Rothman, J. E., & Orci, L. (1990) *FASEB J. 4*, 1460-1468.
Schwarzmann, G., & Sandhoff, K. (1987) *Methods Enzymol. 138*, 319-341.
Schwarzmann, G., & Sandhoff, K. (1990) *Biochemistry 29*, 10865-10871.
Shayman, J. A., Mahdiyoun, S., Deshmukh, G., Barcelon, F., Inokuchi, J.-i., & Radin, N. S. (1990) *J. Biol. Chem. 265*, 12135-12138.
Shayman, J. A., Deshmukh, G. D., Mahdiyoun, S., Thomas, T. P., Wu, D., Barcelon, F. S., & Radin, N. S. (1991) *J. Biol. Chem. 266*, 22968-22974.
Simons, K., & van Meer, G. (1988) *Biochemistry 27*, 6197-6202.
Steinman, R. M., Silver, J. M., & Cohn, Z. A. (1974) *J. Cell Biol. 63*, 949-969.
Sundaram, K. S., & Lev, M. (1984) *J. Neurochem. 42*, 577-581.
Sweeley, C. C. (1985) in *Biochemistry of Lipids and Membranes* (Vance, D. E., & Vance, J. E., Eds.) pp 361-403, The Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA.
Swift, A. M., & Machamer, C. E. (1991) *J. Cell Biol. 115*, 19-30.
Trinchera, M., Fabbri, M., & Ghidoni, R. (1991) *J. Biol. Chem. 266*, 20907-20912.
Uemura, K.-i., Sugiyama, E., & Taketomi, T. (1991) *J. Biochem. 110*, 96-102.
van Echten, G., & Sandhoff, K. (1989) *J. Neurochem. 52*, 207-214.
Vunnam, R. R., & Radin, N. S. (1980) *Chem. Phys. Lipids 26*, 265-278.
Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., & Merrill, A. H., Jr. (1991) *J. Biol. Chem. 266*, 14486-14490.
Wattenberg, B. W. (1990) *J. Cell Biol. 111*, 421-428.
Wieland, F. T., Gleason, M. L., Serafini, T. A., & Rothman, J. E. (1987) *Cell 50*, 289-300.

Dissection of Helix Capping in T4 Lysozyme by Structural and Thermodynamic Analysis of Six Amino Acid Substitutions at Thr 59[†,‡]

Jeffrey A. Bell,[§] Wayne J. Becktel,[∥] Uwe Sauer,[⊥] Walter A. Baase, and Brian W. Matthews*

Institute of Molecular Biology, Howard Hughes Medical Institute, and Department of Physics, University of Oregon, Eugene, Oregon 97403

Received August 30, 1991; Revised Manuscript Received November 22, 1991

ABSTRACT: Threonine 59, a helix-capping residue at the amino terminus of the longest helix in T4 phage lysozyme, was substituted with valine, alanine, glycine, serine, asparagine, and aspartic acid. The valine, alanine, and glycine replacements were observed to be somewhat more destabilizing than serine, asparagine, and aspartic acid. The crystal structures of the different variants showed that changes in conformation occurred at the site of substitution, including Asp 61, which is nearby, as well as displacement of a solvent molecule that is hydrogen-bonded to the γ-oxygen of Thr 59 in wild-type lysozyme. Neither the structures nor the stabilities of the mutant proteins support the hypothesis of Serrano and Fersht (1989) that glycine and alanine are better helix-capping residues than valine because a smaller-sized residue allows better hydration at the end of the helix. In the aspartic acid and asparagine replacements the substituted side chains form hydrogen bonds with the end of the helix, as does threonine and serine at this position. In contrast, however, the Asp and Asn side chains also make unusually close contacts with carbon atoms in Asp 61. This suggests a structural basis for the heretofore puzzling observations that asparagine is more frequently observed as a helix-capping residue than threonine [Richardson, J. S., & Richardson, D. C. (1988) *Science 240*, 1648-1652] yet Thr → Asn replacements at N-cap positions in barnase were found to be destabilizing [Serrano, L., & Fersht, A. R. (1989) *Nature 342*, 296-299]. Threonine and asparagine are shown to require different conformations of the backbone at the end of the α-helix in order to adopt optimal capping conformations. The energetic and structural consequences of substitutions at the N-cap position therefore depend not only on the nature of the substitution and the local environment in which it occurs but also on the backbone conformation that exists at the site of substitution.

A helix N-cap residue is perhaps best defined as the first residue in a helix whose α-carbon is within the cylinder defined by the helix (Richardson & Richardson, 1988). A survey of 215 helices in the structures of 45 different globular proteins (Richardson & Richardson, 1988) found that the most common residues at the N-cap position are Ser, Asn, Gly, Asp, and Thr, in order of decreasing frequency. In the evolution of proteins, some advantage evidently favors the choice of these residues although the reasons are not clear. Both kinetic (protein folding) and thermodynamic (protein stabilizing) roles for N-cap residues have been proposed (Presta & Rose, 1988; Richardson & Richardson, 1988).

The stability of helices in small synthetic peptides is known to be dependent on charged residues at the ends of those helices (Ihara et al., 1982; Shoemaker et al., 1985, 1987), presumably due to a favorable electrostatic interaction with the α-helix dipole (Blagdon & Goodman, 1975; Wada, 1976; Hol et al., 1978). Nicholson et al. (1988, 1991) have also shown that

[†] This work was supported by the National Institutes of Health (GM21967 to B.W.M. and GM20195 to J. A. Schellman) and the Lucille P. Markey Charitable Trust. A USPHS postdoctoral fellowship (GM10378) to J.A.B. is also gratefully acknowledged.

