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Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding

(protein structure/lysozyme/protein design/proline/glycine)

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ABSTRACT **It is proposed that the stability of a protein can be increased by selected amino acid substitutions that decrease the configurational entropy of unfolding. Two such substitutions, one of the form Xaa → Pro and the other of the form Gly → Xaa, were constructed in bacteriophage T4 lysozyme at sites consistent with the known three-dimensional structure. Both substitutions stabilize the protein toward reversible and irreversible thermal denaturation at physiological pH. The substitutions have no effect on enzymatic activity. High-resolution crystallographic analysis of the proline-containing mutant protein (Ala-82 → Pro) shows that its three-dimensional structure is essentially identical with the wild-type enzyme. The overall structure of the other mutant enzyme (Gly-77 → Ala) is also very similar to wild-type lysozyme, although there are localized conformational adjustments in the vicinity of the altered amino acid. The combination of a number of such amino acid replacements, each of which is expected to contribute ≈1 kcal/mol (1 cal = 4.184 J) to the free energy of folding, may provide a general strategy for substantial improvement in the stability of a protein.**

There is considerable interest in enhancing the stability of proteins. In some instances genetic screens have allowed the selection of mutant proteins that are more stable than their parent (1–3). In other cases increased stability has been obtained by rational modifications of the protein structure (4–11). However, general methods of increasing protein stability are lacking.

In this paper it is suggested that entropic effects might be used to increase the thermostability of proteins of known three-dimensional structure. Consider, as an example, the difference between the transfer of a glycine and an alanine from the unfolded to the folded form. Glycine lacks a β-carbon and has more backbone conformational flexibility than alanine. In other words the backbone of a glycine residue in solution has greater configurational entropy than alanine. For this reason more free energy is required during the folding process to restrict the conformation of glycine than alanine. It follows that the stability of a protein should be increased by the judicious replacement of glycines with alanines (or with other residues containing a β-carbon). Potential sites of substitution must be chosen to avoid the introduction of unfavorable steric interactions in the "engineered" protein.

This enhancement of protein stability based on the difference between the backbone configurational entropy of different amino acids is not restricted to replacements involving glycine. Residues such as threonine, valine, and isoleucine, with branched β-carbons, restrict the backbone conformation more than nonbranched residues. Similarly, the pyrrolidine ring of proline restricts this residue to fewer conformations than are available to the other amino acids. As a consequence, there are many possible amino acid substitutions that alter the backbone configurational entropy of unfolding of a protein and may potentially be used to increase protein stability.

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Theory

The stability of a protein structure is determined by the net difference in free energy between the folded and the unfolded forms. Both enthalpy and entropy contribute to the free energy terms. The contribution of any given residue to the configurational entropy of unfolding of the polypeptide backbone of a protein can be estimated as

$$\Delta S_{conf} = R \ln z, \quad [1]$$

where R is the gas constant and z is the number of conformations available to that residue in the unfolded state (12). As discussed above, the value of z is not the same for all amino acids.

An exact evaluation of ΔS_{conf} requires a statistical averaging over all conformations. However, an approximate estimate can be obtained (13) by considering the area that is available to a given amino acid in a Ramachandran *et al.* (14) conformational map. If γ_Y is the relative area in a conformational map accessible to amino acid Y (where $\gamma = 1$ for the entire map) and γ_N is the relative area that corresponds to residue Y in the folded structure, then the entropy of unfolding for residue Y is given (13) by

$$\Delta S_{conf}(Y) = R \ln(\gamma_Y/\gamma_N). \quad [2]$$

From Eq. 2 and the assumption that changes in γ_N are negligible (see below), one can estimate the relative entropy of unfolding of a different type of residue, Z, relative to Y, namely,

$$\Delta S_{Z,Y} = \Delta S_{conf}(Z) - \Delta S_{conf}(Y) = R \ln(\gamma_Z/\gamma_Y). \quad [3]$$

On this basis, Nemethy *et al.* (13) estimated that the backbone contribution to the entropy of unfolding of an alanine relative to a glycine is −2.4 cal/deg·mol (1 cal = 4.184 J). On the same basis, a proline relative to an alanine can be estimated to have a relative configurational entropy of unfolding of about −4 cal/deg·mol. For T4 lysozyme at pH 6.5, −4 cal/deg·mol corresponds to a change of ≈1.4 kcal in the free energy of unfolding and an increase in the melting temperature of about 3.5°C.

