Thermal stability determinants of chicken egg-white lysozyme core mutants: Hydrophobicity, packing volume, and conserved buried water molecules

PHOEBE SHIH,^{1,2,4} DEBRA R. HOLLAND,^{3,5} AND JACK F. KIRSCH^{1,2}

¹ Departments of Chemistry and Molecular and Cell Biology, University of California, Berkeley, California 94720

² Center for Advanced Materials, Lawrence Berkeley Laboratory, Berkeley, California 94720

³ Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97405

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Abstract

A series of 24 mutants was made in the buried core of chicken lysozyme at positions 40, 55, and 91. The midpoint temperature of thermal denaturation transition (T_m) values of these core constructs range from 60.9 to 77.3 °C, extending an earlier, more limited investigation on thermostability. The T_m values of variants containing conservative replacements for the wild type (WT) (Thr 40-Ile 55-Ser 91) triplet are linearly correlated with hydrophobicity ($r = 0.81$) and, to a lesser degree, with combined side-chain volume ($r = 0.75$). The X-ray structures of the S91A (1.9 Å) and I55L/S91T/D101S (1.7 Å) mutants are presented. The former amino acid change is found in duck and mammalian lysozymes, and the latter contains the most thermostable core triplet. A network of four conserved, buried water molecules is associated with the core. It is postulated that these water molecules significantly influence the mutational tolerance at the individual triplet positions. The pH dependence of T_m for the S91D mutant was compared with that of WT enzyme. The pK_a of S91D is 1.2 units higher in the native than in the denatured state, corresponding to $\Delta\Delta G_{298} = 1.7$ kcal/mol. This is a low value for charge burial and likely reflects the moderating influence of the buried water molecules or a conformational change. Thermal and chemical denaturation and far UV CD spectroscopy were used to characterize the *in vitro* properties of I55T. This variant, which buries a hydroxyl group, has similar properties to those of the human amyloidogenic variant I56T.

Keywords: buried water; chicken lysozyme; hydrophobicity; internal packing; site-directed mutagenesis; thermal stability; X-ray structure

The sequestering of nonpolar amino acid residues from solvent is generally regarded as the dominant force in determining protein stability (e.g., Tanford, 1962; Baldwin, 1986; Dill, 1990). This hypothesis has received substantial support from a number of recent investigations employing mutant proteins prepared by recombinant DNA technology (Kellis et al., 1988; Lim & Sauer, 1989; Pace, 1992). Additional stability is gained by direct packing interactions (Richards, 1977; Sneddon & Tobias,

1992). The interiors of proteins are nonisotropic, thus attempts to correlate stabilities of a series of homologous replacements with simple thermodynamic parameters such as free energies of transfer from water to apolar solvent or packing density often fail. Successful examples do exist, such as limited linear free energy correlations with hydrophobicity (Yutani et al., 1987; Matsumura et al., 1988) or packing density (Malcolm et al., 1990; Shortle et al., 1990; Jackson et al., 1993).

Chicken lysozyme, a 129-amino acid protein with four disulfide bonds, undergoes reversible chemical and thermal denaturation transitions that obey a two-state process to a good approximation (Tanford et al., 1966; O'Reilly & Karasz, 1970). The denaturation of wild-type enzyme has been studied extensively (e.g., Foss, 1961; Pfeil & Privalov, 1976a, 1976b, 1976c; Radford et al., 1992), and the accumulated results provide a substantive platform for the interpretation of newer data obtained with recombinant enzymes. In all modern game bird lysozymes, a triplet of structurally adjacent residues comprising either Thr 40, Ile 55, Ser 91 (TIS), or Ser 40, Val 55, Thr 91

Reprint requests to: Jack F. Kirsch, mailing address: Department of Molecular and Cell Biology, 229 Stanley Hall, University of California, Berkeley, California 94720; e-mail: jack_kirsch@maillink.berkeley.edu.

⁴ Present address: Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599.

⁵ Present address: Parke-Davis, Ann Arbor, Michigan 48105.

Abbreviations: r , correlation coefficient; RMSD, RMS deviation; GdnHCl, guanidine hydrochloride; $\Delta\Delta G_{298}$, the difference in Gibbs free energy of denaturation between two protein species at 298 K; T_m , the midpoint temperature of thermal denaturation transition; WT or wt, wild type.

(SVT), is found in an internal core just below the active site (Jollès & Jollès, 1984). (See e.g., Lescar et al. [1994] for a graphic presentation of the triplet.) Four water molecules are associated with the triplet in a completely buried, predominately nonpolar core including additionally Leu 83, Leu 84, Ile 88, Leu 56, and Phe 3 (Blake et al., 1983; Lescar et al., 1994).

Triplet SVT is found in guinea fowl and New World quail lysozymes, and TIS occurs in the remaining game-bird lysozymes. Malcolm et al. (1990) determined the thermal stabilities of all eight combinations of Ser/Thr 40, Ile/Val 55, Ser/Thr 91, to investigate this rare case of coupled amino acid replacements. An unusual linear correlation between T_m , covering a range of $\sim 7^\circ\text{C}$, and the combined side-chain volumes of the triplets emerged (see Table 1, footnote g of this work). Analysis of high-resolution structures for WT and six core variants showed that the structural consequences of these mutations are highly localized (Wilson et al., 1992). Moreover, the above study (Malcolm et al., 1990) uncovered a variant, S91T, with more nearly optimal packing and higher thermostability than those of the WT protein.

The present study relaxes the evolutionary constraints of the earlier investigation to include an analysis of 24 mutants in the core region to explore the T_m and side-chain volume relationship further, and to search for core constructs having yet greater thermostability than the S91T variant. The X-ray structures of S91A (found in duck and mammalian lysozymes) and of I55L/S91T/D101S (containing the most thermostable core combination, I55L/S91T) are solved to 1.9 and 1.7 Å, respectively. The

thermal stability of the I55T analog of human lysozyme mutant I56T is of particular interest. The latter occurs naturally and has been associated with hereditary non-neuropathic systemic amyloidosis (Pepys et al., 1993).

Results and discussion

Thermostability correlations of 40-55-91 core mutants

The side chains of Thr 40, Ile 55, and Ser 91 (TIS) of chicken lysozyme form a cluster just below the active site cleft (Fig. 1). Core constructs are indexed by a triplet of one-letter amino acid codes, representing the sequential order of residues at these three positions. The tolerance of the thermal stability of lysozyme to substitutions in this core was tested by measuring T_m values for 24 mutants (Table 1). All of these variants are catalytically active in the standard bacteriolytic cell wall lysis assay, the least active having a relative specific activity equal to 64% of that of WT (Table 1).

