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Energetics of Complementary Side-Chain Packing in a Protein Hydrophobic Core†

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ABSTRACT: The energetics of complementary packing of nonpolar side chains in the hydrophobic core of a protein were analyzed by protein engineering experiments. We have made the mutations Ile → Val, Ile → Ala, and Leu → Ala in a region of the small bacterial ribonuclease barnase where the major α-helix packs onto the central β-sheet. The destabilization resulting from the creation of cavities was determined by measuring the decrease in free energy of folding from reversible denaturation induced by urea, guanidinium chloride, or heat. The different methods give consistent and reproducible results. The loss in free energy of folding for the mutant proteins is 1.0–1.6 kcal/mol per methylene group removed. This exceeds by severalfold the values obtained from model experiments of the partitioning of relevant side chains between aqueous and nonpolar solvents. Much of this discrepancy arises because two surfaces are buried when a protein folds—both the amino acid side chain in question and the portions of the protein into which it packs. These experiments directly demonstrate that the interior packing of a protein is crucial in stabilizing its three-dimensional structure: the conversion of leucine or isoleucine to alanine in the hydrophobic core loses half the net free energy of folding of barnase with a concomitant decrease in yield of the expressed recombinant protein.

It is of fundamental importance to understand the laws that govern the three-dimensional conformations adopted by proteins. This knowledge will enable the design of novel proteins, the rational alteration of existing proteins, and the deduction of the tertiary structure of proteins from their primary structure. In addition, understanding the precise molecular basis of protein structure–function relationships, such as ligand binding, conformational change, and protein–protein interactions, depends on this knowledge. These goals are far from realization, although progress toward solving the problem of protein folding is being made rapidly from a variety of approaches. Examples of theoretical approaches are the phenomenological, which range from the classification of single amino acids by their tendency to be found in a given type of secondary structure (Chou & Fasman, 1974; Garnier et al., 1978) to the classification of combinations of secondary structural motifs which constitute overall tertiary folding patterns (Levitt & Chothia, 1976; Janin & Chothia, 1980; Sternberg, 1983; Chothia, 1984), and the ab initio and computational methods (Nemethy et al., 1983; Weiner et al., 1984). There are, however, two apparently overwhelming barriers to calculating the conformation of a protein with the lowest free energy. The free energy of folding is the difference in the free energies of the folded and unfolded states and for smaller proteins is generally only 5–20 kcal/mol. As the noncovalent interaction energies in each state are some 10^3 kcal/mol or so, calculation of free energies of folding requires an accuracy of better than ±0.1–1%, which is far beyond the precision of present energy functions. Further, although the structures of many folded proteins are known to high resolution, structures of unfolded proteins are unknown, and so one of the states involved in the calculations is ill-defined.

The advent of protein engineering has provided a direct experimental attack on the protein folding problem by allowing the systematic production and analysis of mutant proteins. The initial applications are typified by the studies on staphylococcal nuclease (Shortle & Meeker, 1986) and bacteriophage T4 lysozyme (Alber et al., 1987). Mutant proteins differing in thermal stability were selected after spontaneous or random mutagenesis and then subjected to thermodynamic and

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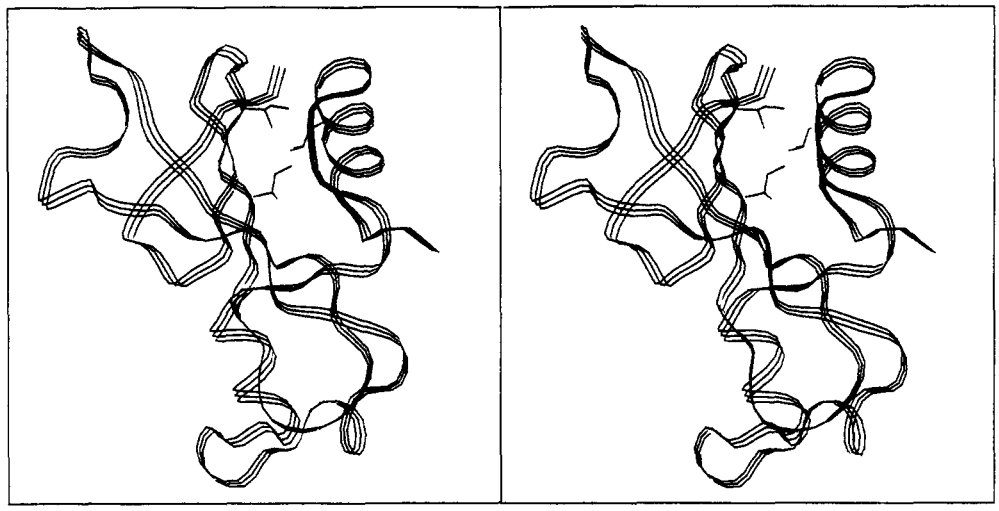

FIGURE 1: Stereo ribbon representation of the backbone of barnase, emphasizing the α-helix composed of amino acid residues 6–18 packed against the face of the five-stranded antiparallel β-sheet composed of residues 50–55, 70–75, 85–91, 94–101, and 106–108. The side chains of the residues mutated in this study are also shown (Leu-14, Ile-88, and Ile-96). The refined atomic coordinates of the protein were provided by Dr. C. Hill, University of York.

structural analysis. Once the temperature-sensitive loci have been identified, they may be studied extensively, for example, by making all 19 amino acid substitutions at that location (Matsumura et al., 1988). In this way, direct experimental data are obtained on how changes in structure cause changes in stability. Empirical rules are obtained on tailoring protein structure, and a data base for calculation is accumulated. A complicating factor is that changes at temperature-sensitive loci frequently involve alteration of several interactions, with perhaps unfavorable interactions being introduced as well as favorable ones being removed.

We are using a complementary approach based on procedures developed during the analysis of the structure and activity of tyrosyl-tRNA synthetase (Winter et al., 1982; Fersht, 1987): the three-dimensional structure of the enzyme is examined for what are suspected to be important and ubiquitous interactions. These noncovalent bonds are removed by making minimal mutations that eliminate the portions of side chains involved (Kellis et al., 1988). The mutations are chosen such that the possibility of additional interactions being made is minimized [so-called "nondisruptive deletions" (Fersht, 1987) are made]. The properties of wild-type and mutant enzymes are then directly compared under the same conditions to relate the change in these properties to the change in structure. The advantage of this approach is that only one set of interactions is changed at a time, namely, the removal of favorable interactions, and so analysis is simplified. What we wish to do is to take an existing protein structure, systematically remove the individual interactions that stabilize it, and quantitate their contributions to the balance between folding and unfolding.