Selection of Substitutions

As an initial test of entropic stabilization, two different types of amino acid substitutions in bacteriophage T4 lysozyme were considered, the first of the form Gly → Xaa and the second of the form Xaa → Pro. In both cases the objective

was to choose substitutions that would cause minimal perturbation of the three-dimensional structure of T4 lysozyme as determined crystallographically (15–17).

There are 11 glycines in T4L, 3 of which (Gly-77, Gly-110, and Gly-113) have conformational angles (ϕ, ψ) that are within the allowed range for amino acids with a β-carbon. Inspection of these glycine sites using the interactive computer program FRODO (18) suggested that residues 77 and 113 could potentially accommodate a β-carbon without interfering with neighboring atoms. As an initial test Gly-77 was replaced with alanine. The choice of alanine is conservative and avoids possible secondary effects that might occur with a larger side chain. Based on the one-letter identification of the amino acids, the mutant protein Gly-77 → Ala is designated G77A.

A proline residue in a polypeptide chain restricts the (ϕ, ψ) values at the proline itself and, in addition, limits the (ϕ, ψ) values of the preceding residue (19). Substitutions of the form Xaa → Pro must be compatible with these constraints. A survey of prolines in several accurately determined protein structures was made to determine the ranges of the conformational angles that occur in actual proteins. Details will be given elsewhere. In the present instance it was required that the residue preceding a potential proline site has (ϕ, ψ) values within the allowed region given by Schimmel and Flory (19). The values of ϕ and ψ at the substitution site itself were