[‡] The coordinates for all T4 lysozyme structures discussed in this report have been deposited in the Protein Data Bank at the Brookhaven National Laboratory.

* Address correspondence to this author at the Institute of Molecular Biology.

[§] Present address: Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY 12180.

[∥] Present address: Department of Chemistry, The Ohio State University, Columbus, OH 43210.

[⊥] Present address: European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, 6900 Heidelberg, Germany.

0006-2960/92/0431-3590$03.00/0

Table I: Data Collection and Refinement Statistics for X-ray Crystal Structure Determinations[a]

protein	cell a, b (Å)	cell c (Å)	total films	R_{merge} (%)	reflections measured total	reflections measured independent	resolution limit (Å)	R_{refine} (%)	rms deviation from ideal geometry: bond length (Å)	bond angle (deg)	trigonal planarity (Å)	group planarity (Å)
WT*	60.9	96.8		4.5		13879	1.75	14.8	0.015	2.08	0.010	0.014
T59A	60.9	97.1	15	6.2	30000	13312	1.8	15.9	0.016	2.24	0.012	0.014
T59D	60.9	96.9	16	8.5	30600	10903	2.0	15.3	0.018	2.34	0.013	0.015
T59G	61.0	96.9	18	7.2	37200	14849	1.7	16.3	0.016	2.20	0.011	0.014
T59N	61.0	97.0	17	6.3	35000	13597	1.8	15.5	0.015	2.14	0.010	0.014
T59S	61.0	97.0	17	7.5	31400	12755	1.8	16.3	0.017	2.40	0.011	0.015
T59V	61.0	97.0	16	5.3	31600	13413	1.8	15.5	0.016	2.21	0.012	0.014

[a] All of the proteins described herein contain the mutations C54T/C97A, which were introduced to remove the two cysteine side chains present in wild-type lysozyme. Refinement of all of the proteins with substitutions at residue 59 was based on the coordinates for the structure of C54T/C97A, which is labeled WT* (Pjura et al., 1990). Numbers of reflections are based on data between 6 Å and the resolution limit shown.

Table II: Changes in Thermal Stability for Variants at Site 59[a]

protein	pH 2.0 ΔT_m (°C)	pH 2.0 $\Delta\Delta G$ (kcal/mol)	pH 6.5 ΔT_m (°C)	pH 6.5 $\Delta\Delta G$ (kcal/mol)
T59N	−2.1	−0.6	−2.8	−1.1
T59S	−2.6	−0.7	−0.4	−0.2
T59D	−3.1	−0.9	−3.1	−1.2
T59G	−7.7	−2.2	−4.1	−1.6
T59V	−10.0	−2.8	−4.0	−1.5
T59A	−10.1	−2.8	−4.0	−1.5

[a] Thermally promoted unfolding experiments were performed in 0.15 M potassium chloride with hydrochloric acid added to pH 2.0 and also in 0.15 M potassium chloride with 0.01 M potassium phosphate adjusted to pH 6.5 (Becktel & Baase, 1987). The change in melting temperature, ΔT_m, of each mutant protein was determined relative to the T_m of the pseudo-wild-type reference protein, WT*, here found to be 41.0 and 63.0 °C at pH 2 and 6.5, respectively. Errors in ΔT_m values are less than 15% except in the case of T59S where the estimated absolute error of ±0.3 °C corresponds to a 75% relative error. The change in free energy of unfolding of the mutant protein relative to wild type, $\Delta\Delta G$, was calculated from $\Delta\Delta G = \Delta T_m \Delta S$, where ΔT_m is the difference in melting temperatures of the proteins being compared and ΔS is the unfolding entropy change of the reference protein, here found to be 280 and 380 eu for low and high pH, respectively (Becktel & Schellman, 1987). ΔT_m is T_m(mutant protein) minus T_m(reference protein) so that a negative value indicates decreased stability relative to WT*.

negative charges introduced near the N-termini of α-helices increase the stability of T4 lysozyme through electrostatic interactions.

Serrano and Fersht (1989) made a number of substitutions at Thr 6 and Thr 26, the N-cap residues for the two α-helices in the enzyme barnase. They observed that the free energy of folding of the protein depended in a fairly consistent manner on the amino acid that was substituted at either of these N-cap positions. The most unexpected, and unexplained, result was that asparagine was energetically less favorable at either site than serine or threonine, even though asparagine is observed at high frequency at the N-cap position.

In this report we investigate the role of the N-cap residue threonine 59 which is at the end of the longest (23 residues) helix in T4 lysozyme. Thr 59 was substituted with six other amino acids, four of which are commonly found as helix N-caps and two of which are rarely found in this role (Richardson & Richardson, 1988). The stabilities of the variants have been measured and the crystal structures determined, allowing the thermodynamic data to be rationalized in structural terms. Because the substituted residue in wild-type lysozyme is threonine, it is possible to compare the present results with the threonine helix-capping replacements reported previously by Serrano and Fersht (1989), subject to certain differences in technique (see legend to Figure 1).