In this study we have focused on hydrophobic interactions. Interactions between nonpolar residues are thought to be among the most important in determining the three-dimensional structure of a protein (Kauzmann, 1959; Tanford, 1980; Jaenicke, 1987), although there are little data on real proteins to support this notion. We have gone straight to where these interactions are expected to be at their most important, the hydrophobic core of a protein where an α-helix packs on a β-sheet by the interdigitation of hydrophobic side chains.

The protein used in our studies is barnase, an extracellular ribonuclease from *Bacillus amyloliquefaciens* (Nishimura & Nomura, 1958). It is an excellent paradigm for protein folding studies for several reasons. It is a small monomeric enzyme of 110 residues, with M_r = 12 382 (Hartley & Barker, 1972). It is composed of a single domain, yet has both α and β structural elements. Accordingly, it can be used to test hypotheses about the formation and stability of these types of secondary structure and the interactions between them. Barnase undergoes reversible solvent-induced and thermally induced denaturation, closely approximating a two-state equilibrium between folded and unfolded conformations (Hartley, 1968). It contains no sulfur atoms, thereby obviating the complications of cysteine and methionine oxidation and disulfide bond formation in folding/unfolding experiments. The crystal structure of the protein has been solved at high resolution (Mauguen et al., 1982), a prerequisite for rational protein engineering experiments, and its solution structure is in the process of being elucidated by high-field proton NMR spectroscopy (M. Bycroft and A. R. Fersht, unpublished results). The gene for this enzyme has been cloned and can be expressed in *Escherichia coli* (Paddon & Hartley, 1986; Hartley, 1988).

The tertiary structure of barnase comprises a C-terminal five-stranded antiparallel β-sheet (residues 50–55, 70–75, 85–91, 94–101, and 106–108) with two N-terminal α-helices, one packed against its face and the other against its edge. The packing of the α-helix composed of residues 6–18 against the face of the β-sheet is a common structural motif which has been described in many proteins [see Chothia (1984) for a review]. The energetics of this interaction have been studied on a theoretical basis (Chou et al., 1985). The main hydrophobic core of barnase is formed where nonpolar side chains of the α-helix and the β-sheet interdigitate and pack closely together (see Figure 1). The mutations designed for this study are truncations at the key area of contact in the core: Leu-14 → Ala (α-helix); Ile-88 → Val/Ala and Ile-96 → Val/Ala (β-sheet). These mutations are among the best choices for nondisruptive deletions; the amino acid side chains are simply shortened, leaving cavities in the hydrophobic core. Here we report experiments in which we examine the thermodynamics of unfolding of these mutants by several approaches, allowing us to extend and generalize our preliminary findings (Kellis et al., 1988).

Experimental Procedures

Materials

Chemicals. The buffer used in the denaturation experiments was 2-(*N*-morpholino)ethanesulfonic acid (MES) from Sigma.

The MES stock was a 1.00 M solution containing 387 mM acid form and 613 mM sodium salt, which gives a pH of 6.3 at 25 °C on dilution to 50 mM. Urea and guanidinium chloride were highly purified Aristar grade, from BDH Limited. Restriction enzymes and molecular biology reagents were obtained from New England Biolabs and Boehringer Mannheim, the radiochemicals were from Amersham International, and SP-Trisacryl was obtained from IBF. All other reagents were purchased from either Sigma or BDH Limited.

Recombinant Barnase. The wild-type barnase gene was cloned into the plasmid pUC19 by Paddon and Hartley (1987). This recombinant plasmid, pMT410, which confers resistance to ampicillin, was generously donated by Dr. Hartley. It contains the structural gene for barnase fused to the promoter and signal sequence of the *E. coli* alkaline phosphatase gene, as well as the gene for barstar [the intracellular inhibitor of barnase (Smeaton & Elliot, 1967)] under the control of its own promoter. These genes are contained on a 1.4-kb *Eco*RI–*Hin*dIII restriction fragment.

For mutagenesis, this fragment was subcloned into bacteriophage M13mp8 (Messing & Vieira, 1982). The sequences of the primers for site-directed mutagenesis of Ile-96 to Val and Ala have been published (Kellis et al., 1988); the following primers were also synthesized with an Applied Biosystems 380B DNA synthesizer and were used to direct the remaining mutations (asterisks follow the mismatches): Ile-88 → Ala, 5′AGTAAAGAG*C*CCGGTCT3′; Ile-88 → Val, 5′AGTAAAGG*AC*CCGGTCT3′; Leu-14 → Ala, 5′ATGTCTGAG*C*ATAATCC3′. Phosphorylation of the mutagenic primers, annealing, extension, and ligation were carried out as described by Zoller and Smith (1983). Mutant bacteriophage were identified by the oligonucleotide hybridization method described by Carter et al. (1984), using the appropriate mutagenic primer, phosphorylated with [γ-^{32}P]-dATP, as a probe. The nucleotide sequences of the mutant genes were verified by dideoxy sequencing (Sanger et al., 1977) after plaque purification of phage from hybridization-positive colonies. The mutant DNA was subcloned to regenerate pMT410, which was used to transfect *E. coli* strain TG2 (Fersht et al., 1988).