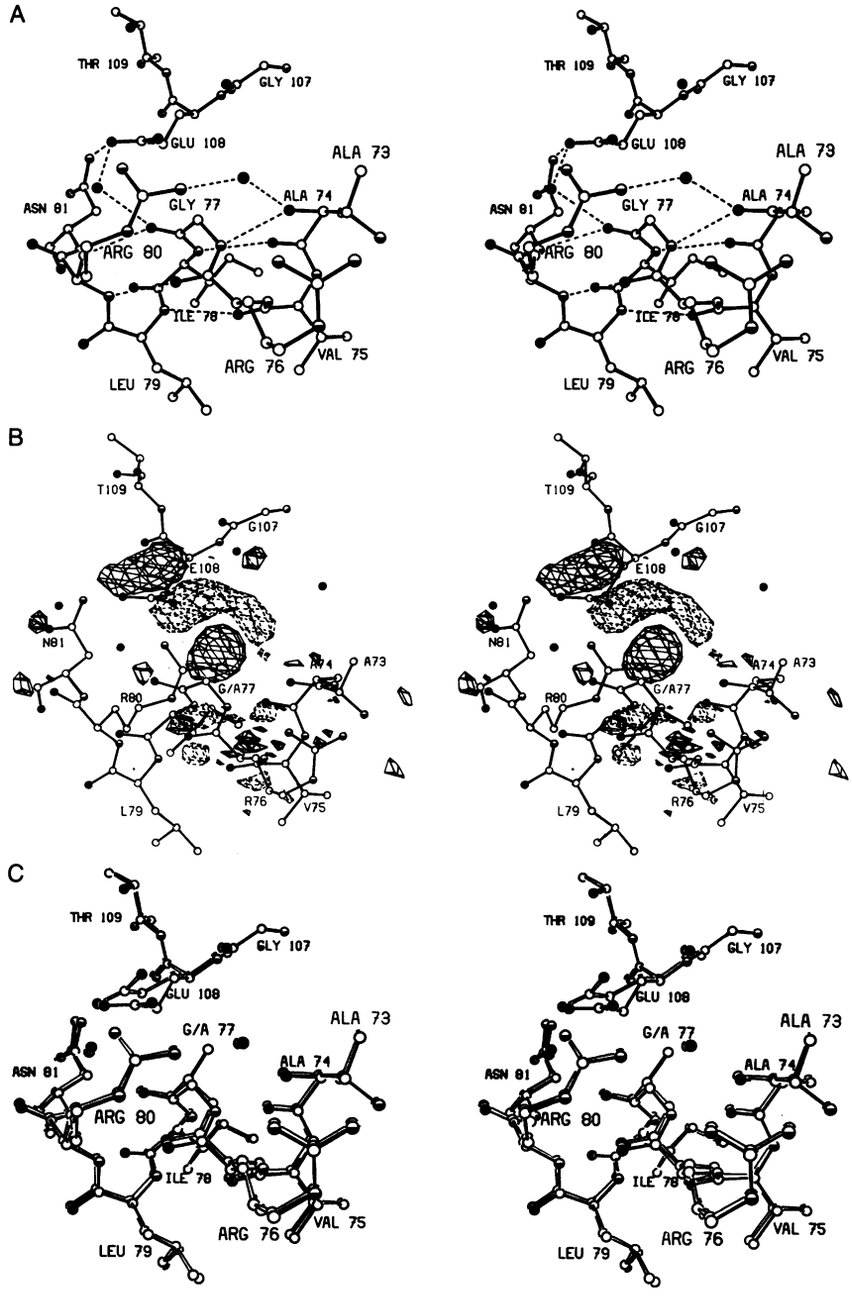

FIG. 1. (*A*) Stereo drawing showing the structure of wild-type T4 lysozyme in the vicinity of Gly-77. Oxygen atoms are solid circles, nitrogen atoms are half-solid circles, and carbon atoms are open circles. Hydrogen bonds are drawn as broken lines. (*B*) Electron density map showing the difference in density between mutant G77A and wild-type lysozyme. Coefficients are ($F_{mut} - F_{WT}$) and phases are from the refined model of wild-type lyosyzme. Resolution is 1.7 Å. Positive contours (solid lines) and negative contours (broken lines) are drawn at levels of $\pm 4\sigma$, where σ is the root-mean-square density throughout the unit cell. The wild-type structure is superimposed. Amino acids are identified by the one-letter code. The positive peak due to the addition of the β-carbon at residue 77 is of height 13σ. (*C*) Superposition of the structures of G77A lysozyme (open bonds) and wild-type lysozyme (solid bonds).

required to be within the regions $\phi = -50°$ to $-80°$, $\psi = 120°$ to 180° or $\phi = -50°$ to $-70°$, $\psi = -10°$ to $-50°$. Of the 164 amino acids in T4L, 17 residues met the above criteria. Two of these are already proline in wild-type lysozyme. Inspection with FRODO (18) was used to eliminate sites where a proline side chain would sterically interfere with neighboring atoms. Sites where the side chain of a residue appeared to participate in intramolecular interactions within the native structure were also removed from consideration. This screening procedure left three preferred candidates for proline substitutions—namely Lys-60, Ala-82, and Ala-93. The mutant Ala-82 → Pro (A82P) was constructed and is described here.

Mutant lysozymes G77A and A82P were obtained by oligonucleotide-directed mutagenesis (20). Procedures for mutagenesis, cloning, DNA sequencing, and protein purification were as described elsewhere (21–24). The lysozyme expression system was developed and kindly provided by D. C. Muchmore (University of Oregon).

Structures of Mutant Lysozymes

Crystals of G77A and A82P were obtained under conditions similar to those used for the wild-type enzyme (16, 17). X-ray diffraction data to 1.7-Å resolution were collected by oscillation photography (17, 25). Each data set consisted of about 14,000 independent reflections with agreement between equivalent intensities of 6–7%.