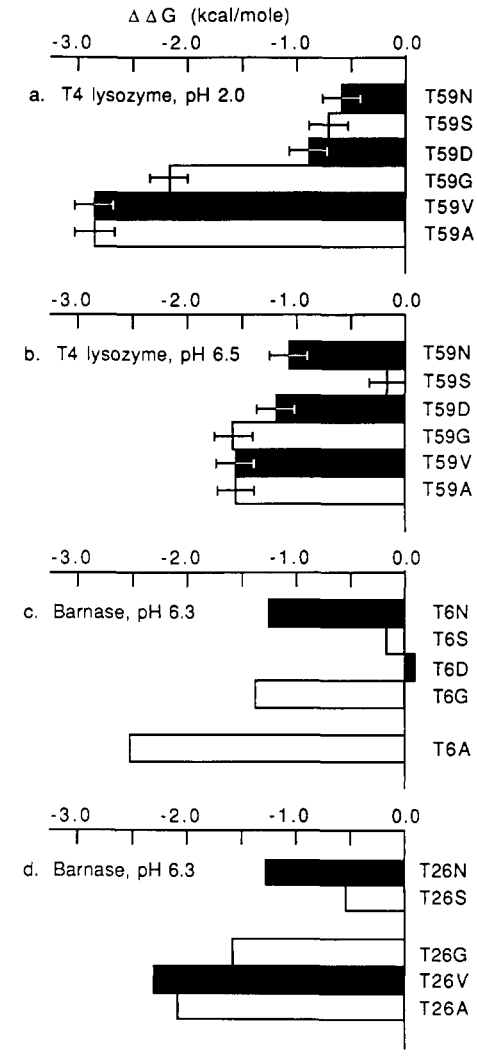

FIGURE 1: The difference between the free energy of unfolding of mutant lysozymes and that of wild-type lysozyme at pH 2.0 and 6.5 ($\Delta\Delta G$ from Table II) is shown respectively in (a) and (b). Errors in $\Delta\Delta G$ were propagated on the basis of a 15% error in ΔT_m and a 10% error in ΔS. This gave an overall error in $\Delta\Delta G$ of 20% except in the case of T59S (see Table II). Changes in stability due to replacements of Thr 6 and Thr 26 of barnase are shown respectively in (c) and (d) [from Serrano and Fersht (1989)]. Serrano and Fersht (1989) estimated the change in free energy of folding of mutant barnase relative to wild type ($\Delta\Delta G$) from the change in the concentration of urea required to unfold the protein. This gives an estimate of $\Delta\Delta G$ at the concentration of urea at which wild-type barnase is 50% unfolded. In the present case, $\Delta\Delta G$ was determined from the change in melting temperature, T_m, of the mutant lysozyme relative to wild type (Table I). This gives an estimate of $\Delta\Delta G$ at the T_m of wild-type lysozyme.

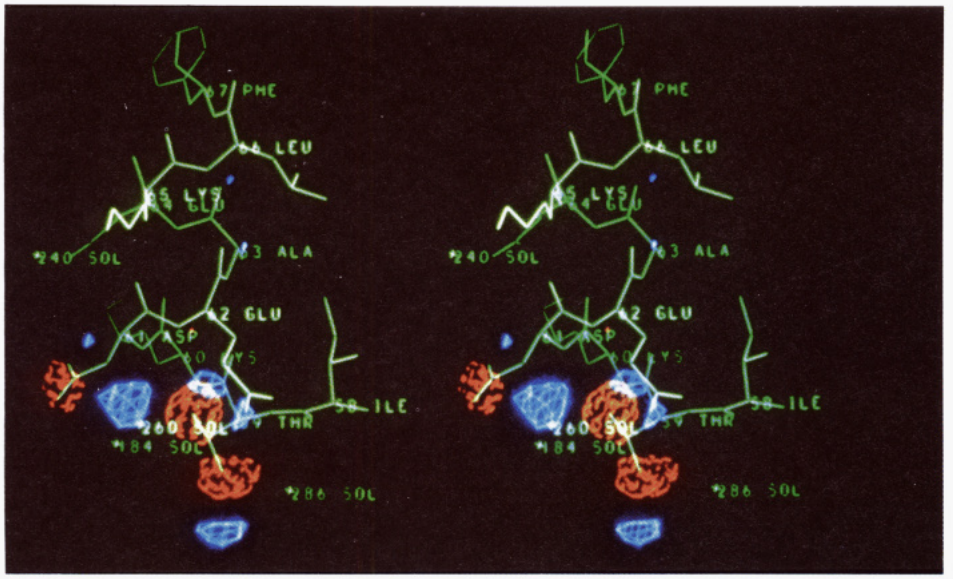

FIGURE 2: Stereoview showing the difference in electron density between T59A and the wild-type structure, superimposed on the structure of the wild-type protein. The figure includes most of the long helix for which residues 59 is the N-cap. Positive contours ($+4\sigma$) are shown in blue and negative contours (-4σ) in red. Amplitudes were $F_{mutant} - F_{WT*}$, where F_{mutant} and F_{WT*} are the structure amplitudes observed for the mutant and pseudo-wild-type lysozyme, respectively. The phases were calculated from the refined model of pseudo-wild-type lysozyme. Difference electron density is largely localized to the end of the helix. The change in conformation of Asp 61 is clearly indicated by the difference electron density.

Table III: Hydrogen-Bonding Geometry for the Different Side Chains at Residue 59 and for Asp 61[a]

	distance (Å)		angles (deg)			
residue at 59 (H-bond residue)	H61–O	H62–O	N61–H–O	N62–H–O	H61–O–C	H62–O–C
Thr 59	3.1	2.3	111	161	95	135
Ser 59	2.9	2.2	112	164	102	140
Asn 59	2.4	1.8	105	155	128	147
Asp 59	2.7	1.9	107	158	118	160
Ala 59 (Asp 61)	1.9	2.4	114	131	109	89
Gly 59 (Asp 61)	1.9	2.2	113	138	95	94
representative value	2.1	2.1	151	151	124	124

[a] The first column shows the residue at site 59. In the case of Ala 59 and Gly 59, the residue whose side chain forms the hydrogen bond with the amide backbone is Asp 61 as shown in parentheses (see Figures 2 and 3). Distances were measured between the amide hydrogens of both residues 61 and 62 and the oxygen hydrogen-bond acceptor on the side chain. The symbol H61–O denotes the distance between the hydrogen on the amide of residue 61 and the oxygen hydrogen-bond acceptor of the side chain. The symbol H61–O–C denotes the angle between the hydrogen on the amide nitrogen of residue 61, the oxygen hydrogen-bond acceptor of the side chain, and the carbon atom to which the oxygen atom is bonded. The analogous definitions hold for angles involving residue 62. Hydrogen-bonding distances and angles were estimated using the program Macromodel 2 (Still, 1988). Amide hydrogen positions were estimated as being 1.01 Å from amide nitrogens, on trigonal planar nitrogen atoms. No other possible hydrogen-bonding interactions between these side chains and protein atoms were observed. The representative values are averages for observed hydrogen bonds between N–H groups and side chains in globular proteins (Baker & Hubbard, 1984).

