Design and structural analysis of an engineered thermostable chicken lysozyme

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Abstract

A hyperstable (hs) variant of chicken egg-white lysozyme with enhanced thermal ($\Delta T_m \approx +10.5^\circ\text{C}$) and chemical (ΔC_m for guanidine hydrochloride denaturation = +1.3 M) stabilities relative to wild-type (WT) was constructed by combining several individual stabilizing substitutions. The free energy difference between the native and denatured states of the hs variant is 3.1 (GdnHCl, 25 °C) to 4.0 (differential scanning calorimetry, 74 °C) kcal mol⁻¹ greater than that of WT. The specific activity of the hs variant is 2.5-fold greater than that of WT. The choice of mutations came from diverse sources: (1) The I55L/S91T core construct with $\Delta T_m = 3.3^\circ\text{C}$ from WT was available from the accompanying study (Shih P, Holland DR, Kirsch JF, 1995, *Protein Sci* 4:2050–2062). (2) The A31V mutation was suggested by the better atomic packing in the human lysozyme structure where the Ala 31 equivalent is Leu. (3) The H15L and R114H substitutions were selected on the basis of sequence comparisons with pheasant lysozymes that are more stable than the chicken enzyme. (4) The D101S variant was identified from a screen of mutants previously prepared in this laboratory. The effects of the individual mutations on stability are cumulative and nearly additive.

Keywords: α -helix propensity; avian lysozyme sequences; chicken lysozyme; human lysozyme; interior packing; protein design; structures; thermodynamic stability

A challenging goal of protein engineering is the design of proteins with significantly enhanced thermo- and chemical stabilities. A variety of successful routes to this objective has been undertaken. These include: (1) introducing disulfide bridges (Matsumura et al., 1989b); (2) increasing internal hydrophobic packing (Malcolm et al., 1990); (3) reducing solvent-exposed nonpolar surface area (Pakula & Sauer, 1990); (4) incorporating a metal association site (Kuroki et al., 1989); and (5) adding charged side chains to interact with the α -helix dipoles (Nicholson et al., 1988). However, the above strategies are not always successful and their pursuit has often yielded proteins that are less stable than the WT progenitors for reasons that are not fully understood (e.g., Karpusas et al., 1989; Matsumura et al., 1989a; Eijssink et al., 1992; Leontiev et al., 1993).

An attractive approach is to take advantage of homologous enzymes found in thermophilic and mesophilic organisms to deduce those changes responsible for thermostability (e.g., Menéndez-Arias & Argos, 1989; Kelly et al., 1993). The three-dimensional folds are quite similar in some cases (Kelly et al., 1993); however, the number of amino acid differences is too large to allow clear-cut delineation of those responsible for the greater stability.

Game bird lysozymes have a high degree of sequence identity, and a wealth of primary and three-dimensional structural information is available. The determination of their differences in thermostability suggested possible site-directed mutations in the chicken lysozyme that might lead to enhanced thermostability. This approach, taken together with that of increasing hydrophobic packing, has been used to construct a hyperstable variant of chicken lysozyme.

Results and discussion

The hs variant of chicken lysozyme

Several thermostabilizing mutations of chicken lysozyme have been combined to produce an hs variant with $\Delta T_m \approx +10.5^\circ\text{C}$ and $\Delta C_m = +1.3$ M GdnHCl relative to WT. Their locations with the WT side chains are shown in Figure 1. The side chains

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Abbreviations: hs, a hyperstable variant of chicken lysozyme; GdnHCl, guanidine hydrochloride; T_m , the midpoint temperature of the thermal denaturation transition; C_m , the midpoint concentration of the GdnHCl denaturation profile; DSC, differential scanning calorimetry; ΔH_{cal} , calorimetrically determined enthalpy of denaturation; ΔC_p , the difference between the heat capacities of the native and the denatured states; WT or wt, wild type.

