Energetics of Side Chain Packing in Staphylococcal Nuclease Assessed by Exchange of Valines, Isoleucines, and Leucines†

Jason B. Holder,‡ Allen F. Bennett,§ Junmei Chen,|| Daniel S. Spencer, Michael P. Byrne,⊥ and Wesley E. Stites*

Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701-1201

Received June 19, 2001; Revised Manuscript Received September 11, 2001

ABSTRACT: To examine the importance of side chain packing to protein stability, each of the 11 leucines in staphylococcal nuclease was substituted with isoleucine and valine. The nine valines were substituted with leucine and isoleucine, while the five isoleucines, previously substituted with valine, were substituted with leucine and methionine. These substitutions conserve the hydrophobic character of these side chains but alter side chain geometry and, in some cases, size. In addition, eight threonine residues, previously substituted with valine, were substituted with isoleucine to test the importance of packing at sites normally not occupied by a hydrophobic residue. The stabilities of these 58 mutant proteins were measured by guanidine hydrochloride denaturation. To the best of our knowledge, this is the largest library of single packing mutants yet characterized. As expected, repacking stability effects are tied to the degree of side chain burial. The average energetic cost of moving a single buried methyl group was 0.9 kcal/mol, albeit with a standard deviation of 0.8 kcal/mol. This average is actually slightly greater than the value of 0.7−0.8 kcal/mol estimated for the hydrophobic transfer energy of a methylene from octanol to water. These results appear to indicate that van der Waals interactions gained from optimal packing are at least as important in stabilizing the native state of proteins as hydrophobic transfer effects.

How important are side chain packing considerations in determining protein structure and stability? One answer to this question is found in the fact that Nakagawa et al. (*1*) discovered the only way to classify vertebrate cytochrome *c* sequences into an acceptable evolutionary cladrogram was to group them by side chain volume and length. This finding that steric factors may be more important in determining the course of protein evolution than functional group conservation or codon similarity is less surprising when one recalls that the hydrophobic cores of proteins are as tightly packed as organic crystals (*2*) and that cavities in protein interiors tend to be few in number and small (*3*−*5*). When a protein is well-packed, it optimizes van der Waals interactions and minimizes the presence of cavities. A cavity represents the loss of potential van der Waals interactions or, as they are sometimes termed, London dispersion forces, and cavity formation also carries an entropic cost.

There is now much evidence that the hydrophobic interior of a protein contains much of the "folding information". Mutational studies show that the tolerance for substitution is clearly lower for buried residues than for surface residues (*6*−*8*). The most extensive demonstration of this principle is in the T4 lysozyme system where Rennell et al. (*9*) assessed the tolerance of 163 of 164 positions for 13 different side chains. The least tolerant sites clearly tended to be buried residues. However, the changes that were tolerated in the protein interior tended to be of one hydrophobic residue for another. Other experimental studies have explored the effects of substituting one buried residue for another (*10*−*24*) but have not yet fully answered the question of how side chain packing dictates what hydrophobic amino acids are acceptable at which buried positions. Reviews (*5*, *25*−*30*) have summarized much of what is known about the effects of perturbing the packing of the hydrophobic cores of proteins by altering side chain size and shape. It is notable that success in predicting the effect of changes in side chain size and shape upon protein stability has been limited.

An obstacle to better theoretical understanding of this problem is the lack of significant numbers of packing mutants with characterized thermodynamic stabilities. While experiments such as those of Rennell et al. (*9*) and Lim and Sauer (*10*, *12*) have examined large numbers of mutant proteins, these proteins have often been examined only by some qualitative functional assay. For this reason, the stability effects of altering side chain packing in the hydrophobic core of staphylococcal nuclease (nuclease) are quantitatively investigated in this work. Specifically, the nine valines of nuclease were individually replaced with isoleucine and leucine; the 11 leucines were substituted with isoleucine and valine, and the five isoleucines, previously substituted with valine (*31*), were substituted with leucine and methionine. In addition, at eight threonine sites earlier replaced with valine (*32*), we have now substituted the larger isoleucine side chain. The thermodynamic stability of each mutant was

† Funding for this work was provided by NIH Grant GM52714.

* To whom correspondence should be addressed. Phone: (501) 575-7478. Fax: (501) 575-4049. E-mail: wstites@uark.edu.

‡ Current address: College of Medicine, University of Arkansas for Medical Sciences, 4301 W. Markham St., Little Rock, AR 72205.

§ Current address: University of Arkansas School of Law, Waterman Hall, Fayetteville, AR 72701.

|| Current address: N1302 Alkek Research Building, One Baylor Plaza, Baylor College of Medicine, Houston, TX 77030.

⊥ Current address: Human Genome Sciences, 9410 Key West Ave., Rockville, MD 20850-3338.