In the map showing the difference in electron density between G77A and wild-type T4L (Fig. 1*B*), the strongest positive feature confirms the addition of the methyl group at residue 77. There are also strong positive and negative features indicating a shift in the nearby side chain of Glu-108. Refinement of the G77A structure (R = 15.7% at 1.7-Å resolution) with the "TNT" package of programs (26) indicates that the carboxyl oxygen of Glu-108 closest to Ala-77 (Fig. 1*A*) moves 1.3 Å relative to its position in wild-type lysozyme (Fig. 1*C*). In addition, several backbone atoms in the vicinity of the substitution site move 0.25–0.35 Å. These shifts are also indicated in Fig. 1*B*. Otherwise, the G77A structure appears to be essentially identical with wild type. As judged by inspection of the three-dimensional structures of G77A and wild-type lysozyme, the movement of Glu-108 does not suggest any structural basis for differences in energy of the respective folded proteins. Although the carboxyl of Glu-108 moves ≈1 Å, it is not in close contact with the α-carbon of Gly-77 of wild-type lysozyme (closest approach, 3.6 Å) or with the β-carbon of Ala-77 of G77A lysozyme (closest approach, 3.9 Å). The only apparent interactions of the carboxyl group of Gly-108 that contribute to the stability of wild-type lysozyme are two hydrogen bonds, one from the side chain of Asn-81 (distance, 2.5 Å) and the other from a bound water molecule (distance, 2.7 Å) (Fig. 1*A*). Both of these hydrogen bonds are retained with respective distances 2.6 Å and 2.7 Å in the mutant structure (Fig. 1*C*).

In the case of mutant A82P, the difference map (Fig. 2*A*) shows the expected positive density corresponding to the addition of the pyrrolidine ring. An adjacent negative feature clearly indicates the displacement of a water molecule (W355) bound to the peptide nitrogen of Ala-82 in wild-type lysozyme. It must be asked whether the displacement of this solvent molecule could cause a difference in stability of the mutant relative to the wild-type protein. The water molecule is bound to wild-type lysozyme and presumably remains bound in the unfolded state. This bound water molecule should contribute approximately equally to the free energy of the two forms. Similarly, in the mutant protein the water molecule cannot bind to either the folded or the unfolded form of the protein and, again, should have no net effect. Refinement of the A82P structure to an R value of 15.8% at 1.7-Å resolution shows that it is virtually identical with