The T_m values vary from 60.9°C (TTS) to 77.3°C (TLT). This range substantially expands the $\sim 7^\circ\text{C}$ variation reported previously (Malcolm et al., 1990). The T_m values of the eight originally investigated mutants are all well correlated ($r = 0.94$) with the combined side-chain volume of the triplet. By contrast, the present study shows only that a subset comprising 16 "conservative replacements"⁶ is satisfactorily correlated with either hydrophobicity or combined side-chain Voronoi volumes (Fig. 2A,B). The correlation with the former variable, $r = 0.81$, is marginally better than that obtained with the latter, $r = 0.75$. Indeed, the T_m values for three additional constructs, TAS, TTS, and TID, that do not meet the conservative replacement criterion are correlated with hydrophobicity and not with combined side-chain volume. Their inclusion improves the correlation with hydrophobicity to $r = 0.91$ (dashed line in Fig. 2A), whereas the T_m values for two "conservative" variants, TMS and TAT show significant deviation from the side-chain volume correlation line in Figure 2B. (Their exclusion improves r from 0.75 to 0.93.) TMS (I55M) is the mutant that best demonstrates that T_m is better correlated with hydrophobicity than with side-chain volume (Fig. 2A,B).

A number of additional contributors to core determinants of global stability include steric complementarity, side-chain entropy, and torsional strain (see reviews by Matthews, 1993; Rose & Wolfenden, 1993; and Richards & Lim, 1993). Nonetheless, the excellent correlation of thermostability with a simple hydrophobic parameter shows that this is the dominant determinant in the lysozyme core.

There is ample literature precedent for decreases in protein stability relative to WT where small aliphatic side-chain amino acids replace the larger ones extant in WT (Kellis et al., 1988, 1989; Sandberg & Terwilliger, 1991; Eriksson et al., 1992). However, examples of increased stability occasioned by substituting larger aliphatic amino acids for the smaller ones in WT proteins

Fig. 1. Ribbon drawing showing the locations of core residues 40, 55, and 91 and the active site amino acids 35, 52, and 101 in chicken egg white lysozyme. The side chains of the core residues and Asp 101 are shown as ball-and-stick models with carbon atoms as gray and heteroatoms as black balls. This figure was made with the MOLSCRIPT program (Kraulis, 1991).

⁶Conservative replacements include those that maintain the same type of functional group at a given position, i.e., aliphatic side-chain amino acids or methionine at position 55, and Thr or Ser at positions 40 and 91. Alanine is allowed in all three positions. Combined mutations that meet these criteria, but involve total side-chain volume changes of more than two heavy atoms from WT, are also excluded.

Table 1. Thermostability, bacteriolytic activity, side-chain volume, and hydrophobicity of the chicken lysozyme core triplet mutants

40-55-91 ^a variant	T_m^b (°C)	$\Delta\Delta G^c$ (kcal/mol) from WT	Specific activity ^d (% WT)	Side-chain volume ^e (Å ³)	Hydrophobicity ^f
TLT	77.3	+1.25	110	213	-4.46
TIT ^g	76.6	+0.99	110	214	-4.32
SIT ^g	75.6	+0.61	100	191	-4.73
TIS (WT)^g	74.0	—	100	191	-4.73
TVT ^g	74.0	0	96	187	-5.11
TIV	73.8	-0.08	74	233	-3.01
TIA	73.6	-0.15	92	183	-4.26
SIS ^g	73.3	-0.27	85	168	-5.14
SVT ^g	73.2	-0.30	90	164	-5.38
TLS	72.8	-0.45	96	190	-4.87
SIV	72.6	-0.53	72	210	-3.42
SIA	71.7	-0.87	98	160	-4.67
TVS ^g	71.6	-0.91	100	164	-5.38
TVA	70.7	-1.25	97	156	-5.05
TVV	70.7	-1.25	64	206	-3.80
SVS ^g	70.5	-1.33	85	141	-5.93
SVA	69.6	-1.67	93	133	-5.46
IIS	68.2	-2.20	71	237	-2.63
TMS	68.0	-2.27	73	193	-5.51
TID	67.9	-2.31	70	216	-5.73
TFS	67.5	-2.46	89	225	-4.74
TAT	66.7	-2.77	93	136	-6.36
TIY	65.9	-3.07	73	295	-3.37
TAS	62.4	-4.40	68	114	-6.77
TTS ^h	60.9	-4.96	91	144	-6.83

^a The core triplet is a cluster of three residues at positions 40, 55, and 91 located at the bottom of the active site cleft. Constructs are referred here by the one-letter amino acid code in sequential order at these three positions.

^b Protein samples were ca. 25 μ M in 66 mM potassium phosphate buffer, pH 6.4. Thermal denaturation was monitored by A_{292} , as described in the Materials and methods. Reported T_m value for each construct is the average of at least three determinations. Experimental errors in T_m were $\sim \pm 0.3$ °C.

^c $\Delta\Delta G$, the change in the free energy of unfolding, ΔG_{mutant} minus ΔG_{WT} at the T_m of WT, was calculated from Equation 4 (Becktel & Schellman, 1987). The value of ΔS_m is 379 cal K⁻¹ mol⁻¹ for WT enzyme at its T_m of 74 °C. This value was obtained from plotting $\Delta S_{native} - \Delta S_{denatured}$ values, obtained from Pfeil and Privalov (1976c), versus temperature. This figure is pH independent and is close to that determined in this study, 384 ± 21 cal K⁻¹ mol⁻¹, from van't Hoff analysis of 20 WT melting curves (data not shown). Because the approximation of Becktel and Schellman assumes a small perturbation in T_m due to the mutation, the error in $\Delta\Delta G$ is likely to increase with increasing ΔT_m .

^d Bacteriolytic activity was measured by the turbidity assay as described in the Materials and methods. Standard deviation for multiple determinations was typically within 10%.

^e Calculated by summing the side-chain volume (the volume of amino acid minus that of glycine) of each residue in the triplet using the Voronoi volume from Chothia (1975).

^f The sum of the hydrophobicity values of each residue in the triplet using the scale, derived from free energies of transfer from octanol to water, determined by Fauchère and Pliska (1983).

^g T_m values of these eight variants were reported by Malcolm et al. (1990). Slight differences from the T_m values determined in this work are mainly due to the method of fitting the data (see the Materials and methods).

^h T_m at pH 6.4 could not be determined accurately due to the tendency of this variant to aggregate. It was determined in 0.1 M Na acetate, pH 5.0, and the T_m at pH 6.4 was estimated from the T_m versus pH profile of WT (Fig. 5A).

are rare (Malcolm et al., 1990). The T_m values of TLT and TIT are 3.3 and 2.6 °C higher than that of WT, respectively (Table 1). Thus, these two constructs fit into the latter group.