10.1021/bi011267t CCC: $20.00
Published on Web 10/26/2001

determined by guanidine hydrochloride denaturation. This is a large increase in the number of thermodynamically characterized packing mutants. These stabilities are compared to those of other substitutions previously made at these same sites (*31*−*33*). Analysis of these data provides insight into how dependent the protein's stability is on the particular amino acid side chain present at a given site and how important packing is relative to other factors that influence protein stability. This work also serves as a foundation for the detailed study of multiple packing mutants described in the articles that immediately follow.

EXPERIMENTAL PROCEDURES

Mutagenesis. Mutants were prepared by the method of Kunkel (*34*, *35*) as previously described (*32*).

Protein Expression and Purification. Protein expression and purification followed the procedures previously described (*31*, *32*).

Protein Stability Determination. The stabilities of each of the mutants in this study were characterized by guanidine hydrochloride denaturation at 20 °C using fluorescence as a probe of structure as previously described (*32*, *36*, *37*). We have found that the storage of staphylococcal nuclease samples in frost-free freezers slowly leads to metastable dimer formation just as it does for some other proteins (*38*). These metastable dimers tend to slightly increase the apparent stability of the protein but can be easily removed by briefly preheating the sample to 40−50 °C (*39*). We have found that for most mutants there is very little difference in apparent stability whether this preheating step is done, presumably because stabilities are usually measured very shortly after proteins are prepared and also because the effects are not large even in cases where prolonged storage leads to large amounts of the dimer. However, for wild-type staphylococcal nuclease, we have in the past routinely stored the protein for fairly long periods and even minor effects are important because we reference stability changes to the stability of the wild type. Our values for the wild-type denaturation have therefore been slightly elevated when our numerous wild-type runs were averaged. Average values of denaturations performed after alteration of our storage conditions and institution of the preheating procedure described above are slightly different from those previously reported (*32*, *39*). We believe this new value for the wild type should be used when calculating changes in mutant stability from our previously published mutant protein stabilities. The estimated errors in wild-type stability remain the same as these are based on the random errors from determination to determination, not the systematic error corrected here.

Solvent Accessible Surface Calculations. The solvent accessible surface was calculated using the Whatif software package (*40*) and the 1.6 Å wild-type staphylococcal nuclease structure [PDB entry 1EY0 (*41*)]. The probe was 1.4 Å, and the contact surface was measured. The percentage accessibility of each wild-type residue was calculated by comparison to the accessibility of the same type of residue in an extended Gly-XXX-Gly tripeptide model (*42*).

RESULTS

Each of the nine valines in wild-type staphylococcal nuclease was substituted with isoleucine and leucine, and each of the 11 leucines was substituted with isoleucine and valine. The five isoleucines, previously substituted with valine (*31*), were substituted with leucine and methionine. Previously published work details the effects of mutating all the valines, isoleucines, and leucines in nuclease to alanine and glycine (*31*) and all the valines to threonine and serine (*32*).

In addition to the substitutions at valine positions, threonines at eight positions were individually replaced with isoleucine. Threonine to valine mutations have previously been constructed and characterized by fluorescence solvent denaturation (*32*). These two residues obviously differ greatly in hydrophobicity, but are very similar in size and shape.

Each of the mutants in this study was subjected to a solvent denaturation using fluorescence as a probe of structure. These data were analyzed as previously described (*32*, *36*) to determine the stability difference in the absence of denaturant between a protein's native and denatured states (ΔG_{H_2O}), the rate of change of free energy with respect to GuHCl concentration [m_{GuHCl} or d(ΔG)/d[GuHCl]], and the concentration of guanidinium hydrochloride at which the protein is half-denatured (C_m). These results are summarized in Table 1. The V66L, I72V, I92V, and I18M mutant proteins have been previously described (*43*) but were remade here because of their importance to subsequent work reported in the articles that immediately follow. Our independently created V66L and I92V mutants had, within experimental error, denaturation behavior identical to that previously reported.