Fig. 1. Ribbon drawing showing the major secondary structural elements and the locations of residues 15, 31, 55, 91, 101, and 114 in chicken egg-white lysozyme. Side chains of the residues are shown as stick models.

of the amino acids at positions 31, 55, and 91 are interior, whereas those at positions 15, 101, and 114 are found at the surface in WT. Data from thermal and GdnHCl denaturation and differential scanning calorimetry (Fig. 2) experiments on the WT and hs variants are summarized in Table 1. The free energy difference between the native and denatured states of hs is 3.1 (GdnHCl, 25 °C) to 4.1 (DSC, 74 °C) kcal mol⁻¹ greater than that of WT. The strategies for the selection of thermostable mutations and the structural analyses of these constructs are described below.

Methods for the selection of thermostable mutations

Interior packing contributions to enhanced thermostability

A comparative investigation of substitutions in the internal core of chicken and quail lysozymes led to the discovery of three combinations of mutants in the chicken enzyme with T_m values greater than that of WT (Malcolm et al., 1990; Shih et al., 1995 [companion paper]). The most thermostable core construct, I55L/S91T ($\Delta T_m = +3.3$ °C), was selected to be combined with the additional substitutions described below.

The human and the chicken lysozyme structures, which were among the earliest protein structures solved to atomic resolution (e.g., Blake et al., 1965; Banyard et al., 1974), were compared with the objective of finding cavities in the former that could be filled in the chicken framework by mutation. Position

31, which is located at the interface between two helices, is occupied by Leu in the human and by Ala in the chicken enzyme. The chicken mutations A31V and A31L exhibited T_m values 3.1 and 4.7 °C greater than the WT enzyme, respectively. Thus, the increased packing due to these substitutions does result in a significant increase in thermostability.

Fig. 2. DSC profiles of hs and WT lysozymes. Protein samples were ca. 120 μ M in 20 mM sodium acetate, pH 5. Scan speed was 42 °C h⁻¹. Experimental error in T_m is ± 0.5 °C.

Table 1. Summary of thermal^a and GdnHCl^b denaturation and DSC^c data for the WT and the hs variant of chicken lysozyme

	WT	hs
Thermal denaturation		
T_m (°C) by Abs ₂₉₂	74.0 ± 0.3	84.5 ± 0.3
$\Delta\Delta G_{A292}^{347K}(hs-WT)$ (kcal mol ⁻¹)		-4.0
DSC		
T_m (°C)	76.2 ± 0.4	86.4 ± 0.5
ΔH_{cal} (kcal mol ⁻¹)	130	150
$\Delta\Delta G_{cal}^{347K}(hs-WT)$ (kcal mol ⁻¹)		-4.0
GdnHCl denaturation		
m (kcal M ⁻¹ mol ⁻¹)	2.4 ± 0.2	2.3 ± 0.2
C_m (M)	4.12 ± 0.06	5.43 ± 0.05
ΔG_{N-D} (kcal mol ⁻¹)	10.2 ± 0.7	13.3 ± 0.8
$\Delta\Delta G_{GdnHCl}^{298K}(hs-WT)$ (kcal mol ⁻¹)		-3.1

^a mM potassium phosphate, pH 6.4. Experimental errors in T_m were monitored by A₂₉₂. Protein samples were prepared in 66 ± 0.3 °C. $\Delta\Delta G$, the change in the free energy of unfolding, at the T_m of WT, i.e., 347 K, was calculated from equation 1 (Becktel & Schellman, 1987). See the Materials and methods.

^b Followed both by fluorescence emission intensity at 360 nm and by CD ellipticity at 222 nm. $\Delta\Delta G_{GdnHCl}^{298K}(hs-WT)$, ΔG_{N-D} (kcal mol⁻¹), C_m , m , were calculated from Equations 13–15 in Shih et al. (1995).

^c Ca. 1.5 mg/mL protein samples prepared in 20 mM Na acetate, pH 5.0 were used. These T_m values differ by ~2 °C from those determined by A₂₉₂, mainly due to the difference in the pH values between the two experiments (see Fig. 5B in Shih et al. [1995]). $\Delta\Delta G$, the change in free energy of unfolding at 347 K was calculated from equation 2 (see the Materials and methods).