Solvent-inaccessible residues tend to be nonpolar (Cantor & Schimmel, 1980); however, the buried TIS core contains two polar side chains that are surrounded by a number of hydrophobic residues including Phe 3, Leu 8, Leu 56, Leu 83, Leu 84, and Ile 88. It might have been anticipated that replacement of T40 and S91 with aliphatic side-chain amino acids would therefore yield lysozymes with enhanced thermostabilities. The opposite was observed, because the T_m values for T40I, S91V, I55V/

S91V, and T40S/S91V all fall well below the correlation lines of Figure 2A and B. Triplets containing the S91V substitution will be discussed further below. The Thr 40-O^γ atom is hydrogen bonded with the Lys 1-N atom ($r = 2.86$ Å), the T40I mutation results in the loss of this hydrogen bond and an increase of 31 Å³ in the side chain (Richards, 1974). These two factors are likely to be responsible for the observed decrease in stability of T40I. Steric considerations are particularly prominent for those triplets with aromatic substitutions, I55F and S91Y. The core cavity in the WT crystal structure has insufficient room to accommodate the bulky aromatic rings without introducing

Fig. 2. Thermostability of core triplet variant lysozymes versus (A) combined hydrophobicity and (B) Voronoi side-chain volume (see Table 1 for details and definitions). Conservative substitutions (see text) from WT are denoted by filled circles and others are shown as open circles. Error bars represent deviations of T_m from multiple determinations. Solid lines represent the least-squares fits through the filled symbols. The dashed line in A depicts the linear fit through the filled symbols and the three open symbols for the T_m values of the TTS, TAS, and TID constructs. The slopes are (A) 4.1 ± 0.8 (solid line, $r = 0.81$) and 5.3 ± 0.6 (dashed line, $r = 0.91$) $^{\circ}T_m$ per hydrophobicity unit; (B) 0.086 ± 0.021 $^{\circ}T_m$ per $\text{\AA}^3$ (or 2.25 ± 0.53 $^{\circ}T_m$ per methylene equivalent of $26 \text{ \AA}^3$) ($r = 0.75$). Van der Waals' volumes (Richards, 1974) yield a similar plot with $r = 0.74$.

compensating strain in the adjacent regions of these two core mutants.

X-ray structures of S91A and I55L/S91T/D101S

S91A

Although the 40-55-91 core triplet is occupied by either TIS or SVT in lysozymes of game birds (order Galliformes), the duck and mammalian enzymes have Ala at position 91. The S91A replacement resulted in only very slight changes in the protein structure. The space created by the lack of the O^{γ} serine atom is partially filled by a water molecule that shifts from its position in WT toward this vacated space. The adjustments to the structure in the vicinity of residue 91 are illustrated in the mutant minus WT difference electron density map shown in Figure 3A. Negative features (stippled lines) indicate atoms in the WT structure that are removed or shifted by the mutation. Positive difference features (solid lines) denote positional shifts of atoms introduced by the mutation.

The S91A replacement removes a hydroxyl group from this site. The lack of this functional group is clearly indicated by the presence of a negative difference density corresponding to the Ser 91- O^{γ} of WT in Figure 3A. Three other features dominate the difference map in this region. First, there is a large positive peak near water 201 (W201) that indicates its shift away from the WT position and toward the mutation site at 91. This water molecule shifts by nearly $1 \text{ \AA}$, as shown by comparison of the mutant and WT models. Second, a positive feature near the carbonyl group of residue 83 suggests that this part of the protein main chain shifts slightly as well. Finally, a negative peak near the main chain of residue 54 indicates movement. No significant difference electron density features were found away from the mutation site.

Three water molecules (W201, W212, W215) interact with Ser 91- O^{γ} in the WT structure. They remain in S91A and persist as part of a hydrogen bonding network that includes water mol-

ecules 201, 215, 212, 198, as well as Ile 55-N and Ile 88-O. However, the S91A mutation disrupts a long hydrogen bond between Ser 91- O^{γ} and Leu 83-O ($\text{dist}_{O-O} = 3.41 \text{ \AA}$, $\text{angle}_{C^{\beta}-O^{\gamma}-O} = 161^{\circ}$). With Ser 91- O^{γ} removed, Leu 83-O shifts away from residue 91 toward Ile 55-N (Fig. 3A); at the same time, water 201 shifts (by $0.7 \text{ \AA}$) into the cavity left by the vacated O^{γ} . Overall, no significant hydrogen bonds are made or broken in the S91A substitution. This structural result correlates well with the near WT-like thermal stability of the mutant protein.

I55L/S91T/D101S

Two mutations (I55L and S91T) in this triple mutant cluster in the core region, whereas D101S is in a more hydrophilic, surface-exposed part of the molecule (Fig. 1). The core region of I55L/S91T/D101S contains the most thermostable triplet made to date, TLT, which has an increased T_m of 3.3°C from WT. The structural changes associated with D101S are localized near the site of mutation and do not have an effect in the core region. The present discussion is focused on the TLT/D101S core region. The contribution of the D101S structural change is considered in the following paper of this issue.

Both the I55L and S91T substitutions are apparent in the difference electron density map (Fig. 3B) in which strong positive features highlight the newly introduced atoms, and the negative density indicates the removal of the parent atoms. In addition, pairs of difference density features are scattered around the main chain of residues 83-86 and 53-56, corresponding to shifts in the backbone caused by the mutations in this region. Analysis of the superimposition of the entire backbone for TLT/D101S and WT structures (C_{α} RMSD = $0.1 \text{ \AA}$, not shown) shows that significant main-chain deviations are confined to the mutation regions (C_{α} RMS for 53-56 and 83-86 segments = 0.4 and $0.3 \text{ \AA}$, respectively). A shift in the position of water 201, which is hydrogen bonded to Ile 55-N, as indicated by a large positive peak, appears to be a result of the mutation-induced backbone shift in the sequences from amino acid 53 to 56.

Fig. 3. Stereo view of superimposed electron density maps showing the difference in the core region between WT (open bonds) and mutant (solid bonds) lysozymes. **A:** WT and S91A. **B:** WT and I55L/S91T/D101S. (The D101 locus is outside of the core region.) Coefficients are $|F_{o,mut} - F_{c,wt}|$ and phases are from the refined WT model. Positive contours (solid lines) and negative contours (broken lines) are drawn at levels 3.5σ , where σ is the RMS density throughout the unit cell.