Table 1: Guanidine Hydrochloride Denaturation Parameters

	m_{GuHCl}[a]	C_m[b]	ΔG_{H_2O}[c]		m_{GuHCl}[a]	C_m[b]	ΔG_{H_2O}[c]
V23I	6.08	0.86	5.2	L7V	5.90	0.68	4.0
V23L	6.39	0.83	5.3	L14I	7.05	0.56	3.9
V39I	6.61	0.84	5.5	L14V	7.14	0.55	3.9
V39L	6.68	0.68	4.5	L25I	7.05	0.52	3.7
V51I	6.54	0.83	5.5	L25V	6.89	0.53	3.6
V51L	6.28	0.82	5.2	L36I	6.64	0.35	2.3
V66I	5.94	0.74	4.4	L36V	7.35	0.26	1.9
V66L	5.35	0.95	5.1	L37I	6.00	0.57	3.4
V74I	7.01	0.51	3.6	L37V	5.53	0.43	2.4
V74L	6.35	0.66	4.2	L38I	6.47	0.51	3.3
V99I	6.40	0.81	5.2	L38V	6.41	0.81	5.2
V99L	6.27	0.82	5.1	L89I	6.49	0.67	4.3
V104I	6.41	0.87	5.6	L89V	6.69	0.60	4.0
V104L	6.48	0.71	4.6	L103I	6.68	0.65	4.3
V111I	6.74	0.70	4.7	L103V	6.82	0.50	3.4
V111L	6.61	0.69	4.5	L108I	6.02	0.32	2.0
V114I	6.68	0.79	5.3	L108V	6.31	0.25	1.5
V114L	6.48	0.66	4.3	L125I	6.54	0.68	4.4
I15L	6.65	0.72	4.8	L125V	6.57	0.47	3.1
I15M	6.18	0.82	5.1	L137I	6.50	0.71	4.6
I18L	6.45	0.81	5.2	L137V	6.58	0.60	4.0
I18M	6.75	0.69	4.7	T13I	6.50	0.82	5.3
I72L	6.67	0.78	5.2	T22I	6.40	0.74	4.7
I72M	6.99	0.52	3.6	T33I	6.34	0.95	6.0
25I72V	6.94	0.60	4.2	T41I	6.14	1.00	6.1
I92L	6.68	0.73	4.8	T44I	6.48	0.74	4.8
I92M	7.09	0.53	3.8	T62I	5.19	0.72	3.7
I92V	6.68	0.75	5.0	T82I	6.51	0.90	5.9
I139L	6.55	0.81	5.3	T120I	5.99	0.61	3.7
I139M	6.66	0.76	5.0	wild type	6.53	0.82	5.4
L7I	6.30	0.72	4.5				

[a] Slope value (change in free energy with respect to the change in guanidine hydrochloride concentration). Units of kcal mol^{-1} M^{-1}. The error is estimated to be ±0.09. [b] Midpoint concentration (concentration of guanidine hydrochloride at which half of the protein is denatured) in units of molar. Error estimated to be ± 0.01 M. [c] Free energy difference between native and denatured states in the absence of denaturant. Units of kcal/mol. Error estimated to be ±0.1 kcal/mol.

The stability of an independently created I72V mutation proved to be 0.6 kcal/mol greater than the value previously published (*31*). This result was confirmed by a repeat determination of stabilities and a third independent creation of the mutation. We are quite confident that the earlier value is in error. The I18M mutant reported here is slightly less stable, by 0.2 kcal/mol, than that reported previously (*43*).

DISCUSSION

The exchange of a valine, isoleucine, or leucine residue with another one of these aliphatic side chains is the most conservative change that can be made to assess the role of packing in protein stability. With the exception of the truncation of a methyl group that occurs when substituting isoleucine with the smaller valine residue, which can in theory be accommodated without rearrangement of either the main chain or side chains, all other exchanges between these residues would require some change in the packing of adjacent residues.

While we are interested in determining the changes in protein stability due to the packing, it is important to note that this is not the only way that the substitutions made here can affect protein stability. Each of the three side chains (valine, isoleucine, and leucine) has a distinct hydrophobic transfer free energy. Each side chain has different degrees of conformational freedom for the side chain and main chain in the denatured state and thus varies in the entropic cost of freezing into a single conformation in the native state. We can estimate the energetic consequences of these effects. The leucine and isoleucine side chains have similar hydrophobic transfer free energies when being transferred from octanol to water (*44*, *45*), 2.27 and 2.40 kcal/mol relative to glycine, respectively. The energy for the smaller valine side chain is significantly lower, only 1.61 kcal/mol. The energetic costs at 20 °C of changing the entropic freedom of the polypeptide backbone by substitution of a valine, isoleucine, or leucine side chain for one another are negligible, only 0.04 kcal/mol difference between isoleucine and leucine and a 0.02 kcal/mol difference between valine and leucine or isoleucine (*46*), reflecting perhaps the very similar nature of these three side chains. The energetic costs of lost degrees of freedom between the denatured and native states are very similar for the leucine and isoleucine side chains, only 0.03 kcal/mol difference (*47*). However, a valine side chain has even fewer degrees of freedom lost upon folding to the native state, so substitution with either a leucine or isoleucine is expected to destabilize the native state by approximately 0.5 kcal/mol. This cancels out much of the difference in hydrophobic transfer free energies. This simple calculation predicts that substituting a buried valine side chain with a leucine side chain will stabilize the native state relative to the denatured state by 0.18 kcal/mol; substitution with an isoleucine stabilizes the native state relative to the denatured state by 0.32 kcal/mol. Of course, it is debatable whether the transfer free energy values for transfer from octanol to water are correct for side chain burial in a protein (*48*) and equally debatable whether the calculated values for entropy costs are correct. Still, in the absence of packing effects, it seems reasonable to predict substitution of an isoleucine or leucine for a buried valine should slightly stabilize the native state and that exchange of isoleucine and leucine should be energetically neutral.