METHODS

Site-directed mutagenesis was performed on the T4 lysozyme gene cloned into phage M13mp18 (Matsumura et al., 1988, and references therein) using the method of Zoller and Smith (1984). In order to increase the relative yield of isolates bearing the desired mutation, template DNA was grown in *Escherichia coli* strain CJ236 as described by Kunkel et al. (1987). Phage isolates with the T4 lysozyme gene containing the desired mutant sequences were identified by sequencing directly after mutagenesis without selection. Typically ~30% of the isolates sequenced contained the desired mutations.

All variants of T4 lysozyme in this study also contained the substitutions Cys 54 → Thr and Cys 97 → Ala. The resultant cysteine-free lysozyme (C54T/C97A or WT*) has structure and thermodynamic stability similar to that of the true wild-type protein but is more amenable to reversible thermal denaturation (Wetzel et al., 1988; Matsumura et al., 1988). C54T/C97A has been adopted as a "pseudo-wild-type" standard or reference protein.

Mutant lysozymes with Thr 59 replaced by Ala, Asp, Asn, Gly, Ser, and Val were expressed and purified. All could be crystallized from ~2 M phosphate solutions at pH ~6.7 using conditions as previously described (Alber & Matthews, 1987; Muchmore et al., 1989; Weaver & Matthews, 1987).

Measurement of the midpoint of reversible thermal denaturation at pH 2.0 and 6.5 was performed as described by Becktel and Baase (1987).

X-ray diffraction data were collected as described (Weaver & Matthews, 1987; Alber et al., 1987). Crystallographic refinement was carried out using the TNT package of programs (Tronrud et al., 1987). Geometric restraints were used for bond angles, bond distances, planar groups, trigonal planar groups, and short, nonbonded contacts but not for torsion angles.

Refinement was begun with an initial model based on the structure of the pseudo-wild-type T4 lysozyme (Pjura et al., 1990) with both residues 59 and 61 as alanine. Difference maps (see below) suggested that both these residues were subject to conformational change. After six cycles of positional

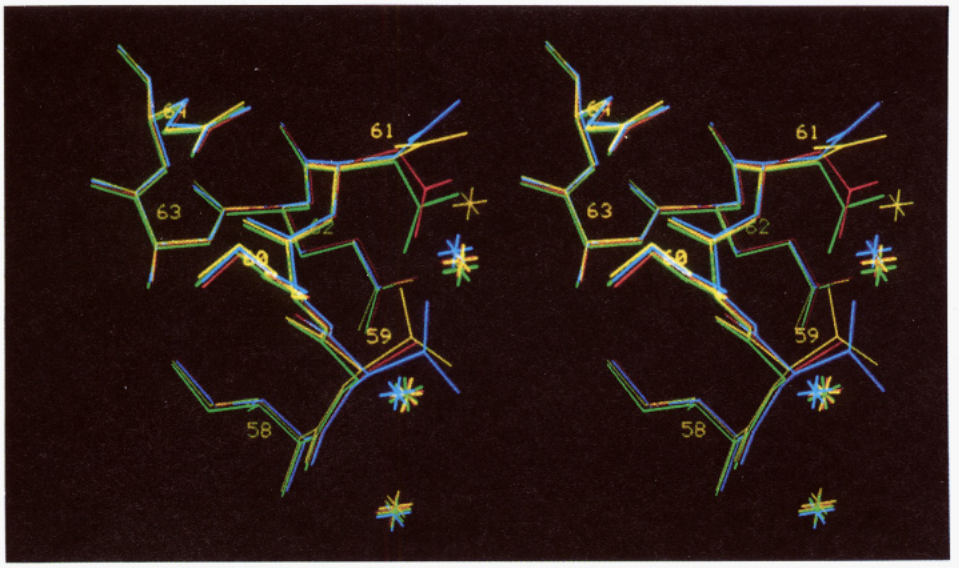

FIGURE 3: Structures of those variants that have a non-hydrogen-bonding side chain at residue 59. The wild-type structure with threonine as residue 59 is shown in yellow. The other structures are T59A (red), T59G (green), and T59V (blue). Note the change in position adopted by Asp 61 in the T59G and T59A structures.