For production of barnase, a 30-mL starter culture of *E. coli* containing the wild-type or mutant plasmid was grown overnight in Luria broth (Lennox, 1955) containing 50 μg/mL ampicillin. This culture was used to inoculate 3 L of low-phosphate medium (Serpersu et al., 1986) containing ampicillin. Phosphate starvation leads to activation of the alkaline phosphatase promoter and, consequently, synthesis and secretion of barnase. Cells were grown for 18 h at 30 °C in Erlenmeyer flasks, with vigorous shaking. Barnase was purified by a method adapted from Paddon and Hartley (1987). All procedures were carried out at 4 °C, until the final chromatography step. The culture medium was chilled, and 55 mL of glacial acetic acid/L of culture was slowly added while stirring. The acidification releases additional barnase from the periplasm into the medium (R. W. Hartley, personal communication). Stirring was continued for 15 min. The culture was centrifuged for 15 min at 10000*g* to pellet the cells. The supernatant fluid was decanted through muslin and combined with 5 mL of SP-Trisacryl cation-exchange resin that had been previously washed with 50 mM sodium acetate, pH 5.0 ("equilibration buffer"). Barnase was allowed to adsorb to the resin for 1 h with gentle swirling. The resin was allowed to settle, and the supernatant fluid was decanted. The resin was washed several times with equilibration buffer and then poured into a chromatography column. The column was washed with equilibration buffer until A_{280} of the eluate was negligible. Barnase was eluted from the column with equilibration buffer containing 0.5 M NaCl. The eluted protein was dialyzed overnight in M_r = 3500 cutoff dialysis tubing (Spectrum Medical Industries, Inc.) against equilibration buffer. The final purification step was carried out at ambient temperature with a Pharmacia FPLC system using a Mono-S cation-exchange column. Barnase was bound to the column in equilibration buffer and was eluted approximately halfway through a linear gradient of 0–0.4 M NaCl. Purified barnase was dialyzed overnight against 50 mM MES, pH 6.3, flash frozen, and stored at −70 °C. The recovery of purified barnase varied from roughly 1–10 mg/L of culture, with less stable mutants giving lower yields. The purified proteins were homogeneous as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Methods

Spectroscopy. UV difference absorption spectroscopy was initially used to monitor urea-induced barnase unfolding (Hartley, 1968; Kellis et al., 1988). Upon unfolding, there is a decrease in absorbance at 286 nm and an increase at 270 nm. $\Delta A_{286} - \Delta A_{270}$ was measured, because this both amplifies the signal and minimizes dilution errors as $\epsilon_{286} \approx \epsilon_{270}$ for barnase. The monitoring of unfolding by fluorescence spectroscopy (Hartley, 1975) was subsequently adopted. The intrinsic fluorescence of barnase decreases by 80% upon unfolding, in contrast with the 18% change in absorption; additionally, only one-tenth as much protein is required. There is a further important advantage of fluorescence when monitoring urea-induced denaturation: the fluorescence yield of the folded and unfolded forms of the protein is constant with respect to urea concentration (up to 6.5 M urea). This gives flat base lines, and the fraction of unfolded protein at a given urea concentration is simply the ratio of the observed fluorescence to the maximal fluorescence change. In contrast, there is a slight decrease in the UV absorbance of the folded enzyme on the addition of urea which requires a correction term (Pace, 1986). Fluorescence spectroscopy was used in experiments involving urea, guanidinium chloride, and thermal denaturation. The excitation wavelength of the spectrofluorometer was 290 nm, and the emission wavelength was 315 nm.

Chemical Denaturation Experiments. Urea and guanidinium chloride solutions were prepared gravimetrically in volumetric flasks with water purified to 15-MΩ resistance by an Elgastat system. The urea solutions were divided into 800-μL aliquots with an SMI (American Hospital Supply Corp.) positive displacement pipetter with repetitive pipetting attachment, frozen in dry ice, and stored at −20 °C to minimize the formation of ammonium cyanate on storage. The guanidinium chloride solutions were stored at room temperature, which is not detrimental. For each data point in the chemical denaturation experiments, 100 μL of barnase stock solution in 450 mM MES was diluted into 800 μL of the appropriate denaturant solution, an SMI positive displacement pipetter being used. The final barnase concentration was 10 μM when absorption spectroscopy was being used and 1 μM in the case of fluorescence spectroscopy. The protein/denaturant solutions were preequilibrated at 25 °C for approximately 1–3 h. The extent of unfolding was found to be constant for 0.5 to 8 h. Spectroscopic measurements were made in thermostated cuvette holders at 25.0 °C. The temperature was carefully monitored by a thermocouple immersed in a neighboring cuvette in the holder. The unfolding was found to be completely reversible, by diluting samples of un-

folded protein to low urea concentration and measuring the recovery of fluorescence.

Thermal Denaturation Experiments. Thermally induced unfolding, monitored by fluorescence spectroscopy, was also carried out with 1 μM barnase in 50 mM MES. The pK_a of this buffer is temperature dependent, and the pH ranged from 6.3 to 5.7 between 25 and 70 °C, which are the extremes of temperature used in these studies. In control experiments at 25 °C, the stability of barnase did not change significantly in this pH range. Jacketed cuvettes were heated by a thermostated circulating water bath. The heating rate was approximately 10 °C/h, and the temperature was carefully monitored by a thermocouple immersed in the cuvette under observation. Thermal unfolding was more than 85% reversible after the slow heating during a typical denaturation experiment.

RNase Assay. RNA hydrolysis by wild-type and mutant barnases was measured at 25 °C in 100 mM Tris, pH 8.5, on the basis of a previously described method (Rushizky et al., 1963), with 4.0 nM enzyme. The reaction was initiated by the addition of 10 μL of a barnase stock solution with a Hamilton syringe to 1 mL of a thermally equilibrated torula yeast RNA solution (2 mg/μL) in a cuvette. The solution was mixed by inverting twice, and the decrease in absorbance at 298.5 nm which accompanies RNA hydrolysis was monitored spectroscopically.

Results

Solvent-Induced Denaturation

Denaturation by Urea. We first compared the relative stabilities of wild-type and mutant barnases by analyzing their unfolding by urea, as monitored by the decrease in intrinsic fluorescence of the proteins. The fluorescence of wild-type and several mutant barnases as a function of urea concentration is shown in Figure 2A, where mutation clearly decreases the stability of the protein. Quantification of those decreases is described below.