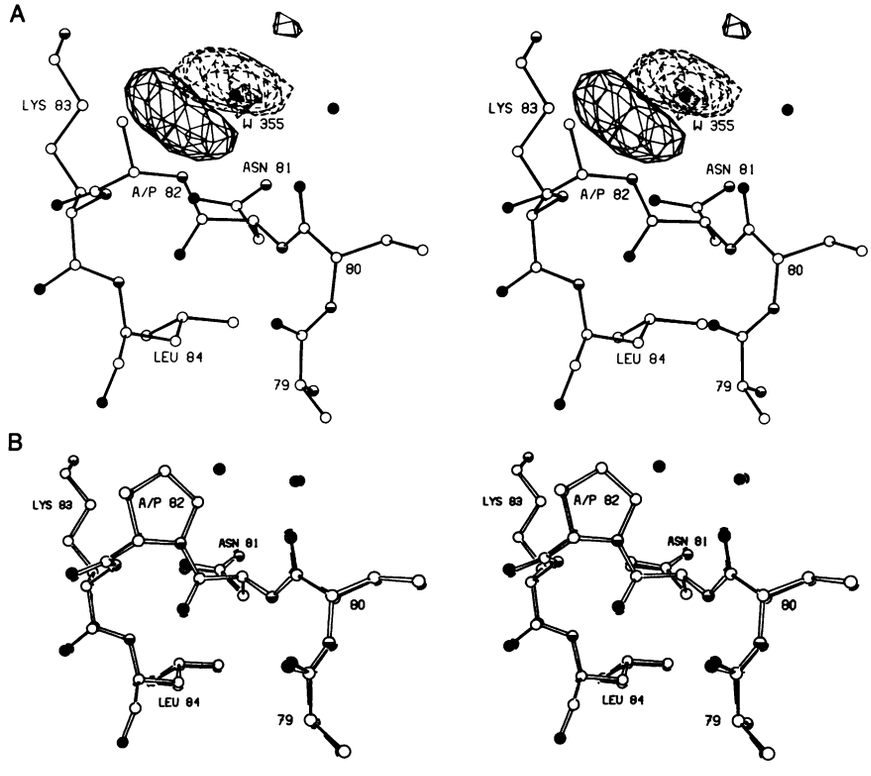

FIG. 2. (*A*) Electron density difference map for A82P lysozyme minus wild-type lysozyme. Coefficients, contour levels, and conventions are as in Fig. 1*B*. The positive peak indicating the addition of the pyrrolidine ring is of height 11σ; the negative peak due to displacement of the bound solvent molecule W355 is -11σ. For clarity, part of the side chains of Leu-79 and Arg-80 have been omitted. (*B*) Superposition of the structures of A82P lysozyme (open bonds) and wild-type lysozyme (solid bonds).

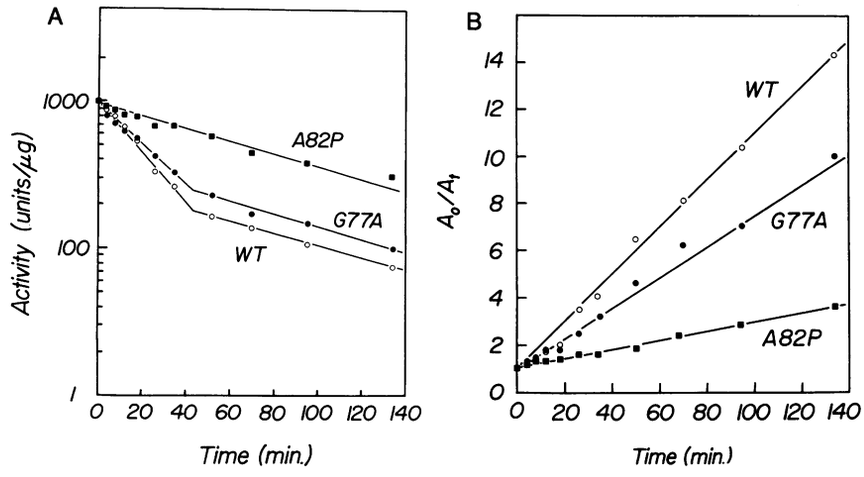

FIG. 3. Kinetics of inactivation of wild-type and mutant lysozymes. Lysozymes were dissolved at 30 μg/ml in 100 mM KH_2PO_4/100 mM KCl/1 mM EDTA, pH 6.5, and 300-μl aliquots were equilibrated at 65°C. After incubation at 65°C, samples were removed, plunged into ice, and then diluted. Specific activity was measured at 22°C in the turbidity assay (27). (*A*) First-order plot (cf. ref. 4). All activities are normalized to 1000 units/μg at zero time. (*B*) Second-order plot. A_0/A_t is the ratio of the initial activity to the activity remaining after time *t*. The respective second-order rate constants are as follows: wild type, 10.4×10^3 $mol^{-1} \cdot sec^{-1}$; G77A, 6.7×10^3 $mol^{-1} \cdot sec^{-1}$; and A82P, 2.1×10^3 $mol^{-1} \cdot sec^{-1}$.

wild-type lysozyme (Fig. 2*B*). The refined crystal structures of G77A and A82P provide experimental justification of the assumption made in obtaining Eq. 3, that the backbone configuration of the native state is not changed by the mutations.