Nevertheless, there is no instance in the experimental data presented in Table 1 where substitution of an isoleucine or leucine for a valine present in the wild-type structure of staphylococcal nuclease results in a more stable protein. Indeed, the larger leucine and isoleucine side chains often result in a less stable protein or, at best, one with stability equal to that of the wild type. Similarly, substitution of a wild-type isoleucine with a leucine or a wild-type leucine with a isoleucine is only rarely energetically neutral and is usually unfavorable.

It is tempting to rationalize each result in Table 1. For example, the data show that two of the most destabilized mutations occur at position L108, with stabilities for the isoleucine- and valine-substituted proteins of 2.0 and 1.5 kcal/mol, respectively. Aurora et al. (*49*) have pointed out that L103 and L108 interact in a Schellman motif, capping the carboxyl termini of an α-helix. The low stabilities of the L108 mutations indicate that the requirements at this position are quite specific. Because the difference in stability of the two L108 mutations is small, it may seem reasonable to assume that the packing of the residues in this capping motif is a possible explanation of the abnormally low free energies. However, the L103 substitutions do not show the same sensitivity toward mutation as the L108 position, despite being packed against L108 in this capping motif. This points out the difficulty of attempting to analyze every mutant in detail. Instead, we focus on the broader trends that this large data set may show.

Can the changes in protein stability be attributed largely or solely to packing, or are there other factors, such as secondary structural propensity, that we should consider? To answer this, we first examine the effect of solvent exposure since the effects of packing at exposed sites should be minimized. Summarized in Table 2 are the differences in protein stability between any two side chains at a given site. Also shown in Table 2 is the percentage of the side chain surface that is accessible to solvent in the wild-type structure. This measure of solvent accessibility is plotted against these stability differences in Figure 1.

Figure 1 clearly shows that high degrees of solvent exposure translate into relatively minor stability differences, regardless of the identity of the original side chain at a position. As the side chain becomes more buried, the range of stability differences increases dramatically. This is consistent with the expected effects of packing. As pointed out in the introductory section, good packing optimizes van der Waals interactions and minimizes cavities. Neither of these is relevant in a solvent-exposed position, but both are critical in a fully buried residue. For example, the loss of hydrophobic transfer free energy when either an isoleucine or a leucine is replaced with a valine would also be more important as the side chain becomes more buried, and this complicates interpretation of the observed stability differences. The simple analysis presented earlier of hydrophobic transfer free energy and entropic effects predicts little difference between a buried valine, isoleucine, and leucine, but nevertheless, comparison of valine to isoleucine or leucine seems more problematic.

We therefore focus on the difference in stability between isoleucine and leucine at various positions. As pointed out above, isoleucine and leucine should have very little difference in hydrophobicity and loss of degrees of freedom upon

Table 2: Relative Stability of Proteins with Different Side Chains at a Given Position

position	$\Delta\Delta G_{V\rightarrow I}$[a]	$\Delta\Delta G_{V\rightarrow L}$[b]	$\Delta\Delta G_{I\rightarrow L}$[c]	$\Delta\Delta G_{WT\rightarrow A}$[d]	side chain SASA[e]
V23	−0.2	−0.1	0.1	−2.9	0.8
V39	0.1	−0.9	−1.0	−2.2	0.0
V51	0.1	−0.2	−0.3	−0.3	60.3
V66	−1.0	−0.3	0.7	−2.2	0.5
V74	−1.8	−1.2	0.6	−3.1	0.0
V99	−0.2	−0.3	−0.1	−3.2	0.5
V104	0.2	−0.8	−1.0	−2.9	0.0
V111	−0.7	−0.9	−0.2	−4.7	0.5
V114	−0.1	−1.1	−1.0	0.0	8.8
I15	0.8[f]	0.1	−0.6	−2.7	30.3
I18	1.1[f]	0.8	−0.2	−2.5	20.9
I72	1.2	1.0	−0.2	−5.1	5.7
I92	0.4	−0.2	−0.6	−4.0	0.6
I139	1.5[f]	1.3	−0.1	−3.5	18.6
L7	0.5	1.4	0.9	−1.6	17.2
L14	0.0	1.5	1.5	−2.3	15.3
L25	0.1	1.8	1.7	−2.7	0.0
L36	0.4	3.5	3.1	−3.5	0.4
L37	1.0	3.0	2.0	−1.7	8.3
L38	−1.9	0.2	2.1	−1.7	2.6
L89	0.3	1.4	1.1	−2.6	19.1
L103	0.9	2.0	1.1	−4.6	0.0
L108	0.5	3.9	3.4	−5.8	6.7
L125	1.3	2.3	1.0	−4.9	0.0
L137	0.6	1.4	0.8	−2.3	32.2
T13[g]	0.1	−	−	−0.7	79.2
T22	0.0	−	−	−1.6	8.8
T33	0.2	−	−	−1.4	26.6
T41	−0.3	−	−	0.0	0.0
T44	−0.9	−	−	−0.4	26.0
T62	−1.7	−	−	−2.4	0.6
T82	0.1	−	−	−0.9	56.7
T120	−0.1	−	−	−1.2	29.5