Data Analysis. The equilibrium constant for unfolding, K_U, in the presence of a denaturant may be calculated from eq 1,

$$K_U = (F_N - F)/(F - F_U) \quad (1)$$

where F is the observed fluorescence and F_N and F_U are the values of the fluorescence of the native and unfolded forms of the protein, and the transition corresponds to a two-state process. The values of F_N and F_U during urea-induced denaturation are independent of the concentration of urea up to 6.5 M, and so eq 1 is simply applied. However, on guanidinium chloride mediated or thermal denaturation monitored by fluorescence (or the equivalent terms in urea-mediated denaturation monitored by UV absorption) either one or both the F_N and F_U for barnase vary with the changing conditions. The values of F_N and F_U at each point in the transition region must be found by extrapolation from the values of F_N and F_U which are determined outside the transition region. [See Pace (1986) for a review of this method.] This complication lowers appreciably the accuracy of data fitting.

It has been found experimentally that the free energy of unfolding of proteins in the presence of urea or guanidinium chloride is linearly related to the concentration of denaturant (eq 2; Pace, 1986). The value of m and ΔG_{H_2O}, the apparent

$$\Delta G_U = \Delta G_{H_2O} - m[\text{denaturant}] \quad (2)$$

free energy of unfolding in the absence of denaturant, may be calculated from eq 1 because $\Delta G_U = -RT \ln K_U$. We directly fitted the entire data set from the fluorescence-monitored urea denaturation with the nonlinear regression analysis program EnzFitter (published by Elsevier-Biosoft, Cambridge), using eq 3 (which is derived from eq 1 and 2). For wild-type

$$F = F_N - (F_N - F_U)\exp[(m[\text{urea}] - \Delta G_{H_2O})/RT]/\{1 + \exp[(m[\text{urea}] - \Delta G_{H_2O})/RT]\} \quad (3)$$

barnase and each of the mutants, the linear plots of ΔG vs [urea] are shown in Figure 2b, and the resulting values of ΔG_{H_2O} and m are given in Table I.

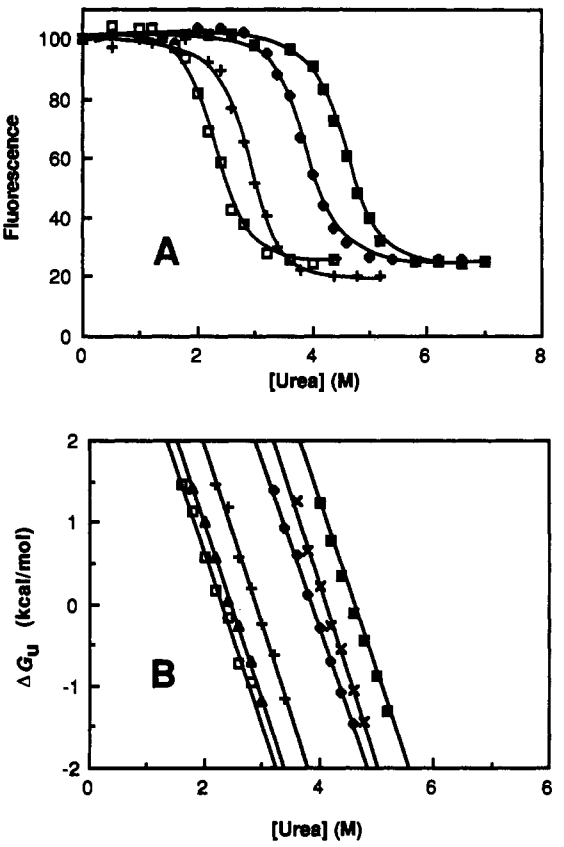

FIGURE 2: Urea-induced unfolding of wild-type and mutant barnases. The methods are described under Experimental Procedures, and the calculations are described under Results. In this and the following figures and in all tables, mutant proteins are designated by the wild-type amino acid residue, its position in the sequence, and the mutant amino acid residue, e.g., Ile-96 → Val = I 96 V. The proteins are designated by the following symbols: wild type (■); I 96 V (×); I 88 V (◆); I 96 A (+); I 88 A (▲); L 14 A (□). (A) Intrinsic fluorescence of barnase as a function of urea concentration. The solid lines are only for visual aid. The data for I 96 V and I 88 A have been omitted for clarity. (B) Free energy of unfolding as a function of urea concentration around the midpoint of the transition.

Calculation of $\Delta\Delta G_U$. $\Delta\Delta G_U$, the difference in free energy of unfolding of wild-type and mutant enzymes, may be calculated from $\Delta\Delta G_U = (\Delta G_{H_2O})_{\text{wild type}} - (\Delta G_{H_2O})_{\text{mutant}}$. This makes the assumption that eq 2 holds over the complete range of urea concentrations. Our data show that the value of m (Table I) is constant over a wide range of concentrations of urea, there being no systematic variation of m, which ranges less than ±3% from the mean. Although reasonably accurate values of ΔG_{H_2O} may be derived from eq 2, the differences between ΔG_{H_2O} for wild-type and mutant enzymes are not sufficiently precise for calculating $\Delta\Delta G_U$. This is because it is a long extrapolation to 0 M urea from the measurements made over a relatively narrow range in the region of 4 M urea. Further, the least accurate calculations of K_U are when it is greater than 10 or less than 0.1 (i.e., when $F \approx F_N$ or F_U), and it is precisely those extreme values of K_U that are most important in determining the slope m and the extrapolation back

Table I: Changes in Free Energy of Unfolding of Barnase Caused by Deletion of Methylene Groups from the Hydrophobic Core of the Protein As Determined by Reversible Chemical Denaturation

denaturant	mutant	ΔG_{H_2O} (kcal/mol)[a,b]	m (kcal mol^{-1} M^{-1})[b,c]	$[D]_{50\%}$ (M)[b,d]	$\Delta\Delta G_U$ (kcal/mol)[e]
urea	wild type	9.42	2.06	4.57	
	I 96 V	8.68	2.11	4.11	0.98
	I 88 V	7.95	2.06	3.87	1.49
	I 96 A	6.29	2.18	2.89	3.52
	I 88 A	5.34	2.16	2.47	4.46
	L 14 A	4.77	2.08	2.29	4.80
guanidinium chloride	wild type	8.88	4.51	1.97	
	I 96 V	8.19	4.64	1.76	0.95
	I 96 A	6.16	4.87	1.26	3.33