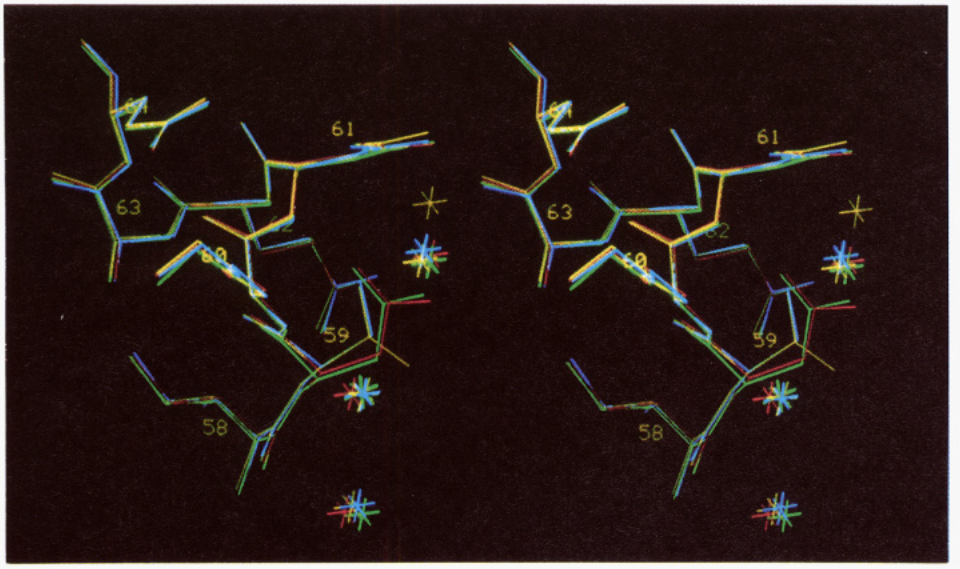

FIGURE 4: Structure of variants at residue 59 that have a hydrogen-bonding side chain. Wild-type lysozyme with threonine as residue 59 is shown in yellow. The other structures are T59S (blue), T59N (green), and T59D (red). The structures are shown as a stereopair.

refinement with moderate weight on geometric restraints, examination of difference maps allowed the positioning of the appropriate side chains at residues 59 and 61. Additional refinement with progressively tighter weights on geometry as well as additional cycles of temperature factor refinement was carried out essentially as described (Bell et al., 1991) to obtain a minimal *R*-factor consistent with satisfactory bond lengths and angles (Table I).

RESULTS

Thermostability. The change in the midpoint for reversible thermal denaturation, ΔT_m, for each of the proteins with substitutions at residue 59 is shown in Table II. Figure 1 summarizes the changes in the free energy of folding, $\Delta\Delta G$. All six replacements decrease the thermostability of T4 lysozyme. The changes are smaller for Asn, Ser, and Asp and are larger for Ala, Gly, and Val, especially at pH 2.0.

Structures of Mutant Lysozymes. Figure 2 shows the difference in electron density between the lysozyme with alanine substituted at position 59 (T59A/WT*) and the pseudo-wild-type lysozyme. The negative features superimposed on the side chain of Thr 59 are consistent with the replacement of the threonine with alanine. Positive and negative features near Asp 61 show that in the mutant structure this side chain no longer occupies the highly solvent-exposed position on the outside of the helix that it does in wild-type lysozyme. Alterations in the structure are localized to the region near the end of the helix (as they are for all of the other replacements of Thr 59). In this variant (T59A) the side chain of Asp 61 is located over the end of the helix where it might be considered to serve as a "substitute" for the truncated (i.e., Thr → Ala) N-cap side chain. The average temperature factor for the atoms in the side chain of Asp 61 remains almost as high in the mutant structure (B = 52 Å^2) as in the wild type (B = 63 Å^2), showing that the side chain is not well ordered in either structure. Consistent with the high mobility of Asp 61 in the mutant structure, the geometry of the side chain suggests that it is not placed to accept a hydrogen bond from any of the amide nitrogens at the end

of the helix. Table III shows the angles between potential hydrogen-bond donors and acceptors at the end of this helix. The side chain of Asp 61 is oriented so that the planar carboxylic group is approximately normal to the N–H bond. Good hydrogen-bond geometry requires that the plane of the carboxylic acid be approximately parallel to the N–H bond (Baker & Hubbard, 1984). The angle between amide nitrogen, hydrogen, and oxygen acceptor is also unusual for a hydrogen-bond interaction (Table III).

The position of Asp 61 in the mutant structure T59A is more clearly seen in Figure 3. The wild-type structure is shown in yellow, and the variant T59A is shown in red. The structure of variant T59G (Figure 3, green bonds) is similar to that of T59A. The side chain of Asp 61 again is oriented over the end of the helix but in a slightly different position. In the Ala 59 structure there is a steric contact between the β-carbon and the γ_1-oxygen of Asp 61 (distance 2.9 Å). Removal of this β-carbon in the Gly 59 structure allows the carboxylate to move slightly further over the end of the α-helix.

In addition to alanine and glycine, valine is the other substitution at position 59 with a side chain which cannot form a hydrogen bond. Figure 3 includes the T59V structure in blue bonds. The valine adopts a conformation very similar to that of the wild-type threonine. Small changes in backbone dihedral angles in the vicinity of residue 59 allow the Val 59 side chain to be displaced away from the amide nitrogens of residues 60 and 61, with which it would otherwise have unacceptably close contacts. Asp 61 retains a position close to that in wild-type lysozyme.

Figure 4 superimposes the structures of those substitutions at position 59 which could, at least in principle, make hydrogen bonds to the end of the helix (Thr, Ser, Asp, and Asn). Analysis of these variants shows that such hydrogen bonds do, in fact, occur. The structure of T59S (shown in blue) is essentially identical to the structure of the wild-type protein (shown in yellow). Asparagine (green) and aspartic acid (red) are very similar to each other. The side chains are positioned so as to accept a hydrogen bond from the amide nitrogen of Glu 62 (Table III). The geometry for a possible second H-bond from the amide nitrogen of Asp 61 is poor (Table III).

Four solvent molecules occur in the wild-type structure near Thr 59: 184, 260, 284, and 286. Solvent molecule 260 is only observed in the wild-type structure (albeit with high thermal factor, B = 59 Å^2), where it is hydrogen-bonded to the γ-oxygen of Thr 59. Solvent molecule 184 always has its closest contact with the side chain of Asp 61. In the structures of T59A and T59G, however, the side chain of Asp 61 has a very different conformation so that the interaction between solvent molecule 184 and this side chain is quite different (Figure 3). Solvent molecules 284 and 286 are seemingly not affected by the replacements for Thr 59. No evidence was observed for additional solvent molecules near the end of the helix in any of the Thr 59 variants examined.