Two prominent features in the structures help to explain the increase in stability of TLT relative to WT. First, introduction of a methyl group by the S91T substitution, as in TIT (Wilson et al., 1992), increases the hydrophobic contacts in the core region and partially fills the largest cavity in lysozyme, decreasing the cavity volume by 6 Å³ (or 8%). The added C^γ methyl group in the S91T mutation engages in van der Waals inter-

actions with Leu 56-C^β and Leu 56-C^{δ2}. Second, although Ile and Leu side chains at position 55 occupy nearly the same space but adopt different χ_1 torsion angles (-70° versus -154°), the mutated Leu 55 gains several new hydrophobic contacts with neighboring residues Ile 88 and Thr 40. The introduced hydrophobic interactions between Leu 55-C^{δ1} and Ile 88-C^{δ2} cause the polypeptide chain including Leu 55 to shift away from its WT-

Fig. 4. Core region structures comparing the packing interactions of (A) TIT and (B) TLT/D101S. (The D101 locus is outside of the core region.) Dot spheres representing the van der Waals surface of Thr 40-C γ^2 , Ile 88-C γ^2 , Leu 56-C β^2 , Thr 91-C γ^2 , and side-chain atoms of 55 (Ile in A and Leu in B) are shown. The TIT structure is from Wilson et al. (1992), and the TLT/D101S structure was determined in this study. PDB codes are 1HEM and 1LSM, respectively.

like position in order to maintain reasonable van der Waals distances. This backbone shift involving residues 53–58 and 81–85 results in the most significant changes when comparing the two structures (Fig. 3B).

Although the T_m value of the single mutant I55L (TLS) is 1.2 °C lower than that of WT (TIS), the same I55L substitution in the TIT framework yields TLT, which has a 0.7 °C higher T_m than the parent TIT. The context dependence of the I55L mutation may be understood by comparing the crystallographically determined packing interactions for side-chain 55 of TIT and TLT. The 3.9-Å Ile 55-C γ^2 to Thr 91-C γ^2 contact in TIT is absent in the TLT structure but is largely compensated by Leu 55-C β , which partially occupies the space vacated by Ile 55-C γ^2 . By doing so, a three-way hydrophobic packing arrangement is formed by the Leu 55-C β , Leu 56-C β^2 , and Thr 91-C γ^2 atoms in TLT, which mimics the direct Ile 55-C γ^2 to Thr 91-C γ^2 contact in TIT. As a significant stabilizing effect, a new hydrophobic contact between Ile 88-C γ^2 and Leu 55-C γ^1 (3.6 Å) is present in the TLT structure. This interaction fills a part of the cavity between residues Ile 88, Leu 8, and Thr 40 that is unoccupied in either the WT or TIT structure (Fig. 4A,B). As a result, the hydrophobic cavity is smallest for TLT. Overall, the volume decreases from 75 Å³ and 69 Å³ in WT and TIT, respectively to 64 Å³ in TLT.

Although the structure of TLS is not available, the above considerations are consistent with the lowered thermostability of TLS from WT. The size of the cavity created between residues 55 and 91 in going from TIT to TIS is increased when Ile 55 is replaced by Leu due to the vacating Ile-C γ^2 methyl group. Unlike in the TLT structure, this packing defect in TLS is not compensated due to the lack of the methyl group from Thr 91-C γ^2 .

Conserved buried water molecules in the lysozyme core

The roles of conserved water molecules in a number of different proteins have been discussed (e.g., Wlodawer et al., 1987; Meyer, 1992; Berghuis et al., 1994). Zhang and Matthews (1994) observed that those most conserved are frequently internal and are characterized by low crystallographic thermal factors. It has been suggested that well-ordered, internal water molecules are significant factors in determining structural stability (Alber et al., 1987; Williams et al., 1994) or function (Alexander et al., 1991; Meyer, 1992). The subject has been reviewed by Westhof (1993) and by Teeter (1991).

Four buried water molecules (198, 201, 212, and 215; numbered according to the WT structure by Wilson et al. [1992]) are found in the chicken lysozyme core. They are conserved in all four crystal forms of chicken lysozyme (Blake et al., 1983) and in all nine mutant structures determined to date as well as in other avian lysozymes (Lescar et al., 1994). They form an extended hydrogen bonding network that decreases the volume of the core cavity by 113 Å³. These four water molecules are well ordered with thermal factors ranging from 9 to 30 Å² in all WT and mutant structures.

Although all four water molecules in the core are within 4 Å of the Ser 91 side chain, their presence is not dependent on the serine. In addition to the mutant structures discussed above, they are found in guinea fowl [Thr 91] (Lescar et al., 1994) and human [Ala 92] (Blake et al., 1983) lysozymes, as well as in α -lactalbumins [Asp 88] (Acharya et al., 1989). (The residues in brackets are equivalent to Ser 91 in chicken.) α -Lactalbumin is evolutionarily related to chicken lysozyme by the 35% amino acid sequence identity and the structural similarity of the two proteins (Acharya et al., 1989). The invariant carboxylate group of Asp 88 in the α -lactalbumins serves as a coordination site for calcium. Two of the six remaining ligands come from the oxygens of the internal water molecules (Stuart et al., 1986). Thus, the network of water molecules around 91 in chicken lysozyme, which is occupied by two water molecules and a calcium ion in α -lactalbumin, appears to be structurally important because it is subjected to strong evolutionary conservation.

The importance of a polar amino acid at position 91

Threonine is readily accommodated in place of Ser 91. (The T_m of S91T is 2.6 °C higher than that of WT.) Substitution with Val, a near isostere of Thr (side-chain volume of Thr = 51.4 Å³ and of Val = 63.8 Å³ [Richards, 1974]), allows the testing of the importance of polarity at this position. Ser 91-O γ makes a long hydrogen bond with Leu 83-O in the WT structure. In addition, Ser 91-O γ in WT and Thr 91-O γ in S91T are within hydrogen bonding distance from water molecules 201 and 215. These interactions are probably not important for stability because the T_m of S91A is only 0.4 °C less than that of WT. However, the three core variants, TIV, TVV, and SIV, have Val at position 91 and are less stable than the same constructs with Ser 91 and Thr 91, with an average of 0.6 °C and 3.0 °C reductions in T_m , respectively. Therefore, it appears important that the β -OH be

preserved even though the hydrogen bond is not energetically important. It is possible that the Val hydrocarbon side chain perturbs the hydrogen bond network by displacing water 201. Disruption of a hydrogen bonded water network has also been observed in crystal structures of subtilisin mutants T71A and T71I. These mutations result similarly in decreased thermostability from that of the WT protein (Pedersen et al., 1994).