[a] Difference between the apparent stability of the protein with an isoleucine side chain and the stability of the protein with a valine substitution at a given position ($\Delta\Delta G_{V\rightarrow I} = \Delta G_{Ile} - \Delta G_{Val}$). The error in each mutant stability was estimated to be ±0.1 kcal/mol, and the error in the difference is estimated to be ±0.17 kcal/mol. [b] Difference in free energy between the free energy of the protein with a leucine side chain and the free energy of the protein with a valine substitution ($\Delta\Delta G_{V\rightarrow L} = \Delta G_{Leu} - \Delta G_{Val}$). The error is estimated to be ±0.17 kcal/mol. [c] Difference in free energy between the free energy of the isoleucine substitution and the free energy of the leucine substitution ($\Delta\Delta G_{I\rightarrow L} = \Delta G_{Leu} - \Delta G_{Ile}$). The error is estimated to be ±0.17 kcal/mol. [d] Difference in free energy between the free energy of the protein with an alanine side chain (*31*) and the free energy of the wild type ($\Delta\Delta G_{WT\rightarrow A} = \Delta G_{Ala} - \Delta G_{WT}$). The error is estimated to be ±0.17 kcal/mol. [e] Solvent accessible surface area of the side chain in the wild-type structure. [f] The free energy data of proteins with isoleucine to valine substitutions are from Shortle et al. (*31*), except for those redetermined here and listed in Table 1. [g] The free energy data for threonine to valine substitutions are from Byrne et al. (*32*), and the free energy data for threonine to alanine substitutions are from Green et al. (*33*).

folding. Other factors should dictate the stability differences. In some cases, this difference, $\Delta\Delta G_{I\rightarrow L}$, is negative. This indicates that the isoleucine side chain results in a more stable protein; in other cases, a positive value indicates that leucine is better at a given position. Regardless of sign, the range of stability changes between isoleucine and leucine clearly increases as the extent of side chain burial increases, consistent with what would be expected if packing were at the root of these stability differences. No other factor which would depend on side chain burial suggests itself. It seems clear that packing is critical to protein stability.

The next issue is comparison of the relative energetic importance of packing and hydrophobicity, the latter commonly held to be the principal driving force for protein

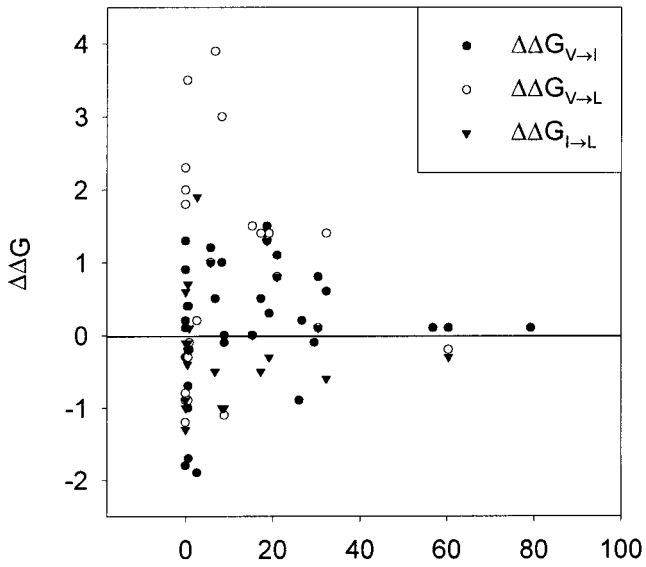

FIGURE 1: Value of $\Delta\Delta G$ plotted against the solvent accessible surface area of that residue of the side chain in wild-type staphylococcal nuclease.

folding. Again let us focus first upon $\Delta\Delta G_{I\rightarrow L}$, an energetic change likely to reflect primarily the effects of repacking. The largest observed value is −3.4 kcal/mol, at position 108. This is a very large change in stability, resulting from moving the position of a single methyl group. Unmistakably, packing is of major importance in this case. However, neither this extreme case nor the other extreme where there is no stability difference is indicative of the more normal course of events. Average values should be considered.