Discussion

In the discussion we will consider in turn the consequences of the different threonine replacements, comparing the present results with those reported by Serrano and Fersht (1989). The changes in energy observed for the two proteins agree in some, but not all, respects. Also it should be remembered that different techniques were used in the two cases (see caption to Figure 1).

Thr 59 → Ser. The substitution of threonine with serine at residue 59 in T4 lysozyme had relatively little effect on thermostability, especially at pH 6.5. This agrees well with the very small decrease in thermostability observed by Serrano and Fersht (1989). In structural terms, this substitution also had little effect. Tables III and IV show that the hydrogen-bonding geometry and χ_1 torsion angles are very similar for both serine and threonine.

Table IV: Torsion Angles (Degrees) at Residue 59 and at Asp 61

amino acid at site 59	residue 59				Asp 61			
	ϕ	ψ	χ_1	χ_2	ϕ	ψ	χ_1	χ_2
Ala	−94	163			−54	−41	54	−51
Gly	−101	170			−56	−44	47	−12
Asn	−99	169	76	38	−57	−44	−74	−1
Asp	−94	168	68	39	−60	−43	−72	−8
Ser	−85	164	83		−62	−43	−71	−7
Thr	−91	166	70		−58	−46	−80	3
Val	−98	165	−57		−55	−43	−72	−8

Thr 59 → Asp. For the Thr → Asp substitution, there is a substantial disagreement between the results obtained for T4 lysozyme and for barnase (Serrano & Fersht, 1989). In helix 6–18 of barnase this substitution was slightly stabilizing, while in T4 lysozyme it is destabilizing. This discrepancy may be rationalized on the basis of unfavorable electrostatic interactions with adjacent negatively-charged residues. Both T4 lysozyme helix 59–81 and barnase helix 6–18 have an aspartic acid as the N2 residue [as defined by Richardson and Richardson (1988); i.e., Asp 61 and Asp 8, respectively]. However, T4 lysozyme helix 59–81 also contains a glutamic acid at position N3 (i.e., Glu 62). The substituted aspartate at position 59 may destabilize the protein through unfavorable electrostatic interactions with Asp 61 and Glu 62, but another factor may also be important (see the discussion for T59N below).

The occurrence of two negatively-charged residues at the end of helix 59–81 of T4 lysozyme was the reason why this site was not chosen as a candidtate in attempts to stabilize the protein by the addition of negatively-charged residues near the beginning of helices (Nicholson et al., 1988, 1991). The destabilizing effect of the Thr 59 → Asp substitution helps validate the selection criteria used for finding appropriate sites for such substitutions.

Thr 59 → Asn. Substitution of Thr 59 with Asn is somewhat destabilizing at both pH 2.0 and pH 6.5. In T4 lysozyme, the Asn 59 substitution decreased the stability of the protein by 1.1 kcal/mol at pH 6.5, compared to 1.3 kcal/mol found by Serrano and Fersht (1989) for the two helices in barnase. These repeated observations in both barnase and T4 lysozyme that Thr → Asn substitutions are destabilizing were at first unexpected since Asn is frequently found as an N-cap residue (Richardson & Richardson, 1988). Serrano and Fersht (1989) suggest that Asn might not be as good an N-cap as Ser or Thr but do not offer an explanation why this might be the case. We would like to suggest an alternative interpretation, namely that Asn can be inherently as good an N-cap as Ser or Thr, but it requires a change in the backbone dihedral angles of the N-cap residue.

If a model-building experiment is performed in which the side chain of Thr 59 of T4 lysozyme is replaced with asparagine (or aspartic acid), then no combination of side-chain torsion angles can be found that will permit the formation of a satisfactory hydrogen bond between the side-chain oxygen and the amide nitrogens at the end of the α-helix. The difficulty is not so much to achieve acceptable hydrogen-bond geometry but rather to avoid an unacceptably short contact between the side-chain oxygen of the introduced asparagine (or Asp) and the α- and β-carbons of the residue in the N2

Table V: Close Contacts Involving Hydrogen-Bonding Side Chains at Residue 59[a]

residue at site 59	hydrogen-bonding atom	interacting atom (distance, Å)			
		$C^{\beta}61$	$C^{\alpha}61$	C61	N62
Asn	OD1	3.0*	3.3	3.5	2.8 (H)
Asp	OD1	3.2*	3.6	3.7	2.9 (H)
Thr	OG1	3.7	4.0	4.1	3.3 (H)
Ser	OG1	3.7	3.9	4.0	3.2 (H)

[a] The letter (H) in parentheses indicates a hydrogen-bonding interaction. The asterisk denotes the close approach shown in Figure 5b.