Thermal Stability

The specific activity of both mutant lysozymes, measured at 22°C in the standard turbidity assay (27, 28), is very close to wild type. Stability toward irreversible inactivation was assessed by incubating the proteins at 65°C at pH 6.5 and measuring the activity remaining as a function of time (4, 27, 28). When displayed as a first-order plot (Fig. 3*A*) (4, 29), the loss of activity of wild-type lysozyme appears to be approximately biphasic. However, at the suggestion of T. Alber, the thermal inactivation is better described as being second order in protein concentration (Fig. 3*B*). This result needs to be confirmed by additional measurements, but, at face value, suggests that irreversible loss of activity is due to a bimolecular process such as intermolecular crosslinking. Perry and Wetzel (29) have, in fact, shown that the products of thermal, oxidative inactivation of wild-type lysozyme are disulfide-linked oligomers. Whether considered as a first- or second-order process, the loss of activity of mutants G77A and A82P is significantly slower than wild-type lysozyme.

Phage T4 lysozyme can be unfolded reversibly under controlled conditions (30). The transitions were monitored as the change in dichroism at 223 nm, as has been described (30). At pH 6.5 for both replacements and at pH 2 for A82P the net free energy change introduced is positive and the mutant proteins are more stable than the wild type (Table 1). The enhancement of thermal stability is due to a decrease in entropy rather than changes in enthalpy (Table 1). This is as expected for these mutations, but it must be emphasized that the numerical values of entropy and enthalpy are subject to relatively large experimental error. Since the changes in both the enthalpy and entropy are negative, however, it is the decrease in the entropy that results in the increase in free energy and the higher melting temperature.

At pH 6.5 the observed increase in thermodynamic stability of each mutant structure relative to wild-type lysozyme is 50–60% of that expected theoretically (Table 1). Considering the simplifications that underlie the theory, the agreement is remarkably good. The theoretical estimate for the change in free energy assumes that this change is solely entropic. Compensation by changes in enthalpy would lessen the stabilization. In the case of A82P, the structure of the mutant lysozyme is virtually identical to wild type (Fig. 2*B*), and the observed increase in stability can reasonably be attributed to the change in backbone configurational entropy. For G77A, however, the substitution of alanine for glycine results in localized changes in the protein structure (Fig. 1*C*) that may either offset or enhance the entropic contribution to the free energy of unfolding. It should also be noted that the crystal structures are determined at pH 6.7 and are, therefore, relevant to the enhanced stability of G77A at pH 6.5 but do not necessarily reflect the structure of lysozyme at pH 2.0 where Glu-108 is protonated and the mutant protein is less stable than wild type.

Table 1. Thermodynamic parameters for wild-type and mutant lysozymes

Protein	T_m, °C	ΔT, °C	ΔH, kcal/mol	ΔS, cal/deg·mol	$\Delta\Delta H$, kcal/mol	$\Delta\Delta S$, cal/deg·mol	$\Delta\Delta G$, kcal/mol	$-T_m\Delta S_{Z,Y}$, kcal/mol
			Denaturation at pH 2.0					
Wild type	41.9 ± 0.4	—	89 ± 5	282 ± 16	—	—	—	—
G77A	40.5 ± 0.7	−1.4 ± 0.8	85 ± 4	270 ± 13	−1 ± 6	−3 ± 21	−0.4	0.8
A82P	42.7 ± 0.1	0.8 ± 0.4	90 ± 5	283 ± 16	−1 ± 7	−2 ± 23	0.3	1.3
			Denaturation at pH 6.5					
Wild type	64.7 ± 0.5	—	129 ± 9	381 ± 27	—	—	—	—
G77A	65.6 ± 0.2	0.9 ± 0.5	125 ± 9	368 ± 27	−6 ± 13	−18 ± 38	0.4	0.8
A82P	66.8 ± 0.2	2.1 ± 0.5	126 ± 9	371 ± 26	−7 ± 13	−22 ± 37	0.8	1.4

