PACKING AS A STRUCTURAL BASIS OF PROTEIN STABILITY: UNDERSTANDING MUTANT PROPERTIES FROM WILDLTYPE STRUCTURE

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Modeling of protein core mutations using sidechain packing can forecast their effects on stability. We have assessed the structural basis of this approach, by evaluating the accuracy of our 1991 model of a three-site mutant of λ repressor (V36L / M40L / V471), against the recently reported crystal structure. The three mutated residues matched the crystal structure to within 0.89Å (1.11 Å for sidechain atoms), giving fairly accurate sidechain placement and packing (81-99th percentile rank in coordinate accuracy). However, the model used different sidechain torsional angles than seen in the crystal structure at residues 36 and 40, apparently to compensate for the backbone shifts present in the actual mutant structure, but not accounted for in our modeling method. To understand the structural basis of stability across a set of λ repressor core mutants, we have analyzed the mutant models, revealing several simple packing effects: V36L, predicted to be stabilized by filling a hydrophobic cavity; M40V, destabilized by a steric clash with the unusual structural demands of a helix-turn transition. These effects illustrate how mutant stability can often be understood directly from scrutiny of wildtype structure. Simply adding the calculated energies of neighboring point mutations predicts the stability effect of the combined mutant relatively well, with little apparent cooperativity, yielding simple rules for each site’s amino acid preferences. Our treatment of core packing indicates that it can permit a large fraction of sequences to fit the native fold, as observed experimentally, far more than indicated by rotamer hard-sphere models.

I Introduction

Historically, the complexity of proteins has made it very hard to predict functional properties like stability from the details of three-dimensional structure. Predicting functional properties of mutants adds an extra layer of complexity in that different mutants may change the structure in unknown ways. For this reason, researchers often use non-structural models based on intrinsic properties of amino acids, such as hydrophobicity and packing volume, for explanations of mutant stability. Such models capture important bulk phenomena, without requiring consideration of structural details. For example, burying more hydrophobic surface area can confer increased stability. Similarly, the packing volume model asserts that a change in the overall volume packed inside the core should cause a proportionate loss in stability. Unfortunately, these models give an incomplete and often very poor accounting of protein stability and other functional properties (1-3).

Recently, approaches have been developed that can predict energetic effects like protein stability and binding specificity from high-resolution structural information, by predicting the structural changes caused when a mutation is introduced (4-10). We have developed calculations that predict the minimum packing energy for the...
mutated sidechain(s) and surrounding sidechains, which correlates well with stability in a set of λ repressor core mutants (1, 11) and T4 lysozyme mutants (12, 13). In this approach, the protein mainchain is held fixed to the wildtype structure, and the mutated sidechain(s) and surrounding sidechains repacked as well as possible in that rigid context. This "best native fit" calculation identifies mutations that cannot fit into the native fold (without significant backbone distortions), but also mutations that improve packing (11).

In this paper we show how these "best native fit" structures can provide insights into the structural basis of the mutants' stability effects. Two types of evidence support the validity of this analysis: i) the calculated energies of these models correlate closely with the mutants' stabilities; ii) models of a series of T4 lysozyme mutants based on the wildtype structure matched crystal structures to 0.68 - 0.89 Å rms on the predicted sidechains (13). This is not unreasonable in light of the extensive evidence showing little backbone shift in mutated proteins (14-16), but would not hold true for large mainchain movements, as observed between globins (17).