Natural variation in avian lysozyme sequences related to thermostability

Complete amino acid sequences of more than 30 c-type lysozymes are known. Twelve of these are from the order Gallifor-

mes (game birds) (Araki et al., 1994). Sequence comparison of selected Galliformes lysozymes and their T_m values relative to that of the chicken enzyme are listed in Table 2. Several pheasant lysozymes have higher T_m values ($\Delta T_m \leq +3$ °C) than the chicken enzyme. Although the magnitude of the increases in the T_m values of pheasant lysozymes is not in itself remarkable, the high amino acid sequence identity ($\geq 92\%$ compared to the chicken enzyme) facilitated the task of pinpointing the substitutions responsible for the differences in thermostability. In an attempt to determine the changes between chicken and pheasant enzymes that contribute to the differences in T_m values, the six mutations, H15L, F34Y, R73K, N77H, G102R, and R114H, were constructed and analyzed for their thermostabilities (Table 3). The two most thermostable mutants are H15L ($\Delta T_m = +2$ °C) and R114H ($\Delta T_m = +1.8$ °C). The H15L substitution is also found in human lysozyme and is discussed more fully below. Gly 102 is a C-cap+2 residue (as defined by Richardson & Richardson [1988]), and the positively charged Arg might be expected to interact favorably with the partial negative α -helix dipole at the C-terminus. The mutant G102R has a T_m 1 °C higher than that of WT. Three additional chicken-to-pheasant substitutions Q41H, Q121S, and A122V, which may also influence thermostability, were not investigated.

Additional lysozyme mutants suggested by comparisons of sequences from different species (Table 2) were available from a previous, unrelated study (Kam-Morgan et al., 1993). The T_m and $\Delta\Delta G$ values of F3Y, N19K, R21Q, R68K, D101G, G102V, and Q121H are included in Table 3. The largest favorable change in T_m of this series is exhibited by the D101G mutation ($\Delta T_m = +1.2$ °C) found in the turkey enzyme. This observation prompted a wider exploration of this surface locus unrestrained by considerations of natural variation. The D101S mutation was thus found to give the largest enhanced thermostability ($\Delta T_m = +2.2$ °C).

The six substitutions, H15L, A31V, I55L, S91T, D101S, and R114H, were combined to give the hs variant. The cumulative effects in T_m of several combinations of these individual mutations are shown in Figure 3. Of the six residues, only positions 55 and 91 are in van der Waals contact with each other. The

Table 2. Protein sequence differences in avian lysozymes^a

Species	Amino acid position																				T_m^b (°C)	ΔT_m (°C)	
	Δ	-1	3	15	19	21	34	41	68	73	77	79	99	101	102	103	113	114	121	122			124
Chicken	0		F	H	N	R	F	Q	R	R	N	P	V	D	G	N	N	R	Q	A	I	74.0	—
Bob. quail ^c	4								K													73.2	-0.8
Cal. quail ^c	4																		H			72.1	-1.9
Jpn. quail	6		Y		K	Q									V	H			N			71.8	-2.2
Turkey	7		Y	L				H		K			A	G					H			74.7	+0.7
Rn. pheasant	10	G	Y	M				G		K	H						K	H	N	V		76.8	+2.8
S. pheasant ^d	9		Y	L			Y	H	K		H								S	V	T	77.0	+3.0
G. pheasant	6		Y	L				H			H								N		T	74.2	+0.2
Re. pheasant	7		Y	L				H			H	S			R				N			76.2	+2.2

^a Sequences are taken from Jollès et al. (1979) and Araki et al. (1990). Bob., bobwhite; Cal., California; Jpn., Japanese; Rn., ring-necked; S., silver; G., golden; Re. Reeves'; Δ is the number of changes from the chicken sequence. ΔT_m is relative to the chicken enzyme. The two thermostabilizing substitutions that were incorporated into the hs variant are highlighted in bold.

^b See Table 1 footnote a for conditions.

^c These two lysozymes also have T40S, I55V, and S91T substitutions (Jollès et al., 1979).

^d The sequence of silver pheasant lysozyme is not known, but it is likely to be similar to that of Kalij pheasant enzyme, whose sequence is given here, based on their close phylogenetic relationship (Johnsgard, 1986).

