, while on average are somewhat smaller, they are comparable in magnitude to the energetic effects of changes in the core of the protein. However, just as in the core, the magnitude of the destabilization or even stabilization due to a hydrophobic surface substitution is largely context dependent. For example, it appears that hyperexposed and exposed surface sites are better able to tolerate a large hydrophobic residue than accessible and marginally accessible surface sites. This indicates that, despite the apparently greater freedom of the motion of surface residues, steric and packing considerations can be important on the surface of a protein. Another example of context dependence is demonstrated by the wide range of helical propensities for surface helical sites in nuclease, indicating that the idea of measuring helical propensities using just one site in a protein or peptide should be viewed with caution.

Little evidence was found for the occurrence of an energetically significant reverse hydrophobic effect at exposed and hyperexposed residues. Such an effect may be operating, but does not generally appear to cause unfavorable energy changes of more than a few tenths of a kcal/mol. Therefore, in the absence of another plausible mechanism attributing the destabilizing effects of solvent-exposed hydrophobic residues to their hydrophobic character, we attribute the majority of the generally unfavorable energetic changes found when phenylalanine substitutions are made to unfavorable steric and packing interactions. This is consistent with the greater energetic effects found at less solvent-exposed sites and the comparable effects of alanine mutations at all sites.

Specific examples of the generation of favorable packing and hydrophobic interactions on the surface of proteins are found in the literature. However, the formation of such small exterior hydrophobic clusters does not appear to occur readily in nuclease upon phenylalanine substitution. It does seem likely that the unfavorable stability changes observed here act as a selective pressure to prevent the appearance of large hydrophobes on the surface. We see no reason, a priori, why stabilizing hydrophobic and van der Waals interactions could not be formed by optimizing the packing of side chains on the surface through further substitutions. The evolution of well-packed, optimized hydrophobic exteriors is more likely prevented, or favored, by the effects of protein association and aggregation. Therefore, we again conclude that although phenylalanine is not frequently found on the surface of proteins, it is not generally because of some destabilizing effect due to residue hydrophobicity.

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SUPPORTING INFORMATION AVAILABLE

A table containing information about the hydrogen bonding and electrostatic interactions of residues substituted with phenylalanine (3 pages). Ordering information is given on any current masthead page.

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