II Results and Discussion

Recently, the crystal structure of one of the modeled λ repressor mutants, V36L M40L V47I ("LLI"); we shall refer to the mutants by the amino acid letter codes for residues 36, 40 and 47), has been solved (18), giving a direct test of the accuracy of the original model (fig. 1) (11). The overall RMS of the model vs. the crystal structure was 0.99 Å (1.38 Å for sidechain atoms only), and 0.89 Å for the three mutated residues (1.11 Å for their sidechain atoms). Two of the mutated sidechains (Leu 36, Leu 40) superimpose with reasonable accuracy on the crystal structure (1.27 Å, 1.36 Å sidechain atom RMS, respectively), while the remaining mutated sidechain fits the crystal structure very closely (0.47 Å sidechain RMS). As Lim et al. remarked in their evaluation of the model, the model deviates substantially from the crystal structure in the sidechain torsions at residues 36 and 40 (18). However, these alternative conformations give similar placement of the 36 and 40 sidechains to the crystal structure, and a similar overall space-filling contour. The modeled Leu 36 differs from the crystal structure primarily at the distal, δ-methyls, which are rotated around the sidechain axis. Statistically, this conformation is more accurate (by RMS) than 83% of the possible leucine sidechain conformations at this residue. The modeled Leu 40 superimposes one Cδ close to the crystal structure, but has the other reversed. Its statistical rank against all possible leucine conformations at this residue is 81st percentile. These are significantly better than simply choosing the best rotamer (66%), and correspond to quality factors of four to five times better than random (see (13)). Ile 47 matches the crystal structure very closely by both
RMS and sidechain torsional angles; its statistical rank was >99th percentile. The probability of obtaining the observed level of accuracy for the three mutated residues by chance (i.e. by random sidechain rotamers) is $1.9 \times 10^{-4}$. Thus, this result is statistically significant.

**Figure 1**: Comparison of the LLI mutant model (dark) vs. crystal structure (light).

To assess the level of error in the model caused by the mutant mainchain shifts, we have repeated our modeling procedure starting from the mutant crystal structure backbone, instead of the wildtype backbone. The resulting model is significantly more similar to the LLI crystal structure at residues 36 and 40 ($0.39$ Å and $0.66$ Å sidechain RMS, respectively), but only marginally better at Ile 47 ($0.26$ Å RMS). The improved accuracy at 36 and 40 corresponds to statistically near-optimal rankings (>99th, and 98th percentile, respectively). Thus, while our original LU model was reasonably accurate, its fixed-backbone approximation appears to responsible for nearly all of the error observed versus the crystal structure.

The predictions based on the fixed-backbone approximation appear to compromise between two general trends: a) minimal coordinate error: the modeled mutations superimpose well but not precisely on the mutant crystal structure; b) minimal strain with the fixed backbone: the predicted conformation is selected based on minimal overall strain, which can actually prevent it from superimposing precisely on the mutant crystal structure coordinates. In the LLI model, Leu 36 and Leu 40 conformations closer to the crystal structure would create steric clashes with surrounding atoms, that could only be relieved by backbone shifts. To extract
meaningful energy information about the fit of the mutations within the structure, the model must avoid these anomalous strains as much as possible, by minimizing the structure within the fixed backbone context. These calculations correctly distinguished between mutant LLI (predicted to be slightly more stable than wildtype; experimentally it was the most stable mutant in the set) versus mutant FFI (predicted to be much less stable than wildtype; experimentally it was the least stable mutant). By contrast, standard sequence-based measures consider leucine and phenylalanine to be similar, predicting that these mutations should have similar effects on stability (19). The calculations' ability to perceive that leucine has the flexibility to fit the 36 and 40 sites with minimal disturbance, while phenylalanine does not, probably comes at some cost to its accuracy in predicting coordinates, particularly the sidechain torsional angles.