Table 3. Thermostability of chicken lysozyme mutants suggested by natural variation in other avian lysozymes^a

Variant	F3Y	H15L	N19K	R21Q	F34Y	R68K	R73K	N77H	D101G	G102V	G102R	R114H	Q121H
T_m (°C)	72.8	76.0	71.2	73.6	74.5	73.9	74.6	73.0	75.2	73.9	75.0	75.8	72.8
$\Delta\Delta G$ (kcal mol ⁻¹)	-0.45	0.76	-1.06	-0.15	0.19	-0.04	0.23	-0.38	0.45	-0.04	0.38	0.68	-0.45

^a See Table 1 footnote a for conditions.

combination of I55L and S91T mutations resulted in a non-cumulative effect on T_m (Shih et al., 1995). The thermostabilizing effects of the remaining substitutions are nearly additive. The sum of the ΔT_m values from WT for the individual mutations in hs (with I55L and S91T considered as a unit) is 12.5 ± 0.7 °C, which is close to the ΔT_m of hs from that of WT (10.5 ± 0.3 °C). Wells (1990) has pointed out that the effects of separate mutations are more often additive if they are spatially remote. Serrano et al. (1993) similarly imported the stabilizing substitutions from binase to the highly homologous barnase and improved its stability in urea by 3.4 kcal mol⁻¹ with six amino acid replacements.

Structural analyses of individual thermostabilizing mutations

Improved internal packing by the Ala 31 substitutions

The side chain of Ala 31 in chicken lysozyme points toward the interior of the protein in the middle of helix B that includes residues Leu 25–Ser 36. Helix B is one of the four α -helices, which together with a C-terminal 3_{10} -helix, constitute the α -domain of the protein. Nonpolar amino acids are generally preferred in similar locations in other proteins (Richardson & Richardson, 1988). Leu replaces Ala 31 in most mammalian lysozymes (Jollès & Jollès, 1984). Figure 4A and B shows the structures of the human and chicken enzymes, respectively, in this region. Internal packing at this locus is more dense in the human than in the chicken enzyme. Hooke et al. (1994) suggested that Leu 31 (human) versus Ala 31 (chicken) is responsible for the faster rate of core formation from helices A and B in the human relative to the chicken enzyme (Miranker et al., 1991; Radford et al., 1992).

Fig. 3. Cumulative effects of thermostabilizing mutations in the hs variant of chicken lysozyme. Thermal denaturation was monitored by A_{292} in 66 mM potassium phosphate, pH 6.4. The hs variant combines the H15L, A31V, I55L, S91T, D101S, and R114H substitutions.

Imoto et al. (1987) found that chicken lysozyme containing the A31V mutation could not be refolded following expression as an inclusion body in *Escherichia coli*, although they were able to refold WT enzyme and other mutants. The presently employed yeast expression system produced soluble A31V protein directly. The mutant has a T_m value that is 3.1 °C greater than that of WT. A31V was thus incorporated into the hs architecture. Subsequently, further exploration of heat stability related to residue 31 showed that A31I and A31L substitutions yielded lysozymes with even greater thermostabilities than A31V with ΔT_m from WT = 3.6 °C and 4.7 °C, respectively (Table 4). Indeed, A31L is the most thermostable single mutant of chicken lysozyme made to date. The A31L mutation has yet to be evaluated in the context of the hs framework.

It is likely that a combination of packing density and α -helical propensity is responsible for the observed dependence of the T_m value upon the amino acid at position 31. Table 4 shows that packing, i.e., side-chain volume is qualitatively correlated with T_m . The higher T_m value of A31L versus A31I may reflect the higher helical propensity of the former. Figure 5A compares the far-UV CD spectra of WT and variants at position 31. The spectra of WT, A31V, and A31L are quite similar, but that of A31I exhibits a small reduction in ellipticity in the α -helical region. Figure 5B shows the fluorescence emission spectra of A31 variants, hs, and WT. The fluorescence emission intensity of hs is about 30% less than that of WT. This reduction can be attributed solely to the A31V mutation because the fluorescence emission spectra of the remaining mutations in hs are superimposable with that of WT (data not shown). Ala 31- C^β is ~ 6 Å from the indole ring of Trp 108, which is responsible for a major portion of the lysozyme emission (Formoso & Forster, 1975). The analogous emitter in the human enzyme, Trp 109, is ~ 4 Å from both the $C^{\delta 1}$ and $C^{\delta 2}$ atoms of Leu 31. The relationship between the newly introduced geometry and the decreased emission of lysozyme is not understood.