Fig. 5. Thermal stability as a function of pH. **A:** A comparison of T_m values of WT (●) and S91D (○). **B:** The difference in thermostability, $T_m^{WT} - T_m^{S91D}$. The curve is a nonlinear least-squares fit to Equation 2, yielding $pK_{app} = 4.71 \pm 0.05$. **C:** The ratio of equilibrium constants, $K_{S91D}^{N-D} / K_{WT}^{N-D}$. The curve depicting the fit to Equation 3 gives $pK_{a(native)} = 5.20 \pm 0.08$ and $pK_{a(denatured)} = 3.97 \pm 0.07$ for Asp 91.

The pH dependence of T_m for S91D:

Effects of buried charge

The ionized form of S91D introduces a charged group in the core. The associated energetic penalty is reflected in the 6.1 °C decrease in T_m compared to WT at pH 6.4 (Table 1). The pH dependencies of T_m for WT and S91D are shown in Figure 5A. The T_m value of WT serves as a control for proton dissociations not involved with the S91D mutation; therefore,

$$\frac{[S91D_{(COO^-)}]}{[S91D_{(COOH)}]} = \frac{(\Delta T_m^{obs} - \Delta T_m^{low\ pH})}{(\Delta T_m^{high\ pH} - \Delta T_m^{obs})}. \quad (1)$$

$\Delta T_m^{low\ pH}$ represents the difference in thermal stability between protonated aspartic acid and serine in S91D and WT, respectively. Similarly, $\Delta T_m^{high\ pH}$ reflects the difference between unprotonated aspartic acid in S91D and serine in WT. Substitution of the right side of Equation 1 into the Henderson-Hasselbalch equation followed by rearrangement yields

$$\Delta T_m^{obs} = \frac{\Delta T_m^{low\ pH} + [\Delta T_m^{high\ pH} \times 10^{(pH-pK_a)}]}{1 + 10^{(pH-pK_a)}}. \quad (2)$$

ΔT_m^{obs} is plotted as a function of pH in Figure 5B, yielding a macroscopic pK_a value of 4.72 ± 0.05 . The thermodynamic cycle linking the ionization of S91D with the stability of lysozyme is depicted at the left of Figure 6. K_1 and K_2 are the equilibrium constants for the unfolding of the protonated and unprotonated forms of S91D, respectively. K_{a1} and K_{a2} are the ionization constants for proton dissociation from Asp 91 in the folded and the unfolded species of S91D, respectively. K'_1 represents the equilibrium constant for the denaturation of WT enzyme and is independent of ionization at position 91. The apparent equilibrium constant for the denaturation of S91D includes both the upper and lower branches of the left side of Figure 6. The ratio of equilibrium constants for the unfolding of S91D relative to WT is therefore

$$\frac{K_{S91D}^{N-D}}{K_{WT}^{N-D}} = \frac{K_1 [H^+] + K_{a2}}{K'_1 [H^+] + K_{a1}} = \exp(\Delta\Delta G_{S91D-WT}^{N-D} / -RT). \quad (3)$$

The values of $\Delta\Delta G_{S91D-WT}^{N-D}$ at each pH were approximated from Equation 4 (Becktel & Schellman, 1987), where ΔT_m is

$$\Delta\Delta G \approx \Delta T_m \Delta S_m \quad (4)$$

Fig. 6. The thermodynamic cycle linking the ionization of S91D with the folding-unfolding transition of lysozyme is depicted at the left. K_1 and K_2 are the equilibrium constants for the unfolding of the protonated and unprotonated forms of S91D, respectively. K_{a1} and K_{a2} are the ionization constants for proton dissociation from Asp 91 in the folded and the unfolded species of S91D, respectively. The denaturation of the WT enzyme, which is depicted at the right, is independent of ionization at position 91 with the equilibrium constant K'_1 .

the difference in T_m of S91D from WT and ΔS_m is the entropy of unfolding of WT at T_m (see Table 1, footnote c, and the Materials and methods for discussions on $\Delta\Delta G$ determinations using the approximation of Becktel and Schellman). $K_{S91D}^{N\rightarrow D}/K_{WT}^{N\rightarrow D}$ was then obtained from Equation 3. That the protonated form of aspartic acid is not a perfect substitute for serine is shown by the 1.9 °C ΔT_m between S91D and WT observed at low pH. (The $\Delta T_m^{low\ pH}$ value was obtained from Equation 2.) This figure gives $K_1/K'_1 = 2.95$ from Equation 3. A plot of $K_{S91D}^{N\rightarrow D}/K_{WT}^{N\rightarrow D}$ as a function of pH is depicted in Figure 5C. The values of K_{a1} and K_{a2} , the microscopic dissociation constants of S91D in the folded and unfolded states, respectively, were calculated from nonlinear least-squares regression on Equation 3. Table 2 summarizes the pH-dependent T_m values and the fitted parameters associated with the ionization of S91D. The energetic cost of sequestering the charged carboxylate of S91D is reflected by the 1.2 pH unit elevation in pK_{a2} relative to pK_{a1} . The 3.97 ± 0.07 value for the latter is identical, within error, to that reported for the pK_a of an aspartate in an unstructured peptide (Huyghues-Despointes et al., 1993). This agreement supports the approach used here.

The pK_a determined for the conserved, buried aspartic acid in thioredoxin is increased by approximately 3 pH units ($\Delta\Delta G = 4.6$ kcal mol⁻¹) in the folded state (Langsetmo et al., 1991). Similarly, two charge burial core mutants in T4 lysozyme (Daopin et al., 1991) exhibit >10 °C decreases in T_m 's relative to

WT ($\Delta\Delta G \approx 5$ kcal mol⁻¹). In contrast, the calculated energetic penalty for "burying" Asp 91 in chicken lysozyme is only 1.7 kcal mol⁻¹.

Why is there such a small perturbation in the pK_a of Asp 91 in the folded protein? One possibility is that there is a modest conformational change that requires little energy yet is sufficient to expose the carboxylate group to some solvent water. The tryptophan emission spectrum of S91D showed only a small reduction in intensity and no λ_{max} shift compared to that of WT (data not shown, Ser 91-O^γ is ~9 Å away from the indole ring of the major fluorophoric group W108 in lysozyme, Formoso & Forster [1975]), and the specific bacteriolytic activity of S91D is 70% of WT. These observations argue against an S91D-induced major change in structure.