[a] Data were obtained at 25.0 °C as described under Experimental Procedures and under Results. ΔG_{H_2O} was determined by denaturation of the proteins and linear extrapolation of the data to zero denaturation. [b] Any discrepancies are due to rounding errors. [c] m is the slope of the linear denaturation plot, $-d\Delta G_U/d[\text{denaturant}]$ (eq 2). [d] $[D]_{50\%}$ is the concentration of denaturant at which 50% of the protein sample is unfolded. [e] $\Delta\Delta G_U$ was determined by direct comparison of the free energy of unfolding at denaturant concentrations where the data overlap, except in the case of guanidinium chloride denaturation of I 96 A, where the data did not overlap, and $\Delta\Delta G_U$ was calculated from eq 5.

to 0 M urea. K_U is most accurately measured at values around unity because K_U is at its least sensitive to errors in F, F_N, or F_U at this point where folded and unfolded forms are at equal concentration. Indeed, the most reproducible quantity from repetitive experiments over a period of time and experimental methods is the concentration of denaturant at 50% unfolding ($[D]_{50\%}$, Table I).

The two following procedures have been employed to minimize errors in calculation of $\Delta\Delta G_U$. The first is to measure $\Delta\Delta G_U$ directly at each concentration of urea from eq 4 (Kellis et al., 1988).

$$\Delta\Delta G_U = RT \ln \{([\text{folded}]/[\text{unfolded}])_{\text{wild type}}/([\text{folded}]/[\text{unfolded}])_{\text{mutant}}\} \quad (4)$$

Application of eq 4 requires that the ratio $([\text{folded}]/[\text{unfolded}])_{\text{mutant}}$ can be measured accurately under the same conditions as $([\text{folded}]/[\text{unfolded}])_{\text{wild type}}$. This occurs when $\Delta\Delta G_U$ is small. When $\Delta\Delta G_U$ is large and eq 4 cannot be directly applied, a series of mutants can be used where $\Delta\Delta G_U$ is small between each, to scale one against the other. The advantages of this method are that no assumption is made about the precise law governing the dependence of ΔG_U on [urea] and the differences in properties of wild-type and mutant proteins can be measured directly under identical solvent conditions.

A second procedure that was found to be reliable takes advantage of the reproducibility in measuring the concentration of urea for 50% unfolding, $[\text{urea}]_{50\%}$. Equation 2 may be used for a short interpolation for wild-type and mutant proteins to give

$$\Delta\Delta G_U = 0.5(m_{\text{wild type}} + m_{\text{mutant}})\Delta[\text{urea}]_{50\%} \quad (5)$$

where $\Delta[\text{urea}]_{50\%}$ is the difference between $[\text{urea}]_{50\%}$ for wild type and mutant.

Both these procedures measure $\Delta\Delta G_U$ in the presence of denaturant. The presence of urea should not affect the interactions of fully buried hydrophobic groups in the folded state but is known to increase the solubility of hydrophobic side chains when exposed in the unfolded state (Nozaki & Tanford 1963). At the concentrations of urea where $\Delta\Delta G_U$ is measured, 3–4 M, there is a negligible effect on the solubility of Ile vs Val, but Ile is stabilized in solution by 0.15 kcal/mol relative to Ala. Thus, $\Delta\Delta G_U$ is lowered by 0.1–0.2 kcal/mol for the mutation Ile → Ala measured in urea relative to it being measured in water alone.

Table II: Thermodynamic Data for Unfolding of Wild-Type and Mutant Barnases[a]

mutant	T_m (°C)	ΔH_U (kcal/mol)	ΔS_U (kcal mol^{-1} deg^{-1})	$\Delta\Delta G_U$ (kcal/mol) van't Hoff	$\Delta T_m \Delta S_U$	direct
wild type	53.9	125	0.383			
I 96 V	51.5	121	0.374	0.9	0.9	0.9
I 88 V	51.0	117	0.361	1.1	1.1	1.1
I 96 A	44.9	112	0.351	3.3	3.3	3.2
I 88 A	42.7	106	0.335	4.0	4.1	3.9
L 14 A	42.0	108	0.343	4.3	4.3	4.2

[a] These values were determined by thermal denaturation of the proteins as described under Experimental Procedures and under Results. T_m is the temperature at which half of each protein sample is unfolded. ΔH_U and ΔS_U values were calculated at the T_m for each protein by van't Hoff analysis. $\Delta\Delta G_U$ values were calculated at 48 °C (a value intermediate among the T_m values for the group of proteins) from temperature-compensated $\Delta\Delta H_U$ and $\Delta\Delta S_U$ values (van't Hoff), from the relation $\Delta\Delta G_U \approx \Delta T_m \Delta S_U$, and from the data in Figure 3C (direct).

Precision of Data. Values of m were reproducible to within ±3%, and values of $[\text{urea}]_{50\%}$ were reproducible to within ±1% when the same stock solutions of urea were used. This implies a precision of better than ±0.1 kcal/mol when $\Delta\Delta G_U$ is calculated from eq 5, and we find that the data are reproducible within these limits. Earlier experiments (Kellis et al., 1988) using UV absorbance to monitor denaturation gave values of m within 10%, of $[\text{urea}]_{50\%}$ within 2%, and of $\Delta\Delta G_U$ within 10% of those determined by fluorescence (Table I) despite different urea solutions, a 10-fold higher concentration of enzyme, and much smaller signals being monitored in the UV experiments.

Denaturation by Guanidinium Chloride. Guanidinium chloride was also used as a denaturant for wild-type barnase and the Ile-96 mutants, with results comparable to those found with urea (Table I). All of the values are slightly lower, ranging from 94 to 98% of those determined with urea denaturation.

Thermal Denaturation

We also analyzed the thermodynamics of unfolding of these proteins by subjecting them to reversible thermal denaturation. The fluorescence of wild-type and several of the mutant barnases as a function of temperature is shown in Figure 3a. The mutations cause a decrease in T_m, the temperature at which the proteins are half unfolded, and the rank order of thermal stabilities (see Table II) is the same as that determined by urea denaturation. The ratio of folded to unfolded forms of the enzyme may be calculated by eq 1 as above. It is seen in Figure 3A that outside the unfolding transition the fluorescence of the native and unfolded forms of the protein varies linearly with temperature, and so the values of F_N and F_U must be estimated for each temperature by extrapolation, thus lowering the accuracy relative to that of the calculations from the urea-mediated denaturation.