position (i.e., Asp 61). Such close contacts are, in fact, seen in the T59N and T59D crystal structures (Table V). These close contacts do not, however, have to occur when asparagine or aspartic acid forms the N-cap of an α-helix. For example, Asn 2, Asn 81, and Asp 92 of T4 lysozyme are all N-cap residues that hydrogen bond to the end of the helix and whose closest approach to the C^{α} or C^{β} atom of the N2 residue is 3.8 Å. The key to avoiding a close contact appears to be a rotation of the backbone dihedral angle, ψ, of the N-cap residue by approximately 40° (Figure 5). In the present case, the ψ angle at residue 59 hardly changes at all when Asn or Asp is substituted for Thr (Table IV; Figures 3 and 4). The α-carbon of residue 59 does appear to move a few tenths of an Angstrom, but an unfavorable contact remains between the introduced side chain of residue 59 and the α- and β-carbons of Asp 61. This is especially obvious in the T59N structure (Table V) and provides a structural basis for the loss of stability associated with the Thr → Asn substitution. In the case of T4 lysozyme, other interactions within the protein apparently do not allow the ψ angle of residue 59 to relax when an asparagine or aspartic acid is substituted at this position. These results suggest why asparagine (and perhaps aspartic acid) are frequently observed as N-cap residues yet may not be suitable N-cap replacements at some sites. The optimal backbone ψ angle for an N-cap residue may be different depending on whether that residue is, on one hand, serine or threonine or, on the other hand, asparagine or aspartic acid (Figure 5). In the case of T59N and T59D, the backbone conformation seems not to be appropriate for Asn and Asp and results in strain which is presumably distributed throughout the structure, but is most obvious as a close contact.

This hypothesis predicts that, in proteins in general, certain ψ angles will be preferred for Asp or Asn as the N-cap residue, while other angles will be preferred for Ser or Thr. Further, the distribution of expected ψ angles for Ser or Thr (~150–180°) should be smaller than for Asn or Asp (~60–150°) since the side chains of the former two residues have fewer degrees of freedom than do the side chains of the latter two residues. Indeed, just such distributions were found in a survey of N-cap residue conformation in a set of well-refined protein structures (S. Dasgupta and J. A. Bell, unpublished results). This hypothesis could be further tested by the introduction of serine, threonine, and asparagine N-caps at sites with various backbone conformations.

Thr 59 → Val. This substitution resulted in adjustments in the structure of the protein (Table IV; Figure 3), apparently to prevent an unacceptably close contact between the valine γ_1-carbon atom and the end of the helix. In the T59V structure there is no apparent hydrogen-bonding acceptor for the amide nitrogen of residue 62 (cf. the other variants in Table IV). The presence of this unsatisfied hydrogen-bonding potential explains, at least in part, the destabilization associated with the Thr 59 → Val replacement. Also the shift in position of the α- and β-carbons of residue 59 is greater for Val 59 than for any other replacement, suggesting that backbone strain might also contribute to the loss of stability in this case.

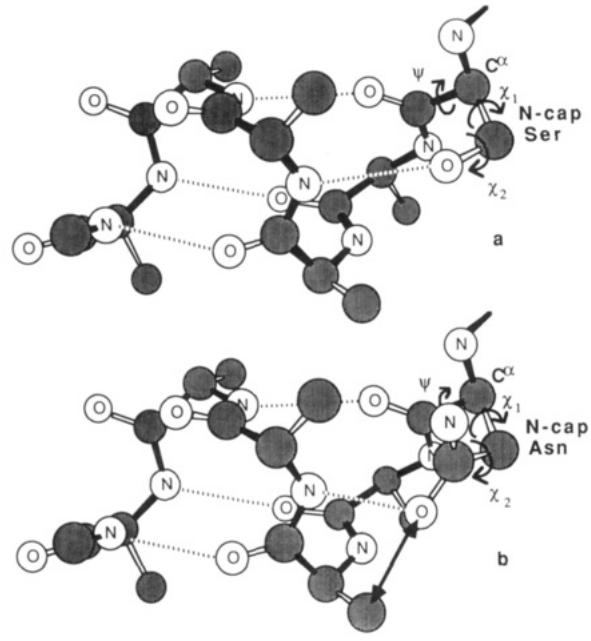

FIGURE 5: An α-helix composed of alanine, except for the N-cap residue, which is serine, is shown in (a). This serine hydrogen bonds (dotted line) to the amide nitrogen of the (N-cap + 3) residue of the helix. Since the conformation within the helix is highly restricted, rotation around two bonds, the backbone angle ψ and the side-chain torsion angle χ_1, largely determines the relative position of the hydrogen-bond donor and acceptor. A similar situation occurs if the N-cap residue is threonine. If the N-cap residue is asparagine, shown in (b), or aspartic acid, then the side chain will have an additional degree of freedom, namely rotation about χ_2. If the ψ value of the Asp or Asn residue is fixed at the value observed for Thr 59 in T4 lysozyme and then rotated about χ_1 and χ_2, steric interference (indicated by the arrowheads) prevents the formation of a stereochemically reasonable hydrogen bond (cf. Table V). Rotation about ψ is also required.

Thr 59 → Gly. Glycine is one of the most destabilizing subsitutions, in the range of 1.3–2.2 kcal/mol, for both T4 lysozyme (Figure 1) and barnase (Serrano & Fersht, 1989). In the crystal structure of T59G, the removal of the side chain of the residue 59 allows the side chain of Asp 61 to change conformation and to move over the end of the helix. For other helices not containing an N-cap residue with a side chain that can form a hydrogen bond, polar residues at the N1, N2, or N3 position often hydrogen-bond back to the end of the helix (S. Dasgupta and J. A. Bell, unpublished results).

In T59G, a hydrogen-bonding interaction may be possible between Asp 61 and its own amide nitrogen, but the geometry for this interaction is very poor (Tables III and IV). The presence of this unsatisfied hydrogen-bonding potential presumably destabilizes the Gly 59 variant. Also, the replacement of any residue with glycine tends to be destabilizing because of entropic effects (Matthews et al., 1987).