Table 1: Best native fit predictions and analyses for λ repressor core mutants

<table>
<thead>
<tr>
<th>Mut.</th>
<th>ΔT</th>
<th>ΔE 36</th>
<th>ΔE 40</th>
<th>ΔE 47</th>
<th>ΔE 1</th>
<th>ΔVol</th>
<th>Comments (* see text)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLI</td>
<td>4</td>
<td>-2.4</td>
<td>+2.6</td>
<td>-0.6</td>
<td>-0.4</td>
<td>+</td>
<td>stabilized by 36, 47 *</td>
</tr>
<tr>
<td>Inv</td>
<td>3</td>
<td>-0.8</td>
<td></td>
<td>-0.8</td>
<td>-0.8</td>
<td>+</td>
<td>stabilized by cavity-filling *</td>
</tr>
<tr>
<td>vnv</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(wildtype)</td>
</tr>
<tr>
<td>LVI</td>
<td>-2</td>
<td>-2.5</td>
<td>+6.8</td>
<td>-1.2</td>
<td>3.2</td>
<td>+</td>
<td>Val40 clash in helix-turn *</td>
</tr>
<tr>
<td>IVI</td>
<td>-2</td>
<td>-0.6</td>
<td>+6.8</td>
<td>-1.2</td>
<td>4.9</td>
<td>+</td>
<td>Val40 clash in helix-turn *</td>
</tr>
<tr>
<td>IVL</td>
<td>-4</td>
<td>-0.6</td>
<td>+6.8</td>
<td>+0.9</td>
<td>7.1</td>
<td>+</td>
<td>added torsional strain of Leu 47</td>
</tr>
<tr>
<td>vVL</td>
<td>-5</td>
<td>+6.8</td>
<td>+0.9</td>
<td>7.8</td>
<td></td>
<td>-</td>
<td>loss of packing stabilization at 36</td>
</tr>
<tr>
<td>FLV</td>
<td>-4</td>
<td>+5.1</td>
<td>+2.6</td>
<td>7.7</td>
<td></td>
<td>+</td>
<td>36 clashes with 42, 65, 68</td>
</tr>
<tr>
<td>FFI</td>
<td>-9</td>
<td>+8.8</td>
<td>+5.2</td>
<td>-1.1</td>
<td>13</td>
<td>+</td>
<td>36, 40 clash with 42, 61, 64, 65, 68</td>
</tr>
<tr>
<td>vVI</td>
<td></td>
<td>+6.8</td>
<td>-1.6</td>
<td>5.2</td>
<td></td>
<td>+</td>
<td>destabilized vs. IVI by loss of Ile 36</td>
</tr>
<tr>
<td>Ivv</td>
<td>-0.6</td>
<td>+6.8</td>
<td></td>
<td>6.2</td>
<td></td>
<td>-</td>
<td>destabilized vs. IVI by loss of Ile 47</td>
</tr>
<tr>
<td>vVVv</td>
<td></td>
<td>+6.8</td>
<td></td>
<td>6.8</td>
<td></td>
<td>-</td>
<td>destabilized by loss of Ile 36 &amp; 47</td>
</tr>
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</table>

Predicting and Understanding Mutant Stabilities from Wildtype Structure

Table 1 summarizes the "best native fit" models for the 36-40-47 series of λ repressor mutations. Throughout this discussion, we will contrast our predictions with simple packing volume considerations (column ΔVol in Table 1), which predict that altering core sidechains' sizes is destabilizing, especially if larger residues than wildtype are introduced.

The packing-volume model, and other astructural models (19), predict that mutating Val 36 → Ile should destabilize the protein by overpacking the core with one extra methyl group. However, our model (fig. 2a) indicates that the extra
methyl gives *increased* stability, due to a cavity apparent in the wildtype structure, into which the extra methyl can be packed in a good rotamer.

A second mutant, IVI, illustrates complex secondary structural effects on stability. This mutant differs from wildtype by three mutations, two of which (Val 36 → Ile, Val 47 → Ile) each add an extra methyl group, while the mutation at residue 40 (Met → Val) subtracts a methyl. Packing-volume considerations would predict destabilization by overpacking due to the extra methyls.

However, IVI's "best native fit" model indicates an opposite effect (fig. 2b). Residue 40 lies at the boundary between a helix and a turn, and this transitional role disfavors the β-branched sidechain of valine. The helix penalizes the sidechain gauche+ and gauche- rotamers (they collide with the carbonyl of the i-4 residue). Val 40 also lies at the start of a turn, bending its own carbonyl away from the helical conformation, into a position that impinges upon the *trans* rotamer. These contradictory demands create steric strain between the two γ-methyls of the β-branch and the protein backbone. The predicted *trans* conformation, which places its Cγ2 too close to Ile 68's Cδ1 (2.9Å). 434 repressor (26% identity versus λ repressor; PDB code 1R69 (20)) has valine at the equivalent of position 40, and has a number of structural changes to accommodate it: the carbonyl oxygen of residue 40 bends 20° further away from its sidechain atoms, compared with that in λ repressor; Ile 68 is replaced by Ala; and 64 Leu moves 1.0Å further away from residue 40. This interpretation is supported by the observation that all the mutants with valine at position 40 experimentally have lower T_m than wildtype.