Calculation of $\Delta\Delta G_U$ from Thermal Denaturation. (A) van't Hoff Equation. The enthalpy, ΔH_U, and the entropy, ΔS_U, of unfolding for the series of proteins are given from classical thermodynamics by eq 6 and 7. The van't Hoff plots

$$\Delta H_U = -R[d \ln K_U/d(1/T)] = RT^2(d \ln K_U/dT) \quad (6)$$

$$\Delta S_U = \Delta H_U/T_m \quad (7)$$

of thermal denaturation (Figure 3B) are approximately linear

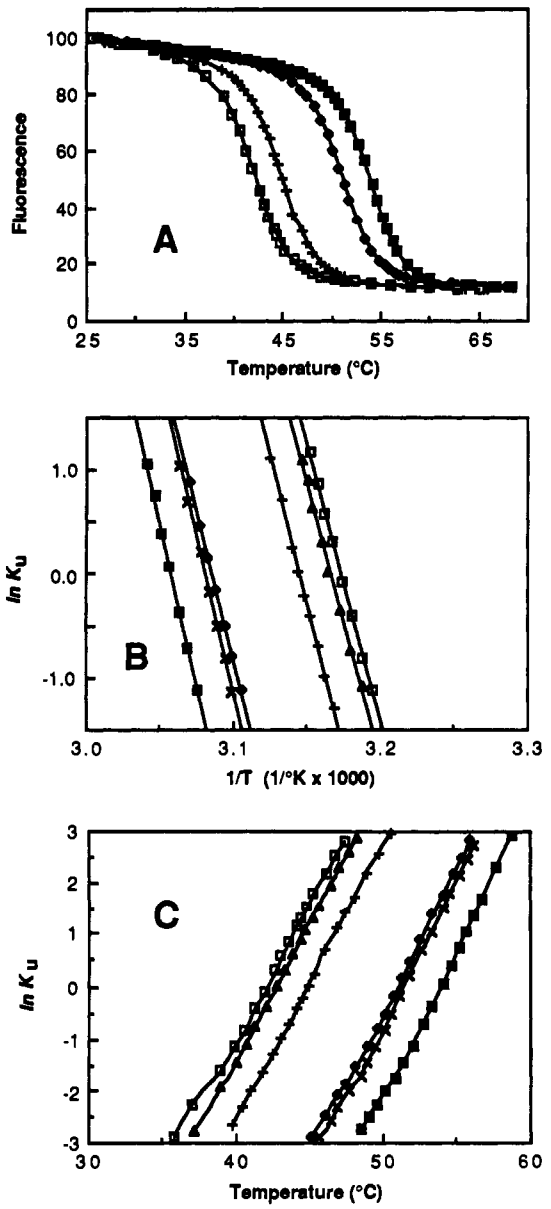

FIGURE 3: Thermal denaturation of wild-type and mutant barnases. The methods are described under Experimental Procedures, and the calculations are described under Results. The proteins are designated by the same symbols as in Figure 2. (A) Intrinsic fluorescence of the proteins as a function of temperature. The data for I 96 V and I 88 A have been omitted for clarity. (B) van't Hoff plots of the denaturation data around the transition. (C) Natural logarithm of the equilibrium constant for unfolding as a function of temperature.

through the T_m region, thus allowing an estimation of the enthalpy and entropy of unfolding at T_m (Table II). Plots over a wider range of temperatures are expected to be curved because ΔH_U and ΔS_U vary with temperature as given in eq 8,

$$\Delta C_p = (\partial \Delta H/\partial T)_p = T(\partial \Delta S/\partial T)_p \quad (8)$$

where ΔC_p is the change in heat capacity at constant pressure which accompanies the unfolding of a protein. ΔC_p is large and positive (Privalov, 1979); therefore, as can be seen in Table II, the enthalpy and entropy of unfolding decrease dramatically with decreasing stability, i.e., with the decreasing T_m at which they were determined. Thus, the value of ΔG_U at a given temperature cannot be simply calculated from ΔH_U and ΔS_U, without allowing for their variation because of ΔC_p. An estimate of ΔC_p for barnase can be obtained from the data of Hartley (1969), where he determined T_m and ΔH_U over a range of pH values. There is good evidence that $\Delta C_p = d\Delta H_U/dT_m$, for variation of pH in the acid limbs of denaturation profiles, because the enthalpies of ionization of the side chains in this region are close to zero (Privalov, 1979). The data of Hartley yield a value of $\Delta C_p = 1.20$ kcal deg^{-1} mol^{-1}. This value, which corresponds to 0.10 cal deg^{-1} g^{-1}, is reasonable for a small globular protein, and there is ample evidence for the constancy of ΔC_p for many proteins between 20 and 80 °C (Privalov, 1979). Consequently ΔH_U and ΔS_U for the barnase mutants can be calculated at other temperatures from eq 9 and 10 [cf. Baldwin (1986)]. We calculated values

$$\Delta H_T = \Delta H_{T_m} + \Delta C_p(T - T_m) \quad (9)$$

$$\Delta S_T = \Delta S_{T_m} + \Delta C_p \ln (T/T_m) \quad (10)$$

of ΔH_U and ΔS_U for each of the proteins (not shown) at 48 °C, which is intermediate among the values of T_m for these proteins. The resulting values of $\Delta\Delta H_U$ and $\Delta\Delta S_U$ allow the calculation of $\Delta\Delta G_U$ at that temperature, because $\Delta\Delta G_U = \Delta\Delta H_U - T\Delta\Delta S_U$; these $\Delta\Delta G_U$ values are given in Table II. Extrapolated $\Delta\Delta H_U$ and $\Delta\Delta S_U$ values may also be used to calculate $\Delta\Delta G_U$ at 25 °C; the results (Table III) are remarkably close to the values determined by urea denaturation at this temperature.

(*B*) *Approximate Method from* ΔT_m. $\Delta\Delta G_U$ can also be estimated from the relationship

$$\Delta\Delta G_U \approx \Delta T_m \Delta S_U \quad (11)$$

where ΔT_m is small (Becktel & Schellman, 1987). This is a reasonable assumption in the present case because the largest ΔT_m is less than 4% of T_m. $\Delta\Delta G_U$ values have been calculated from ΔS_U of wild-type barnase at 48 °C and are given in Table II.