Side-chain interaction between residues 114 and 34

Residue 114 is on the surface of the molecule at the carboxyl end of a short two-turn α -helix (109–114). The WT side chain, Arg, is exposed to solvent with its side chain parallel to that of Phe 34. Residue 114 is usually occupied by Arg or His, whereas Phe, Tyr, or Trp are found at position 34 in most c-type lysozymes (McKenzie & White, 1991). The R114H mutation in the chicken enzyme yields a 1.8 °C increase in T_m . Because the side chain of Phe 34 is the only one that is in close contact with that of residue 114, the thermostability enhancement of the R114H substitution may derive from the interaction between the His 114 and the Phe 34 side chains. This possibility was probed by direct NMR determination of the pK_a of the introduced histidine. It is unlikely that both the protonated and the unprotonated forms of histidine would contribute equally to the enhanced

Fig. 4. Stereo plots showing packing interactions at an interior core in the α -domain of (A) human lysozyme (31 = Leu) and (B) chicken lysozyme (31 = Ala). Chicken enzyme structure from Wilson et al. (1992); human enzyme structure from Artymiuk and Blake (1981). PDB codes are 1HEL and 1LZ1, respectively (Bernstein et al., 1977).

T_m , therefore, the pK_a should be perturbed when Phe 34 is replaced by Leu. (See Chakrabartty and Baldwin [1995] and Honig and Yang [1995] for recent reviews on the electrostatic/ pK_a -linked effect on protein stability.)

WT chicken lysozyme contains a single histidine group at position 15. In the triple mutant, H15L/A31V/R114H, the WT histidine is removed and a new His is introduced at position 114. The titration profiles of the N2-H protons of WT and in H15L/A31V/R114H are shown in Figure 6A. The pK_a values are 5.53 ± 0.05 (His 15) and 6.28 ± 0.08 (His 114). The former is

in good agreement with the literature value of 5.52 ± 0.04 (Takahashi et al., 1992). The titration profiles of N2-H protons of the two histidines (His 15 and His 114) in the double mutant F34L/R114H are shown in Figure 6B. The pK_a values are 5.58 ± 0.07 and 6.36 ± 0.10 , respectively. Thus, there is no significant perturbation in the pK_a of His 114 by the F34L mutation, an observation that argues against a significant interaction between the two residues. The energetically favorable histidine-aromatic interaction had been noted previously (Loewenthal et al., 1992; Armstrong et al., 1993). In the latter study, the perturbations in the His pK_a value upon substitution of the interacting aromatic residue were correlated with the free energies of unfolding of the protein variants.

The X-ray structure of guinea fowl lysozyme, which has Phe 34 and His 114, has been published recently (Lescar et al., 1994). The imidazole ring of H114 engages in a face-to-face contact with the Phe 34 ring at a distance of ca. 4 Å between the two planes, close enough to form a π -complex. A similar interaction was observed in the R115H (equivalent to R114H in the chicken enzyme) mutant of human lysozyme where residue 34 is Trp (Harata et al., 1993). If that orientation persists in the R114H construct of the chicken enzyme, it does not contribute significantly to ΔT_m according to the pK_a perturbation arguments stated above.

Exchange of favorable hydrophobic contacts for a hydrogen bond in the H15L substitution

His 15 in chicken lysozyme is located near the surface of the protein at the C-terminus of helix A, which comprises residues Gly 4 to Gly 16. N $^{\epsilon 2}$ of the imidazole ring is hydrogen bonded to O $^{\gamma}$ of Thr 89 ($r = 2.71$ Å) in the WT structure. Replacement

Table 4. Thermostability determinants at position 31 of chicken lysozyme

	Ala (WT)	Val	Ile	Leu
T_m ($^{\circ}\text{C}$) ^a	74.0	77.1	77.6	78.7
$\Delta\Delta G_{\text{mutant-WT}}$ (kcal mol $^{-1}$)		1.2	1.4	1.8
P_{α} helix parameter ^b	1.60	1.09	1.31	1.50
Hydrophobicity ^c	-2.08	-0.83	-0.04	-0.18
Side-chain volume (Å 3) ^d	28.5	79.9	106.6	106.6

^a See Table 1 footnote a for conditions.