For purposes of evaluating the importance of packing, we are not particularly interested in which amino acid is more stable at a given position. The most stable side chain is nearly always the wild-type side chain. The magnitude of the difference is of more importance. It is likely that the identity of the wild-type side chain affects the magnitude of the energy differences discussed here as well, but this is a more subtle effect that we ignore in this broad analysis of the overall magnitude of packing energetics. We therefore took the absolute value of each $\Delta\Delta G_{V\rightarrow I}$, $\Delta\Delta G_{V\rightarrow L}$, and $\Delta\Delta G_{I\rightarrow L}$ value and averaged them. It is possible that in partially exposed residues there is a change in the amount of buried hydrophobic surface upon mutation, complicating interpretation. Somewhat surprisingly, there is relatively little difference in the average energetic change whether all residues are included in this calculation, just those residues with <10% solvent exposure, or just those that are fully buried. Nevertheless, it is possible that in partially exposed residues there is a change in the amount of buried hydrophobic surface. Accordingly, it seems appropriate to center attention on buried residues. The average value and standard deviation of $\Delta\Delta G_{I\rightarrow L}$ absolute values at the six residues fully buried in the wild type is 1.1 ± 0.3 kcal/mol. If we slightly broaden the definition of buried to include positions with <1% solvent exposure in the wild type, 12 residues in all, the average and standard deviation of $\Delta\Delta G_{I\rightarrow L}$ absolute values is 0.9 ± 0.8 kcal/mol.

In short, moving a single methyl group in the protein interior costs on average ∼1 kcal/mol. The average absolute values of $\Delta\Delta G_{V\rightarrow L}$ and $\Delta\Delta G_{V\rightarrow I}$ reinforce this conclusion. There is a change in hydrophobicity here to complicate

interpretation, but at positions with <1% solvent exposure in the wild type, the average absolute values and standard deviations of $\Delta\Delta G_{V\rightarrow L}$ and $\Delta\Delta G_{V\rightarrow I}$ are 1.2 ± 1.0 and 0.7 ± 0.6 kcal/mol, respectively. It is interesting to note that the average energetic difference between valine and isoleucine is smaller than that of the valine to leucine exchange. Both valine and isoleucine are β-branched so the packing rearrangements required for the latter exchange would be predicted to be larger, and this is reflected in the energetics.

It might be argued that these results are of limited generality since we are comparing only conservative aliphatic for aliphatic substitutions at sites which are originally optimized for an aliphatic residue. Mutations to isoleucine and valine at sites which are threonines in the wild type provide a test of this argument. Presumably, none of these wild-type threonine positions is somehow optimized for any of these hydrophobic side chains. In this set of mutants, we see little difference between the stability of isoleucine- and valine-substituted proteins at solvent-exposed sites. The energetic changes at the two threonine sites with <1% solvent exposure in the wild type are in line with the results at positions which are valine, isoleucine, or leucine in the wild type. Similarly, the effects of the methionine substitution, still hydrophobic but containing a much more polarizable sulfur atom, at isoleucine positions are quite comparable to the effects of valine or leucine substitution at those same sites. In other words, this is evidence that the importance of packing is general and dependent primarily upon the degree of burial of a side chain, rather than upon the hydrophobicity, charge, hydrogen bonding, or other properties of either the wild type or mutant side chain at that site.

The hydrophobic transfer free energy gained when a single methylene unit moves from octanol to water can be estimated from the data of Wimley et al. (*45*) as approximately 0.7−0.8 kcal/mol. Numerous caveats apply to this estimate (*45*, *48*), but it appears that the average energetic cost of repacking a buried methyl group within the core of staphylococcal nuclease is actually greater than the hydrophobic transfer free energy gained from burying that same methyl group.

A well-packed protein optimizes van der Waals interactions, and while there is no doubt that appreciation of the importance of packing has been growing (*5*, *25*−*30*), it seems fair to say that the commonly held point of view has been that the principal driving force for protein folding is the hydrophobic effect (*50*). In part, this may be because it was not clear whether the van der Waals interactions afforded by the close packing of native protein interiors are a net contributor to protein stability because the van der Waals interactions of the unfolded protein with solvent might be thought to be of roughly equivalent energy. It is precisely for this reason that Dill in his oft-cited 1990 review said of van der Waals interactions and hydrogen bonding that their "magnitudes, among all the types of force contributing to protein folding, are currently perhaps the most difficult to assess" (*50*). These results show that that such an assumption is incorrect for the average buried side chain in staphylococcal nuclease. Close packing of the protein seems to be a net stabilizing factor of magnitude similar to the hydrophobic effect.

Finally, we compare the sensitivity of a site to repacking as measured by methyl group rearrangement to the effects of truncating the wild-type side chain by mutating it to alanine. The free energy changes for mutation to alanine are taken from earlier work (*31*) and are shown in Table 2. The values of $\Delta\Delta G_{WT\rightarrow A}$ are plotted against $\Delta\Delta G_{I\rightarrow L}$ in Figure 2. Clearly, there is very little correlation between these two values. In other words, the sensitivity or lack of sensitivity of a given valine, leucine, or isoleucine site to substitution with a much smaller residue poorly predicts the sensitivity to mutation to a similarly sized residue. This indicates that the plasticity of structure that accommodates the subtractive change to alanine apparently does not necessarily translate into the plasticity of structure that can accommodate a larger side chain or even one of the same size, but altered geometry.