Figure 2: Steric analysis of mutant models a. IMV. Ile 36's added methyl group (Cδ1) packs into a cavity present in the wildtype structure. b. IVI. Val 40 packs poorly, because the backbone carbonyl (O) is turned away from the helix axis (the helix carbonyls point vertically in this figure), towards the β-branched Val sidechain.
By contrast, the two Val → Ile mutations are predicted to stabilize the fold. Ile 47 takes advantage of the cavity created by the shortening of residue 40's sidechain, to pack the "new" methyl group C\textsuperscript{δ1} in a good rotamer, while Ile 36 is predicted to fill the same cavity it does in IMV. These predictions could be tested by reverting Ile 36 and Ile 47 to wildtype (see VVI, IVV, VVV, table 1). The packing volume model predicts that relieving the "overpacking" at 36 and 47 should alleviate IVI's destabilization. Intuitively, it makes sense that returning towards the wildtype sequence should move back towards the wildtype stability. However, our calculations indicate that these mutants (VVI and IVV) would be somewhat further destabilized, due to loss of the Ile C\textsuperscript{δ1}'s attractive van der Waals interactions, with the double mutant VVV least stable.

Analyses of these and other mutants suggested that the unbranched sidechain of Met 40 plays a special role in the stability of λ repressor. We have compared how strongly each of the three sites (36, 40, and 47) are sterically constrained to their wildtype amino acid, by calculating packing energies for all single-site mutants substituted at one of these sites with Leu, Ile, Met, or Val (fig. 3a). These calculations indicate that packing imposes little constraint on sequence at positions 36 and 47—in both cases, some mutants have steric energy as low as wildtype. By contrast, at residue 40 no mutation fits as well as methionine. Leucine, the next best residue, is more than 3 kcal/mol higher in packing energy. The site's helix-turn backbone disfavors β-branched residues. For γ-branches, the \( \chi_2 \) gauche+ rotamer position hits Leu 64 C\textsuperscript{β} (2.6 Å) on a neighboring helix, while the gauche- position hits 40's own carbonyl oxygen (2.6 Å). Thus only one good position (trans) is available for a δ-methyl. Since Val and Ile are β-branched, and Leu, His, Phe, Trp and Tyr are γ-branched, Met, a rare amino acid, is the only large hydrophobic that answers this steric prescription well, via the unusual structural virtue of possessing torsional flexibility at each of the atoms in its long sidechain.

**Cooperativity and Steric Coupling**

In proteins as small as λ repressor, energies do not necessarily correlate closely with melting temperature (\( T_m \)); however for these mutants this comparison is possible because the calculated multisite mutant energies correlate well with both the experimental \( T_m \) and \( \Delta G_u \) (8). We have calculated the predicted packing effects of point mutations at positions 36, 40 and 47 (fig. 3a). How well can these independent effects predict the multisite mutants' experimental stabilities? These mutants involve overlapping combinations of mutations at three neighboring sites (Table 1), which might be expected to have strongly cooperative effects that would render this simplistic approach invalid. However, simply adding the energy changes of the separate point mutations correlates well with the multisite mutants'
experimental stabilities (fig. 3b). Comparison with the regular multisite calculations (arrows in fig. 3b, data from Table 1) shows two cooperative effects. In FLV and FFI, Phe 36 forces neighboring residues (especially 42) into strained conformations that open space for mutation to a larger residue at 40. Second, in the wildtype context, Ile 47 is destabilizing because it clashes with Met 40; when 40 is mutated, however, the regular multisite calculations predict a cooperative effect in which Ile 47 becomes strongly stabilizing (in IVI, LVI, LLI, FFI). Experimentally, Val 36 → Ile raises $T_m$ 3.4°C in the wildtype context (VMV → IMV), but only 2.1°C in VVL → IVL, indicating a cooperative effect, that is only weakly observed in our calculations ($\Delta\Delta E_{calc} = -0.8$ kcal/mol for VMV → IMV, vs. -0.6 for VVL → IVL). To a first approximation, however, it appears multisite stability can be uncoupled to the separate "simple rules" for each site's sequence preferences.