Position 91 is an N-cap +2 residue as defined by Richardson and Richardson (1988). Thus, a second possible contributor to the small pK_a change is that due to the pK_a depression resulting from electrostatic interaction between the negatively charged Asp and the partial positive α -helix dipole at the N-terminus. A model study, where the placement of an aspartate was varied in alanine-based peptides, showed that the pK_a of the Asp at the N-cap +2 position is depressed by 0.2 pH units from its normal value (Huyghues-Despointes et al., 1993). Thus, this factor is quantitatively minor in the present instance. A third consideration is that the four buried water molecules (see above) serve to solvate the introduced carboxylate side chain. These solvent molecules are likely to persist in the S91D structure based on their presence in a human lysozyme variant that contains the analogous mutation (Inaka et al., 1991).

Table 2. pH dependence of the thermal denaturation of S91D and WT chicken lysozymes

pH	$\Delta T_m(WT-S91D)$	K_{S91D}/K_{WT}^a
3.42	2.19	3.9
3.69	2.15	3.8
4.07	2.60	5.1
4.52	3.60	9.5
5.04	4.77	19.7
5.40	5.70	35.2
5.56	5.81	37.7
5.69	5.93	40.7
6.05	5.96	41.4
6.46	6.08	44.7
6.98	6.35	52.9

Macroscopic pK_{app} of S91D^b = 4.72 ± 0.05

	ΔT_m	K_{S91D}/K_{WT}
Low pH ^b	1.85 ± 0.10	2.95
High pH ^b	6.29 ± 0.07	48.63

Microscopic pK_a values of S91D^c

pK_{a1} (folded)	5.20 ± 0.08
pK_{a2} (unfolded)	3.97 ± 0.07

$\Delta\Delta G_{(protonated-unprotonated)} = 1.68 \pm 0.14$ kcal mol⁻¹

^a Calculated from Equations 3 and 4.

^b Obtained from the fit shown in Figure 5B to Equation 2.

^c Obtained from the fit shown in Figure 5C to Equation 3.

$\Delta\Delta G_{(protonated-unprotonated)}$ is calculated from the equation, $\Delta\Delta G = 2.303RT(pK_{a1} - pK_{a2})$ at 25 °C.

Characterization of a lysozyme mutant that introduces a polar hydroxyl group into a nonpolar locus

The first report of a lysozyme-associated disease is that either the I56T or D67H mutant in the human enzyme causes hereditary non-neuropathic systemic amyloidosis in two unrelated families (Pepys et al., 1993). Amyloid fibrils extracted from one affected individual consisted of the full-length I56T variant lysozyme. The insoluble crosslinked β -sheets of such protein assemblies generally lead to organ dysfunction (Colon & Kelly, 1992). Human and chicken lysozymes share 60% amino acid sequence identity. The human enzyme has a glycine residue inserted between 47 and 48 in the corresponding chicken sequence. Thus, the relevant chicken mutation is I55T. Because it occurs in one of the core residues, this mutant was characterized in vitro.

This single substitution has a profound effect on the protein. First, the T_m is reduced by 13 °C, and the C_m for GdnHCl denaturation is decreased from 4.1 to 2.6 M (Table 3). Second, the ellipticity in the α -helical region of the far-UV CD spectrum is significantly reduced (Fig. 7).⁷ Third, the mutant appears to be markedly less soluble than WT. More than 90% of WT enzyme is normally found in the supernatant fraction of the yeast expression system. By contrast, 40% of the I55T protein remained in the pellet fraction and could only be recovered by resuspending the cell pellet in 4 M GdnHCl solution. Attempts to deter-

⁷ Spectra shown in Figure 7 were obtained at 25 °C. The decreased helicity of I55T is not due to partial denaturation at this temperature because identical CD spectra were obtained when the cell chamber temperature was changed to either 10 or 40 °C.

Table 3. Thermal^a and guanidinium chloride^b denaturation of WT and I55T mutant of chicken lysozyme

	WT	I55T
T_m (°C)	76.4 ± 0.3	63.3 ± 0.4
m (kcal M ⁻¹ mol ⁻¹)	2.4 ± 0.2	2.6 ± 0.3
C_m (M)	4.12 ± 0.06	2.57 ± 0.09
$\Delta G_{N,D}$ (kcal mol ⁻¹)	10.2 ± 0.7	6.9 ± 0.8

^a Determined in 0.1 M Na acetate, pH 5.0.^b Fluorescence intensity at 360 nm was monitored. See the Materials and methods for sample preparation and data analysis.

mine the I55T T_m value at the standard pH of this investigation of 6.4, were met by aggregation, but this problem was circumvented by lowering the pH to 5.0.

Although the structural basis underlying the properties associated with the I55T mutation has yet to be established, a possible explanation, involving partial β -sheet disruption, might be considered. Ile 55 is located in a turn between two strands of β -sheet, which is a structural motif frequently associated with amyloid fibril formation (e.g., Caputo et al., 1992; McCutchen et al., 1993). Pepys et al. (1993) pointed out that the difficulty in burying the polar hydroxyl group in the nonpolar core could alter the β -sheet region such that the amyloidogenic determinant is exposed. Although the low solubility and reduced α -helicity of chicken lysozyme mutant I55T only suggest that this variant, like its human lysozyme counterpart, is amyloidogenic, the large decrease in stability of this mutant does reflect the profound effect of introducing a hydroxyl group into this nonpolar locus.

Concluding remarks

The present study of lysozyme core variants shows that the thermostability for most mutants is well-correlated with hydrophobicity and to a lesser extent with side-chain volume of constituent residues. However, neither parameter fully accounts for the stability of all variants, e.g., as where steric factors dominate (TFS

and TIY), or where hydrogen bonding interactions are compensated (as in the case of IIS and variants containing the S91V substitution). In addition, the effects of some mutations are context dependent. Steric complementarity can also be an important stability determinant, although it is often overlooked in the absence of a high-resolution structure. The I55L/S91T/D101S structure was essential in deriving an explanation for the noncumulative effect on T_m exhibited by the TLT variant.

Materials and methods

Protein expression and purification

Variants of lysozyme were constructed by site-directed mutagenesis of the cDNA for chicken egg white lysozyme and heterologously expressed in yeast and purified as described in Shih et al. (1993). An additional chromatographic step with hydroxylapatite resin (Bio-Rad) following the CM-Sepharose column (eluted with 50–300 mM potassium phosphate gradient) was sometimes required to purify lysozyme variants to homogeneity. Yeast-derived WT lysozyme was used in all experiments where data for WT are reported.

Activity assay

The specific bacteriolytic activities of lysozyme variants were measured spectrophotometrically by a modification of the turbidity assay of Shugar (1952) as described by Shih et al. (1993). Enzyme concentrations that gave ca. 15 units of activity were used. Protein concentrations were determined from $\epsilon_{280}^{1\text{mg/ml}} = 2.64$ (Aune & Tanford, 1969).