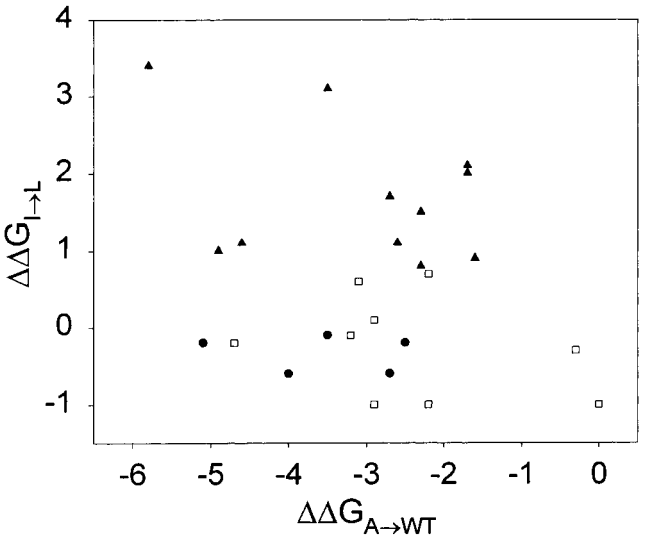

FIGURE 2: Value of $\Delta\Delta G_{WT\rightarrow A}$ ($\Delta\Delta G_{WT\rightarrow A} = \Delta G_{Ala} - \Delta G_{WT}$) plotted against $\Delta\Delta G_{I\rightarrow L}$ ($\Delta\Delta G_{I\rightarrow L} = \Delta G_{Leu} - \Delta G_{Ile}$). Those positions which are leucine in the wild type are shown with black triangles (▼); wild-type isoleucines are shown with black circles (●), and valines are shown with white circles (○). The black symbols all have the same number of atoms lost upon truncation to alanine, whereas the white symbols (valines) have lost one fewer methylene.

This begs the following question: which factors do dictate the energetic response of the protein to packing changes? The answer to that is beyond the scope of this work, but we begin to explore it in the articles which immediately follow in this issue.

CONCLUSION

The exchange of leucine, isoleucine, and valine residues can cause quite large stability changes or have negligible effects. As others have previously observed, solvent-exposed residues usually tolerate substitution well. Buried residues are clearly more sensitive to packing effects, as would be expected. Strikingly, the average magnitude of the packing effects observed when a single methyl group is moved at a buried site appears to be greater than the energetic loss expected from hydrophobic transfer effects when a buried methylene unit is simply removed. This result and recent related findings by other workers (*51*, *52*) call into question the widely held belief that hydrophobic transfer effects are the principal driving force for protein folding. The results presented here certainly seem to indicate that the optimization of van der Waals contacts in the native state of the protein is of at least equal energetic importance to hydrophobicity in maintenance of that native state. However, we temper this

by noting our recent conclusion (*41*) that better packing is not the primary mechanism by which thermophilic organisms achieve high protein stabilities. In short, we feel that while packing is not the single dominant factor in determining protein stability, it plainly ranks as one of several critical factors and one whose importance may have been commonly underappreciated.

ACKNOWLEDGMENT

We thank Lee Manuel and Jeff Schwehm for their help in this work.