(*C*) *Direct Method.* This is analogous to the direct determination by eq 4 used for urea denaturation. The values of K_U were compared at temperatures where the transition regions overlap (with small interpolations where the temperatures are not identical). The plot of ln K_U versus temperature (Figure 3C) allows the determination of $\Delta\Delta G_U$ at a given temperature from $\Delta\Delta G = -RT[(\ln K_U)_{\text{wild type}} - (\ln K_U)_{\text{mutant}}]$, which is analogous to the application of eq 4. This method has the advantage of making no assumptions about the dependence of the thermodynamic parameters on temperature. Values of $\Delta\Delta G_U$ calculated by this method at 48 °C are given in Table II. The values for $\Delta\Delta G_U$ from thermal denaturation calculated by the three different methods are in good agreement with one another and substantiate the validity of these thermodynamic measurements.

Properties of Mutant Enzymes. The yield of purified mutant enzyme decreases with decreasing stability and falls

Table III: Summary of Free Energies of Unfolding of Wild-Type and Mutant Barnase at 25 °C As Determined by Urea-Induced and Thermal Denaturation[a]

	urea denaturation		thermal denaturation	
mutant	ΔG_{H_2O} (kcal/mol)	$\Delta\Delta G_U$ (kcal/mol)	ΔG_{H_2O} (kcal/mol)	$\Delta\Delta G_U$ (kcal/mol)
wild type	9.4		9.5	
I 96 V	8.7	1.0	8.6	0.9
I 88 V	8.0	1.5	8.1	1.4
I 96 A	6.3	3.5	6.2	3.3
I 88 A	5.3	4.5	5.3	4.2
L 14 A	4.8	4.8	5.3	4.2

[a] The values for urea denaturation are from Table I, and the values for thermal denaturation were calculated from the ΔH_U and ΔS_U values of Table II, eq 9 and 10 being used for temperature compensation; $\Delta G_{H_2O} = \Delta H_U - T\Delta S_U$ and $\Delta\Delta G_U = (\Delta G_{H_2O})_{\text{wild type}} - (\Delta G_{H_2O})_{\text{mutant}}$.

Table IV: Ribonuclease Activity of Mutant Barnases[a]

mutant	activity (relative to wild type)
I 96 V	1.0
I 88 V	1.0
I 96 A	0.9
I 88 A	1.0
L 14 A	0.8

[a] RNA hydrolysis was measured at 25 °C by a previously published method (Rushizky et al., 1963), as described under Experimental Procedures.

off at least 10-fold for the larger changes. Measurement of the ribonuclease activity of the various mutants (Table IV) reveals that the mutations have produced no structural changes that significantly decrease enzymatic activity.

Discussion

Validity of Measurements of $\Delta\Delta G_U$ *and* ΔG_{H_2O}. The free energy of unfolding depends on the difference in energy between two states, the folded and the unfolded. A crucial factor in the energetics of unfolding is, therefore, the interaction of exposed side chains in the unfolded state with solvent, solutes, and the rest of the protein. Any specific interactions in the unfolded state will alter the observed value of ΔG_U. Different methods of inducing unfolding have different possible complications. Urea-induced or guanidinium chloride induced denaturation has the reputation of causing more complete unfolding than does thermal denaturation, but there is the possibility of complications from the presence of concentrated solute. Thermal unfolding has a different set of potential complications. In addition to the possibilities of some residual structure in the unfolded state, experiments are performed at differing and elevated temperatures, leading to some irreversible denaturation and the necessity of extrapolation of thermodynamic data to allow for temperature differences. For these reasons, we have (i) made mutations that are likely to make minimal perturbations not only of the folded state but of the unfolded state as well and (ii) performed careful unfolding measurements by two types of solvent-induced denaturation and thermal denaturation. The agreement between $\Delta\Delta G_U$ measured by the three methods is excellent (Tables I–III). There is even excellent agreement between the values of ΔG_{H_2O}, the free energy of unfolding in the absence of denaturant (at 25 °C, Table III) extrapolated from the urea and thermal denaturation experiments. [Measurements from guanidinium chloride denaturation are known to give slightly lower values for ΔG_{H_2O} on extrapolation to the absence of denaturant (Privalov, 1979).] It seems most likely that the measurements reported do represent the true values of free energies, because the different methods of measurements give very similar results.

Loss of Free Energy of Folding on Creating Cavities in the Hydrophobic Core. The free energy of folding of wild-type barnase is approximately 9.5 kcal/mol. Deletion of a single methylene group from the core loses from 1.0 (mutation of Ile-96 → Val) to 1.5 (Ile-88 → Val) kcal/mol. Deletion of three methylene groups (Ile-96 → Ala, Ile-88 → Ala, Leu-14 → Ala) loses 3 times those values. That is, half the stabilization energy of the protein can be lost on what is considered a "conservative" mutation. All of the mutations were designed only to delete groups and not to add unfavorable interactions. For that reason, we did not make the mutation Leu-14 → Val, because there would be a change of position of branching. These values reflect the loss of favorable interactions in the folded states and hydrophobic interactions in the unfolded states.