![Figure 3: a. Comparison of residues 36, 40, and 47's permissiveness to mutation: calculated energies for each of the point mutants. At each position, an arrow indicates the lowest energy mutation. b. Cooperativity of neighboring mutations' stability effects. To test the level of energetic coupling between sites 36, 40, and 47, we summed the point mutant energies shown in a. for the mutations in each of Lim & Sauer's multisite mutants (o); cooperative effects are shown as vectors between these values and the corresponding multisite calculations (Table 1).](image-url)

How strongly coupled are the packing arrangements of core sidechains? When core substitutions are made, does packing force significant rearrangements to accommodate the changed group? To address these questions we have examined the optimized-packing predicted structures of the $\lambda$ repressor mutants for conformational shifts versus the wildtype packing. We have focused on the sidechains neighboring the mutated positions, to assess how their optimal packing conformations change...
due to the mutations. The predicted rearrangements were slight, typically 0.2 - 0.8Å sidechain RMS shift, and consisted of slight rotations of individual residues contacting a bulky substitution, to make room for it. The degree of predicted rearrangement does not correlate with stability effects; for example, LLI (the most stable of the eight mutants) and FFI (the least stable) were predicted to have nearly equal shifts (0.8Å and 0.9Å respectively). Thus, such shifts can yield a well-packed structure, or merely reduce the strain of a poorly packed mutant. The low level of steric change provides a simple explanation for the relative lack of cooperativity in the calculated energetic effects of the neighboring mutations at 36, 40 and 47. Since each substitution causes little steric change, it produces little alteration in the structural environment of a neighboring mutation, and thus little impact on its energetics.

Comparison with Rotamer Modeling

A key issue for structure prediction methods is the combinatorial problem of finding the global energy minimum out of the essentially infinite set of possible conformations. One strategy that has been widely employed is to reduce the number of possibilities drastically, by limiting consideration to rotamer conformations. In this approach, sidechains are treated not as freely rotatable torsional conformers, but as a small set of rotamers (typically three to five for each sidechain, and more in recent rotamer libraries, especially for aromatic residues) from which one is to be chosen as a best conformation. This reduces the total number of conformational permutations by two to three orders of magnitude per residue in the prediction zone, and yields accurate predictions for sidechain packing on wildtype backbones (5, 21-23). Surveys of high resolution crystal structures have found that about 71% of sidechains fit rotamer angles gauche+, trans or gauche- on χ1 to within 20° (24), with >90% classifiable as rotamers within an extended library of 67 total conformations for the 20 amino acids (25).

For predicting mutant structures, however, the challenge of mainchain shifts arises. Current methods neglect mainchain movements, and thus are forced to model the mutant sidechains on the "wrong" backbone, derived from the wildtype structure. In this case the inflexibility of the rotamers is a disadvantage, because no sidechain adjustment is allowed to make up for the fact the backbone is held fixed. In non-rotamer based calculations on T4 lysozyme mutants, we observed that packing optimization used sidechain torsional adjustments departing from rotamers to correctly predict the major structural shifts (13). How well can the λ repressor mutants be represented by rotamers?

To assess this question, we have calculated the deviation of their optimal-packing structures from the closest rotamers in the set compiled by Ponder and Richards (25). We have scored each sidechain as fitting a rotamer if its torsional
angles are within 20° of a rotamer. For the mutated residues 36-40-47, only 46% of the sidechains over the mutant set fit rotamers. Overall, none of the mutants could be represented by rotamers without at least one major deviation from the optimal packing structure, usually at a substituted position. In contrast, 88% of the neighboring, non-mutated sidechains fit rotamers, over the mutant set, and the only deviations occurred in the highly overpacked mutants FLV and FFI. This contrast may suggest that predicting the best packed structure with rotamers may be more difficult for mutated positions than it has proved to be for fitting wildtype sidechains onto the correct wildtype backbone. Mutant sidechains generally do assume standard rotamer conformations within the reference frame of the mutant crystal structure (18, 26). However, in homology modeling based on the wildtype backbone, the inherent mainchain error frequently makes the strict rotamer conformations appear to pack poorly, obscuring the optimal placement of the mutant sidechain atoms and leading to incorrect rotamer selection (13). The lack of backbone movement is the main problem in our calculations; rigid rotamers, used in homology modeling, add to this difficulty by making the sidechains also unable to adjust flexibly in response to mutation. A modeling method for coiled coil proteins has been developed that takes into account both sidechain and mainchain flexibility (27), an important new direction. The occurrence of a non-ideal rotamer conformation in our models appears to be crudely predictive of backbone shifts for the region of that residue.