REFERENCES

1. Nakagawa, H., Koyama, Y., Kawai, T., and Okada, T. (1995) *Int. J. Pept. Protein Res. 46*, 440−451.
2. Richards, F. M. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 151−176.
3. Connolly, M. L. (1986) *Int. J. Pept. Protein Res. 28*, 360−363.
4. Rashin, A. A., Iofin, M., and Honig, B. (1986) *Biochemistry 25*, 3619−3625.
5. Hubbard, S. J., and Argos, P. (1995) *Protein Eng. 8*, 1011−1015.
6. Reidhaar-Olson, J. F., and Sauer, R. T. (1988) *Science 241*, 53−57.
7. Bowie, J. U., Reidhaar-Olson, J. F., Lim, W. A., and Sauer, R. T. (1990) *Science 247*, 1306−1310.
8. Terwilliger, T. C., Zabin, H. B., Horvath, M. P., Sandberg, W. S., and Schlunk, P. M. (1994) *J. Mol. Biol. 236*, 556−571.
9. Rennell, D., Bouvier, S. E., Hardy, L. W., and Poteete, A. R. (1991) *J. Mol. Biol. 222*, 67−88.
10. Lim, W. A., and Sauer, R. T. (1989) *Nature 339*, 31−36.
11. Karpusas, M., Baase, W. A., Matsumura, M., and Matthews, B. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8237−8241.
12. Lim, W. A., and Sauer, R. T. (1991) *J. Mol. Biol. 219*, 359−376.
13. Daopin, S., Alber, T., Baase, W. A., Wozniak, J. A., and Matthews, B. W. (1991) *J. Mol. Biol. 221*, 647−667.
14. Sandberg, W. S., and Terwilliger, T. C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1706−1710.
15. Lim, W. A., Farruggio, D. C., and Sauer, R. T. (1992) *Biochemistry 31*, 4324−4333.
16. Hurley, J. H., Baase, W. A., and Matthews, B. W. (1992) *J. Mol. Biol. 224*, 1143−1159.
17. Baldwin, E. P., Hajiseyedjavadi, O., Baase, W. A., and Matthews, B. W. (1993) *Science 262*, 1715−1718.
18. Anderson, D. E., Hurley, J. H., Nicholson, H., Baase, W. A., and Matthews, B. W. (1993) *Protein Sci. 2*, 1285−1290.
19. Lim, W. A., Hodel, A., Sauer, R. T., and Richards, F. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 423−427.
20. Laub, P. B., Khorasanizadeh, S., and Roder, H. (1995) *Protein Sci. 4*, 973−982.
21. Mollah, A. K., Aleman, M. A., Albright, R. A., and Mossing, M. C. (1996) *Biochemistry 35*, 743−748.
22. Axe, D. D., Foster, N. W., and Fersht, A. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 5590−5594.
23. Skinner, M. M., and Terwilliger, T. C. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10753−10757.
24. Finucane, M. D., and Woolfson, D. N. (1999) *Biochemistry 38*, 11613−11623.
25. Richards, F. M., and Lim, W. A. (1993) *Q. Rev. Biophys. 26*, 423−498.
26. Hurley, J. H. (1994) in *The Protein Folding Problem and Tertiary Structure Prediction*, pp 549−578, Birkhäuser, Boston.
27. Lazar, G. A., and Handel, T. M. (1998) *Curr. Opin. Chem. Biol. 2*, 675−679.
28. Richards, F. M. (1997) *Cell. Mol. Life Sci. 53*, 790−802.
29. Hubbard, S. J., and Argos, P. (1995) *Curr. Opin. Biotechnol. 6*, 375−381.
30. Sandberg, W. S., and Terwilliger, T. C. (1991) *Trends Biotechnol. 9*, 59−63.
31. Shortle, D., Stites, W. E., and Meeker, A. K. (1990) *Biochemistry 29*, 8033−8041.
32. Byrne, M. P., Manuel, R. L., Lowe, L. G., and Stites, W. E. (1995) *Biochemistry 34*, 13949−13960.
33. Green, S. M., Meeker, A. K., and Shortle, D. (1992) *Biochemistry 31*, 5717−5728.
34. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
35. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
36. Stites, W. E., Byrne, M. P., Aviv, J., Kaplan, M., and Curtis, P. M. (1995) *Anal. Biochem. 227*, 112−122.
37. Schwehm, J. M., and Stites, W. E. (1998) *Methods Enzymol. 295*, 150−170.
38. Bennett, M. J., Schlunegger, M. P., and Eisenberg, D. (1995) *Protein Sci. 4*, 2455−2468.
39. Schwehm, J. M., Kristyanne, E. S., Biggers, C. C., and Stites, W. E. (1998) *Biochemistry 37*, 6939−6948.
40. Vriend, G. (1990) *J. Mol. Graphics 8*, 52−59.
41. Chen, J., Lu, Z., Sakon, J., and Stites, W. E. (2000) *J. Mol. Biol. 303*, 125−130.
42. Chothia, C. (1984) *Annu. Rev. Biochem. 53*, 537−572.
43. Shortle, D., and Meeker, A. K. (1986) *Proteins 1*, 81−89.
44. Fauchère, J. L., and Pliška, V. (1983) *Eur. J. Med. Chem. 18*, 369−375.
45. Wimley, W. C., Creamer, T. P., and White, S. H. (1996) *Biochemistry 35*, 5109−5124.
46. Stites, W. E., and Pranata, J. (1995) *Proteins 22*, 132−140.
47. Lee, K. H., Xie, D., Freire, E., and Amzel, L. M. (1994) *Proteins 20*, 68−84.
48. Karplus, P. A. (1997) *Protein Sci. 6*, 1302−1307.
49. Aurora, R., Srinivasan, R., and Rose, G. D. (1994) *Science 264*, 1126−1130.
50. Dill, K. A. (1990) *Biochemistry 29*, 7133−7155.
51. Vlassi, M., Cesareni, G., and Kokkinidis, M. (1999) *J. Mol. Biol. 285*, 817−827.
52. Ratnaparkhi, G. S., and Varadarajan, R. (2000) *Biochemistry 39*, 12365−12374.

BI011267T