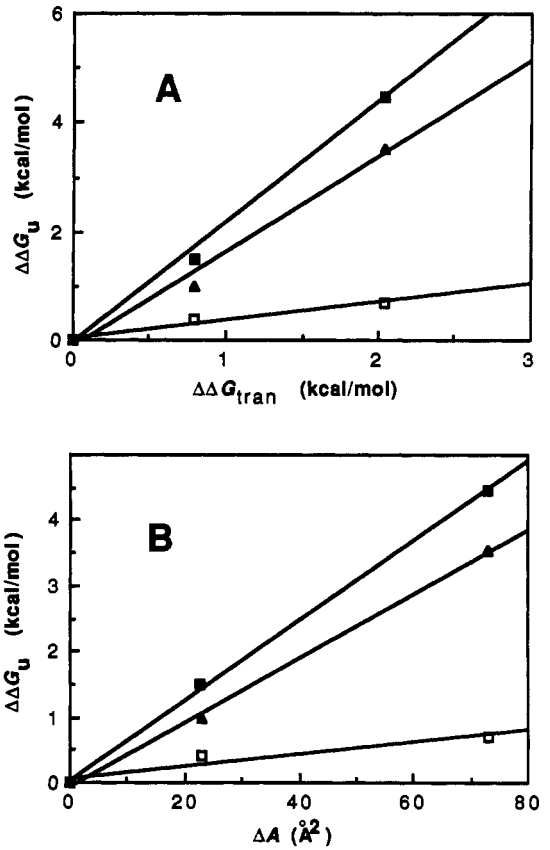

FIGURE 4: (A) Plot of $\Delta\Delta G_U$ versus $\Delta\Delta G$ for transfer from octanol to water for barnase and T4 lysozyme Ile mutants. The mutants are designated as follows: Ile-88 (■); Ile-96 (▲); Ile-3 (T4 lysozyme) (□). The data for $\Delta\Delta G$ of transfer are from Fauchere and Pliska (1983), and the data for $\Delta\Delta G_U$ of T4 lysozyme (at pH 6.5) are from Matsumura et al. (1988). (B) Plot of reduction in free energy of folding versus reduction in solvent-accessible residue surface area for barnase and T4 lysozyme Ile mutants. The symbols are as in (A). The surface area values from which the differences were calculated are defined as the average surface area that residue X has in a Gly-X-Gly tripeptide with the main chain in an extended conformation (Miller et al., 1987). The data for $\Delta\Delta G_U$ of T4 lysozyme (at pH 6.5) are from Matsumura et al. (1988).

A plot may be made for $\Delta\Delta G_U$ on the change Ile → Val → Ala against the free energies of transfer of these side chains from 1-octanol to water (Figure 4A) in a manner analogous to a Hansch plot (Hansch & Coats, 1970). The slopes are 1.75 and 2.20 for mutations at positions 96 and 88, respectively. A plot of $\Delta\Delta G_U$ for these residues against the surface areas of the side chains that are buried (Figure 4B) gives slopes of 49 and 61 cal mol^{-1} Å^{-2}, compared with 20–30 cal mol^{-1} Å^{-2} found for model studies (Chothia, 1975; Eisenberg & McLachlan, 1986; Ooi et al., 1987). Our values, though appearing high, are not inconsistent with the simple model studies which have been used to mimic hydrophobic changes in proteins. This is because there is a crucial difference between transferring a side chain from water to a hydrocarbon liquid and from water to the interior of a protein. When a hydrophobic side chain is buried in a preformed cavity in a protein, two hydrocarbon–water interfaces are eliminated, both that of the side chain and that of the cavity. When a side chain partitions into a hydrocarbon liquid, only the surface of the side chain is removed from water. Hence, to a first approximation, there should be twice the free energy of transfer to a protein than to octanol. Clearly, precise values will depend on the degree of solvation of the protein cavity and closeness of fit of the side chains.

Also plotted in Figure 4 are recent data for the same mu-

tations of Ile-3 of T4 lysozyme [data from Matsumura et al. (1988)]. Here, the slopes are less than one-fifth of those from the mutations of barnase. Perhaps this discrepancy arises because the side chain of Ile-3 is 20% exposed, on the basis of a solvent-accessibility calculation from the crystal structure of T4 lysozyme (Matsumura et al., 1988).

A computer simulation of the mutation of a buried leucine to alanine in trypsin has recently been reported (Bash et al., 1987). These investigators obtained a value for a loss in the free energy of folding of 1.0 kcal/mol, significantly lower than the value of 4.8 kcal/mol that we found experimentally for the same mutation. It has been emphasized, (Fersht, 1987, 1988) that values of $\Delta\Delta G$ from mutagenesis experiments are *apparent* binding energies and are not necessarily equal to real binding energies. Apart from artifacts arising from reorganization of the protein on mutation, the relationship of the apparent binding energies to real binding energies depends crucially on the access of water to cavities created in the enzyme. Nevertheless, whatever the detailed structural effects, the values of $\Delta\Delta G_U$ do accurately measure the stability of mutants compared with wild-type enzyme and, in the present study, the change in energetics on creating cavities in a protein. Comparing the mutants studied in this paper and Ile-3 of T4 lysozyme, it appears that the importance of hydrophobic interactions varies considerably with position within a protein. Our results demonstrate that complementary interactions in the interior of a protein can be far more important than indicated by the experiments on lysozyme and calculations on trypsin and that packing of nonpolar side chains is a major determinant of the three-dimensional conformation adopted by proteins.

Do the Mutant Proteins Have Altered Structures? The data presented here are meant simply to be empirical measurements on the effects of introducing cavities in a hydrophobic core. The possibility exists that the mutations discussed here, although chosen to be nondisruptive, have altered the folded structure of barnase, thus complicating subsequent theoretical analysis. In this context, any structural reorganization of the folded protein on mutation would serve to offset the loss of the interaction, and so the observed value of $\Delta\Delta G_U$ would underestimate the energetics of the interaction that is removed. There is preliminary evidence from NMR measurements that the mutations have not caused large changes in structure. Proton resonances for His-18, at the C-terminal of the α-helix studied in this paper, have been assigned and exhibit ring-current shifts produced by the close proximity of Trp-94 in the β-sheet (Sali et al., 1988; M. Bycroft and A. R. Fersht, unpublished results). These chemical shifts are exquisitely sensitive to the distance and orientation of the aromatic ring that gives rise to them (Cantor & Schimmel, 1980) and are, therefore, indicative of subtle structural rearrangement of the α-helix relative to the β-sheet in the mutant proteins. One-dimensional NMR spectra for mutants at each position studied in this paper show no changes in the chemical shifts of the His-18 resonance, confirming that the mutations have created no significant change in the conformation of the α-helix relative to the β-sheet (M. Bycroft and A. R. Fersht, unpublished results). Full NMR structural studies are in progress on the mutant proteins, which are also being crystallized for X-ray analysis in an effort to determine the precise conformation and packing of the truncated amino acid side chains.

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Registry No. Ile, 73-32-5; Leu, 61-90-5; Val, 72-18-4; Ala, 56-41-7; barnase, 37300-74-6.

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