Thermal stability determination

The transition midpoints (T_m) of lysozyme variants were monitored by the absorbance at 292 nm as a function of temperature with a Gilford UV-visible apparatus equipped with an automatic temperature-stepping module, using the software Response II Thermoprogramming as described by Shih et al. (1993). Approximately 25 μ M lysozymes in solutions of degassed potassium phosphate buffer, pH 6.4 were used. Thermal denaturation of lysozyme variants is nearly fully reversible (the specific activity of heat-denatured samples after cooling is typically within 90% of that of unheated enzyme solutions), provided that they are not heated at temperatures beyond T_m for extended time periods. The thermally induced transition for lysozyme obeys the two-state model to a good approximation (Tanford et al., 1966; O'Reilly & Karasz, 1970). Because the pre- and post-denaturation baselines in melting curves are approximately linearly dependent on temperature, K_{eq} , the equilibrium constant for transition from native (N) to denatured (D) state at each temperature, is related to the experimental observable absorbance by an expression that is analogous to that derived by Santoro and Bolen (1988) for chemical denaturation:

$$K_{eq} = \frac{[D]}{[N]} = \frac{(Abs_N + m_N T) - Abs_{obs}}{Abs_{obs} - (Abs_D + m_D T)}, \quad (5)$$

where Abs_{obs} is the observed absorbance at 292 nm, Abs_N , Abs_D , and m_N , m_D are intercepts and slopes of pre- and post-

Fig. 7. Far-UV CD spectra of WT (●) and I55T (○), the putative amyloidogenic core mutant. Mean residue ellipticity is plotted as a function of wavelength. Protein samples were in 66 mM potassium phosphate, pH 6.4, and at 25 °C.

denaturation baselines. The change in free energy for protein denaturation as a function of temperature (ΔG_T) is described by the Gibbs-Helmholtz equation and related to K_{eq} as follows:

$$\Delta G_T = \Delta H_m \left(1 - \frac{T}{T_m}\right) - \Delta C_p \left[(T_m - T) + T \ln \left(\frac{T}{T_m}\right) \right] \\ = -RT \ln K_{eq}. \quad (6)$$

ΔH_m is the enthalpy change for unfolding measured at T_m , and ΔC_p is the difference in heat capacity between the unfolded and folded states. Combining and rearranging Equations 5 and 6 gives Equation 7

$$Abs_{obs} = \frac{Abs_N + m_N T + \left[(Abs_D + m_D T) e^y \left(\frac{T}{T_m}\right)^{\Delta C_p/R} \right]}{1 + e^y \left(\frac{T}{T_m}\right)^{\Delta C_p/R}}, \quad (7)$$

where

$$y = \frac{1}{R} \left(\frac{T_m \times \Delta C_p - \Delta H_m}{T} + \frac{\Delta H_m}{T_m} - \Delta C_p \right).$$

Thermal denaturation data were fitted to Equation 7 by non-linear least-squares analysis using the general curve fit option of the KaleidaGraph 3.0.1 (Abelbeck software) program to obtain values for T_m and ΔH_m . An example of the fitting of thermal denaturation data to Equation 7 is shown in Figure 8. ΔC_p , a parameter that has been difficult to determine experimentally, is included in Equation 7. Fortunately, the important fitted parameters of ΔH_m and T_m are relatively insensitive to the value of ΔC_p . For example, entry of $\Delta C_p = 1.57 \text{ kcal mol}^{-1} \text{ K}^{-1}$ (Pfeil & Privalov, 1976a) as a nonadjustable parameter gave the fitted $T_m = 347.0 \pm 0.1 \text{ K}$ and $\Delta H_m = 124 \pm 7 \text{ kcal mol}^{-1}$ for WT, whereas entering ΔC_p as an additional adjustable param-

eter gave $T_m = 346.9 \pm 0.1 \text{ K}$, $\Delta H_m = 127 \pm 8 \text{ kcal/mol}$, and $\Delta C_p = 3.1 \pm 5.4 \text{ kcal mol}^{-1} \text{ K}^{-1}$. It follows that accurate determination of ΔC_p from this type of experiment is very difficult. The fitting errors in the T_m and ΔH_m values were typically $\leq 0.1 \text{ K}$ and $\leq 10 \text{ kcal/mol}$, respectively. T_m is the most precisely determined entity from multiple data sets, with standard deviations $\pm 0.3 \text{ K}$, whereas values of ΔH_m have associated standard deviations of $\pm 15\%$. Thus, a significant error can result when ΔG is calculated from the fitted T_m and ΔH_m by the Gibbs-Helmholtz equation due to the error in ΔH_m . The matter is made worse when the error in the respective ΔG value is used for propagation of error in $\Delta \Delta G_{(WT-mutant)}$. For these reasons, we chose to report T_m in our analyses and employed the approximation of Becktel and Schellman (1987) for the estimation of $\Delta \Delta G$.

Preparation and crystallization of mutant lysozymes

Two- to three-milligram samples of each protein were sufficient for crystallographic determination. Crystals were grown under similar conditions to those described earlier (Wilson et al., 1992). Protein was initially dialyzed against 200 mM sodium acetate, pH 4.4, and concentrated to $\sim 20 \text{ mg/mL}$. Using the vapor diffusion method at room temperature, 10- μL drops comprised of 5 μL protein and 5 μL well solution were suspended over 1 mL of well solution. Within 1 week, several multifaceted crystals grew in each drop to sizes averaging 0.6 mm on one side for mutant I55L/S91T/D101S using a well solution containing 20 mM sodium acetate and 4.5% sodium chloride, pH 4.4. Using the same technique, bipyramidal crystals of S91A were grown from 20 mM sodium acetate and 7.5% sodium chloride, pH 4.4, to sizes averaging 0.4 mm in width. Both crystal forms were isomorphous with the WT and mutant crystals used in previous chicken egg white crystallographic experiments (Blake et al., 1965; Wilson et al., 1992) with space group $P4_32_12$.

Crystallographic data collection, model building, and structure refinement

Three-dimensional diffraction data were collected for each crystallized variant using the oscillation method. Data for WT were collected previously by similar methods at 1.7 Å resolution by Wilson et al. (1992). Cell dimensions for the two mutants are summarized in Table 4.