The thermodynamic parameters were derived from van't Hoff analyses of reversible thermal denaturations of the wild-type and mutant proteins. Equilibrium constants were obtained from the fraction of native protein present under a given set of conditions of sequence, temperature, and pH. T_m is the temperature of denaturation, and ΔT is the difference in melting temperature. ΔH is the enthalpy of unfolding, and $\Delta\Delta H$ is the difference in unfolding enthalpy of mutant and wild-type proteins measured at the melting temperature of the wild-type protein. ΔS is the entropy of unfolding, and $\Delta\Delta S$ is the difference in unfolding entropy of mutant and wild-type proteins. The difference between the free energy of unfolding of mutant and wild-type proteins, $\Delta\Delta G$, is the observed free energy of stabilization and can be compared with $-T_m\Delta S_{Z,Y}$, which is the backbone entropic stabilization estimated from Eq. 3. The temperature variation of the enthalpy and entropy of denaturation for these T4 lysozymes, ΔC_p, was determined to be 2.0 ± 0.2 kcal/deg·mol. Additional details will be presented elsewhere.

Inferences for Protein Stabilization

The lysozyme mutants G77A and A82P provide two successful examples of protein stabilization by rational amino acid replacements based on the concept of entropic stabilization. Amino acid substitutions of the form Gly → Ala have been shown to increase the thermal stability of the λ repressor (6) and the neutral protease from *Bacillus stearothermophilus* (8). In these instances the enhanced thermostability was attributed to the replacement within an α-helix of a poor helix-forming residue (glycine) with a good helix former (alanine) (31). Gly-77 of T4L is also in an α-helix so that enhanced protein stability resulting from the Gly-77 → Ala substitution might be attributed to differences in helical propensity (31). However, the concept of entropic stabilization provides a rationalization for the otherwise empirical observation that Gly → Ala substitutions within α-helices can enhance protein stability. It may also explain why glycine occurs less frequently within α-helices than any other amino acid (31), since each such glycine represents a source of instability. Entropic considerations suggest that appropriately chosen Gly → Ala substitutions should increase stability whether or not the glycine is located within an α-helix.

Entropic stabilization has the advantage that it can potentially be applied in different ways at different sites in a protein. Even though T4 lysozyme has only 164 amino acids, it has five potential Gly → Xaa or Xaa → Pro substitution sites. The two substitutions tested to date, Gly → Ala and Ala → Pro, exploit changes in backbone entropy and minimize possible complications due to changes in side-chain entropy. Substitutions such as Ala → Ser, Ser → Thr, Ala → Val, etc., can also be used to decrease the backbone entropy of unfolding (13), but the energy gained may be offset by an increase in the entropy of unfolding of the side chain. In such cases the amino acid to be introduced should, if possible, be chosen so that it will have favorable interactions to compensate for the entropy cost of restricting the position of the side chain. In this context, amino acid substitutions that decrease the backbone entropy of unfolding and, at the same time, introduce new intramolecular interactions in rigid parts of the protein structure are expected to be most effective (32, 33). There is reason to expect that stabilization resulting from independent mutations is additive (6, 34, 35). Therefore, the combination of a number of amino acid substitutions, each of which may cause only a small increase in stability, may provide a strategy for substantial improvement in the stability of a protein.

The suggestion that the irreversible loss of activity of lysozyme might be second order was made by Dr. T. Alber. We also thank Dr. Alber for advice concerning site-directed mutagenesis; Dr. D. C. Muchmore for the lysozyme expression system; J. Wozniak for excellent technical assistance; L. H. Weaver, D. E. Tronrud, K. Wilson, and R. Faber for advice and access to unpublished data; and Drs. T. Alber, F. W. Dahlquist, L. McIntosh, and M. Matsumura for helpful comments on the manuscript. This work was supported in part by grants from the National Institutes of Health (GM21967 and GM20066 to B.W.M.; GM20195 to J. A. Schellman) and the National Science Foundation (DMB8611084 to B.W.M.; DMB8609113 to J. A. Schellman).

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