Packing and Sequence Permissiveness

How strongly does packing constrain sequence within the protein core? The density of packing inside protein cores has long suggested that it may be a very strong constraint (28). Some years ago, Ponder and Richards performed ground-breaking calculations of sequences allowable within protein cores, using exhaustive search of rotamers and assessing packing by hard-sphere cutoffs. These calculations indicated that only a minute fraction (much less than one percent) of possible hydrophobic sequences fit a given protein core well enough to fold, leading to the characterization of this tiny subset as a "template" designating which sequences could adopt its fold (25). However, experimental studies (1, 16, 26, 29-31) have demonstrated that protein cores are surprisingly tolerant to mutation. Fully 70% of Lim & Sauer's hydrophobic sequence variants (mutated at λ repressor residues 36, 40 and 47) possessed detectable biological activity (1). Are these mutations able to fold because the protein backbone can distort significantly to accommodate them, or is a much larger fraction able to pack directly in the wildtype backbone than suggested by previous calculations?

To address this question, we have graphed the calculated "best native fit" energies of all 125 mutants containing Ile, Leu, Met, Phe or Val at residues 36-40-47 (fig. 4), calculated within the unperturbed wildtype backbone. Of these, 4% fit
the native core with packing energy lower than that of wildtype, and more than 20% are lower than that of IVI, whose melting temperature is only 2°C lower than wildtype. Overall, 80% were calculated to have better packing energy than FFI, which has a melting temperature 9°C lower than wildtype. Thus, the energetics of steric fit to the native core do not appear to restrict sequence strongly.

Figure 4: Distribution of packing energies for 36-40-47 Ile-Leu-Met-Phe-Val set

This much greater breadth of acceptable sequences than predicted by previous theory arises primarily in the tools used to locate and assess potential packings. First of all, Ponder and Richards enforced a strict volume requirement: sequences with less total packing volume than wildtype were scored as failing to fold. We simply calculated total packing energy, with no volume constraint. Second, our calculations modeled sidechains with unrestricted structural flexibility, by permitting all torsions to rotate freely (in steps of about 10 degrees), rather than using just a few fixed rotamer conformations. Furthermore, a flexible shell of sidechains surrounding the mutations was included in the molten zone, to allow for structural adjustments in the environment in response to the mutations. Third, to evaluate packing quality we calculated a physically realistic van der Waals potential energy of the system, instead of using a hard-sphere cutoff. Hard-sphere cutoffs neglect the structural adaptability characteristic of proteins, and are unreasonably unforgiving. Furthermore, they don't include attractive van der Waals interactions, which have played a very important role in our calculated stabilization energies. For example, six of the 125 mutants were predicted to be more stable than wildtype, due not to reduced repulsive forces, but to increased attractive interactions. We believe that these new features give a more realistic treatment of core sequence permissiveness. Even with little backbone motion considered, a large number of hydrophobic sequences appear to fit λ repressor core well enough to fold.
III Acknowledgments

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IV Methods

"Best native fit" models and energies were calculated using self-consistent ensemble optimization (SCEO (7, 8, 11, 32)). Coordinates of λ repressor (PDB code 1LRD (33)) were obtained from the Brookhaven Protein Data Bank (34), and sidechain atoms for a molten zone consisting of residues 36, 40, 42, 47, 61, 64, 65 and 68 deleted. The molten zone sidechains were then rebuilt in random conformations, with the desired amino acids at positions 36, 40 and 47. This structure was used as the starting point for 25 cycles of SCEO packing optimization, using linear cooling from 6000K to 298K over the first 15 cycles, followed by ten cycles equilibration at 298K. We followed the standard protocol (8) without modification. The predicted structure for each mutant was generated by selecting each molten zone residue's most frequent conformation in the final, optimally packed ensemble. The packing energy of this final ensemble for each mutant ($E_{calc}$) was taken as the predictor of its stability, relative to that calculated for wildtype. The energy calculations and parameters were exactly as reported previously (4, 11). To make a crude correction for the mutants' self-energies in the unfolded state, we assumed all χ torsional potentials adopt optimal rotamers in the unfolded state, and subtracted these values from the native packing energies. To model the energetics of a given residue in the unfolded state, we employed a simplistic model of an extended tripeptide chain fragment centered on that residue (8). Models were examined and analyzed using the LOOK software suite (Molecular Applications Group, Palo Alto, CA), and evaluated performed as described in (13).

V References