Thr 59 → Ala. For the threonine to alanine substitutions, barnase variants were destabilized by 2.1 and 2.5 kcal/mol, somewhat more than the 1.5 kcal/mol measured for the T59A variant of T4 lysozyme at pH 6.5. The origin of this disagreement may be due to a compensating role played by Asp 61 in T4 lysozyme. In the crystal structure of the T59A variant of T4 lysozyme (at pH 6.7), Asp 61 adopts a conformation such that it interacts with the end of the α-helix. The presumed electrostatic interaction (at pH 6.5) between Asp 61 and the α-helix dipole may, to some extent, offset the hydrogen bond that is lost between the side chain of Thr 59 and the backbone amide of residue 62. As with the T59G

structure, discussed above, the geometry of Asp 61 does not support a strong hydrogen-bonding interaction between this group and the amide nitrogen at the end of the α-helix.

Serrano and Fersht (1989) argued that valine was more destabilizing as a replacement for Thr 26 in barnase than was alanine. This inference seems hardly justified by the data (included in Figure 1) as there is only a marginal difference in stability between the Thr 26 → Ala and Thr 26 → Val replacements. In addition, the Thr 6 → Ala replacement in barnase is actually more destabilizing than Thr 26 → Val. The present observations (Figure 1), together with the data of Serrano and Fersht (1989), suggest that there is essentially no difference in energy between the alanine and valine replacements. Serrano and Fersht (1989) also found that alanine was more destabilizing than glycine at both position 6 and position 26 (data included in Figure 1). It was inferred by Serrano and Fersht (1989) that the difference in stability between Gly, Ala, and Val correlated with the steric bulk of the residue; i.e., glycine allowed better hydration of the end of the helix than did alanine, which, in turn, allowed better hydration than valine. The present results do not support this hypothesis. As noted, replacements with alanine and valine are energetically equivalent (Figure 1). In none of the Gly, Ala, or Val structures is a new water molecule seen to replace the hydrogen-bonding function of the γ-hydroxyl of the wild-type threonine variant. The different conformational changes that are observed in T4 lysozyme for different substitutions at site 59 also indicate that changes in stability cannot be attributed simply to differences in the degree of hydration and they illustrate the critical importance of knowing the structures of mutant proteins in order to understand their properties.

ACKNOWLEDGMENTS

We thank Joan Wozniak for excellent technical assistance and Dr. J. A. Schellman for facilities.

REFERENCES

Alber, T., & Matthews, B. W. (1987) *Methods Enzymol. 154*, 511–533.

Alber, T., Dao-pin, S., Wilson, K., Wozniak, J. A., Cook, S. P., & Matthews, B. W. (1987) *Nature 330*, 41–46.

Baker, E. N., & Hubbard, R. E. (1984) *Prog. Biophys. Mol. Biol. 44*, 97–179.

Becktel, W. J., & Baase, W. A. (1987) *Biopolymers 26*, 619–623.

Becktel, W. J., & Schellman, J. A. (1987) *Biopolymers 26*, 1859–1877.

Bell, J. A., Wilson, K. P., Zhang, X.-J., Faber, H. R., Nicholson, H., & Matthews, B. W. (1991) *Proteins: Struct., Funct., Genet. 10*, 10–21.

Blagdon, D. E., & Goodman, M. (1975) *Biopolymers 14*, 241–245.

Daopin, S., Baase, W. A., & Matthews, B. W. (1990) *Proteins: Struct., Funct., Genet. 7*, 198–204.

Hawkes, R., Grütter, M. G., & Schellman, J. (1984) *J. Mol. Biol. 175*, 195–212.

Hol, W. G. J., van Duijnen, P. T., & Berendsen, H. J. C. (1978) *Nature 273*, 443–446.

Ihara, S., Ooi, T., & Takahashi, S. (1982) *Biopolymers 21*, 131–145.

IUPAC–IUB Commission on Biochemical Nomenclature (1970) *J. Mol. Biol. 52*, 1–17.

Jones, T. A. (1978) *J. Appl. Crystallogr. 11*, 268–272.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367–382.

Matsumura, M., Becktel, W. J., & Matthews, B. W. (1988) *Nature 334*, 406–410.

Matthews, B. W., Nicholson, H., & Becktel, W. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6663–6667.

Muchmore, D. C., McIntosh, L. P., Russell, C. B., Anderson, D. E., & Dahlquist, F. W. (1989) *Methods Enzymol. 177*, 44–73.

Nicholson, H., Becktel, W. J., & Matthews, B. W. (1988) *Nature 336*, 651–656.

Nicholson, H., Anderson, D. E., Dao-pin, S., & Matthews, B. W. (1991) *Biochemistry 30*, 9816–9828.

Pjura, P., Matsumura, M., Wozniak, J., & Matthews, B. W. (1990) *Biochemistry 29*, 2592–2598.

Ponder, J. W., & Richards, F. M. (1987) *J. Mol. Biol. 193*, 775–791.

Richardson, J. S., & Richardson, D. C. (1988) *Science 240*, 1648–1652.

Serrano, L., & Fersht, A. R. (1989) *Nature 342*, 296–299.

Shoemaker, K. R., Kim, P. S., Brems, D. N., Marqusee, S., York, E. J., Chaiken, I. M., Stewart, J. M., & Baldwin, R. L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2349–2353.

Shoemaker, K. R., Kim, P. S., York, E. J., Stewart, J. M., & Baldwin, R. L. (1987) *Nature 326*, 563–567.

Still, C. (1988) *Macromodel 2.0*, Columbia University, New York, NY.

Tronrud, D. E., Ten Eyck, L. F., & Matthews, B. W. (1987) *Acta Crystallogr. A43*, 489–503.

Wada, A. (1976) *Adv. Biophys. 9*, 1–63.

Weaver, L. H., & Matthews, B. W. (1987) *J. Mol. Biol. 193*, 189–199.

Wetzel, R., Perry, L. J., Baase, W. A., & Becktel, W. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 401–405.

Zoller, M. J., & Smith, M. (1984) *DNA 3*, 479–488.