Difference electron density maps were generated as described by Wilson et al. (1992), with the following modification: the WT model lacking mutated side chains (WT') was used to calculate maps with coefficients $|2F_{o,mutant} - F_{c,WT'}|$. The resulting maps were then used to construct models for the new side chains using the FRODO package (Jones, 1982). The mutant structures were refined using the TNT package (Tronrud et al., 1987; Tronrud, 1992) as described by Wilson et al. (1992). The coordinates of WT with the mutated side chains removed were again used as a starting model. After conjugate gradient refinement of the x, y, z and individual B -factors of this starting model, mutant side chain(s) were placed from inspection of the $(2F_{mutant} - F_{WT'})$ electron density map. Refinement statistics are summarized in Table 4. Coordinates for the two structures, S91A and I55L/S91T/D101S, have been deposited in the Protein Data

Fig. 8. An example of thermal denaturation data for WT chicken lysozyme ($\sim 25 \mu\text{M}$ in 66 mM potassium phosphate, pH 6.4). A_{292} is followed as a function of temperature. The solid curve represents the fit of the data to Equation 7 (with ΔC_p as a nonadjustable parameter), yielding $T_m = 347.0 \pm 0.1 \text{ K}$ and $\Delta H_m = 124 \pm 7 \text{ kcal mol}^{-1}$.

Table 4. Data for the crystallographic determination of the chicken lysozyme mutants S91A and I55L/S91T/D101S^a

	S91A	I55L/S91T/D101S
Crystals		
Size (mm)	0.4 × 0.4 × 0.5	0.6 × 0.6 × 0.6
<i>a</i> , <i>b</i> (Å)	79.08	79.08
<i>c</i> (Å)	37.60	37.80
Refinement		
Resolution (Å)	1.9	1.7
<i>R</i> -factor (%)	15.1	16
Reflections	8,921	11,062
Δ _{bond length} (Å)	0.016	0.015
Δ _{bond angle} (°)	2.2	2.3

^a Size is the dimensions of the crystal used for data collection. *a*, *b*, and *c* are the unit cell dimensions. *R*-factor is the standard crystallographic residual. Reflections gives the number of unique structure amplitudes collected. Δ_{bond length} and Δ_{bond angle} are the RMSDs of bond lengths and bond angles, respectively, from ideal values.

Bank (Brookhaven National Laboratory) with codes 1LSN and 1LSM, respectively.

Calculation of solvent-accessible surface area and cavity volumes

The solvent-accessible surface area of the WT lysozyme core region was calculated using the Connolly (1983) algorithm of the InsightII software (Biosym Technology Inc.) on an Indigo2 graphics workstation (Silicon Graphics). A rolling water molecule of radius 1.4 Å was used to determine the solvent-exposed molecular surface area. Cavity volumes were calculated using the PQMS program (Connolly, 1985) with a 1.2-Å probe.

p*K*_a determination of S91D

Thermal denaturation studies of WT and S91D mutant lysozymes were conducted at pH values ranging from 3.4 to 7. Aggregation is pronounced at pH ≥ 7. The buffers used were: pH 3.4–5.0, 0.1 M sodium acetate; pH 4.0–7.0, acetic acid/MES/Tris combination of Ellis and Morrison (1982) with constant ionic strength of 0.1; and pH 5.5–7.0, MES/TAPSO/diethanolamine mixtures with *I*_c = 0.1 (Ellis & Morrison, 1982). Thermal denaturation data were analyzed as described above. *T*_m's were buffer independent within experimental error. Δ*T*_m at each pH was obtained by subtracting *T*_m of S91D from that of WT, and the macroscopic apparent p*K*_a of S91D was estimated by nonlinear least-squares analysis on Equation 2. ΔΔ*G*_(S91D-WT), the difference in the free energy of unfolding of S91D and WT at each pH value was calculated from Equation 4. The Δ*S*_m value used in the calculation (0.37 kcal mol⁻¹ K⁻¹) was obtained from a plot of Δ*S*_m values, obtained from Pfeil and Privalov (1976c), versus temperature at the average *T*_m value (i.e., 72 °C). The ratio of the net protein unfolding equilibrium constant for S91D versus WT, *K*_{S91D}^{N→D}/*K*_{WT}^{N→D}, at each pH was calculated from Equation 3. The microscopic proton dissociation constants for the native and the denatured forms of S91D, *K*_{a1(native)} and *K*_{a2(denatured)} (Fig. 6), were determined from Equation 3.

GdnHCl denaturation

GdnHCl stock solutions were made in 0.1 M potassium phosphate, pH 6.4, and the precise concentration determined from the refractive index (Pace, 1986). Approximately 1.5 μM solutions of lysozyme were equilibrated for at least 3 h at 25 °C in each GdnHCl solution before measurement. Denaturation was monitored by fluorescence intensity with a Perkin-Elmer LS50B luminescence spectrometer. Samples were excited at 280 nm and emission monitored at 360 nm. Excitation and emission band-passes were 2.5 and 5 nm, respectively. The thermostatted cell chamber was maintained at 25 °C unless specified otherwise. GdnHCl denaturation data were analyzed by Equation 8 (Santoro & Bolen, 1988):

$$Y_{obs} = \frac{(\alpha_F + \beta_F[D]) + (\alpha_U + \beta_U[D])\exp\left[-\frac{m[D] + \Delta G_{N \rightarrow D}}{RT}\right]}{1 + \exp\left[-\frac{m[D] + \Delta G_{N \rightarrow D}}{RT}\right]} \quad (8)$$

where *Y*_{obs} is the fluorescence intensity, [*D*] the concentration of GdnHCl, *m* the slope at the midpoint of the transition, α and β the intercepts and slopes of pre- and postdenaturation baselines, and Δ*G*_{N→D} the free energy of denaturation in the absence of denaturant. It is often more valuable to determine *C*_m, the concentration of GdnHCl at which half of the protein is denatured, because *C*_m, like *T*_m, is generally the most precisely determined parameter. Therefore, it provides the most sensitive measure of the difference in stability between WT and mutant proteins. GdnHCl denaturation data were also analyzed by Equation 9 (Jackson et al., 1993).

$$Y_{obs} = \frac{(\alpha_F + \beta_F[D]) + (\alpha_U + W_U[D])\exp\left[\frac{m[D] - C_m}{RT}\right]}{1 + \exp\left[\frac{m[D] - C_m}{RT}\right]} \quad (9)$$

ΔΔ*G*_{N→D}^(*C*_m) is the difference in free energies of denaturation between WT and mutant at a GdnHCl concentration that is intermediate between the two *C*_m values, and is calculated from Equation 10 (Jackson et al., 1993):

$$\Delta\Delta G_{N \rightarrow D}^{(C_m)} = 0.5(m_{WT} + m_{mutant})\Delta C_m \quad (10)$$

CD spectroscopy

CD spectra were taken with an AVIV 62 DS spectrometer equipped with a temperature-controlled cell chamber. Approximately 5 μM lysozyme samples in 66 mM potassium phosphate, pH 6.4, were used.

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