^b Helical preference parameters given by the frequency of an amino acid in α -helices (the three residues at each end are excluded) relative to its frequency in the entire protein (Williams et al., 1987). A higher value denotes a greater helix propensity.

^c Derived from free energies of transfer from octanol to water, determined by Fauchère and Pliska (1983). Less negative values denote greater hydrophobicity.

^d The Voronoi volume of the amino acid minus that of glycine from Chothia (1975).

Fig. 5. **A:** Far-UV CD spectra of WT and A31 variant lysozymes. Protein samples were in 66 mM potassium phosphate, pH 6.4 and 25 °C. **B:** Fluorescence emission spectra of WT, hs, and A31 variant lysozymes. Excitation = 280 nm; ca. 2 μ M protein solutions in 66 mM potassium phosphate, pH 6.4 and 25 °C.

of His 15 with either Met (ring-neck pheasant) or Leu (other pheasants and turkey) would be expected to be unfavorable because these side chains would expose hydrophobic surfaces to the solvent, and they cannot make hydrogen bonds. Most mammalian lysozymes, including the human enzyme, have the H15L substitution, as well.

Banyard et al. (1974) first pointed out that H15L is one of the three substitutions from the chicken to the human enzymes that "result in surface residues becoming internal." A rotation around the β - γ bond of Leu 15 in the human enzyme, "that appears to leave the main-chain conformation essentially unchanged [from the chicken enzyme], largely buries [the side-chain] in the hydrophobic core." This observation suggested that the H15L substitution in the chicken enzyme might yield increased stability. The H15L T_m is 2 °C greater than that of WT, in accord with this expectation. Table 5 compares the side-chain interactions at position 15 in chicken and turkey lysozymes. The turkey enzyme is used for reference instead of the human enzyme because of the much higher sequence identity (95% versus 60%, respectively) compared to the chicken enzyme and the absence of concerted replacements around residue 15. The structural changes associated with the H15L mutation in the chicken enzyme can be inferred from this comparison. If the structural context of the H15L mutation in the chicken enzyme was the same as in

Fig. 6. Histidine pK_a determination of lysozyme variants. Chemical shifts of the C2-H resonances as a function of pD (uncorrected for the isotope effect) were fitted to the equation for a single protonic equilibrium. **A:** H15 in WT ($pK_a = 5.53 \pm 0.05$) and H114 ($pK_a = 6.28 \pm 0.08$) in H15L/A31V/R114H. **B:** H15 ($pK_a = 5.58 \pm 0.07$) and H114 ($pK_a = 6.36 \pm 0.09$) in F34L/R114H.

the turkey structure, one hydrogen bond would be replaced by two nonpolar contacts (Table 5). The higher T_m value supports the idea that the latter dominate in this case. A substitution accompanied by a side-chain rotation from a surface to an internal site has been observed in a thermostable mutant of T4 lysozyme, S117F (Anderson et al., 1993). The enhanced stability of this mutant was attributed to improved hydrophobic core packing and introduced aromatic-aromatic interaction in replacing a hydrogen bond as a result of the side-chain rotation.

Alleviation of a destabilizing steric contact at residue 101

The side chain of Asp 101 makes a hydrogen bond with the saccharide unit in subsite B (Cheetham et al., 1992). Asp 101 is found in most but not all c-type lysozymes (Jollès & Jollès, 1984). For example, it is replaced by Gly in the turkey enzyme. The thermostabilities of a series of position 101 mutations, originally made to analyze contributions to the free energy of substrate association (Kirsch et al., 1989), were evaluated here. Most of the constructs with position 101 replacements are more thermostable than WT (Table 6) with D101S the most stable ($\Delta T_m = 2.2$ °C). The T_m rankings are in descending order: S > A > F > G > R > K > N > D = E > Q. Residue 101 is located near the C-terminus of helix C, which runs from Ile 89 to

Table 5. Comparison of side-chain interactions at position 15 in chicken and turkey lysozymes^a

Chicken (15 = His)			Turkey (15 = Leu)		
Atom	Neighbor	Distance (Å)	Atom	Neighbor	Distance (Å)
His 15-C γ	Val 92-C γ 1	4.4	Leu 15-C γ	Val 92-C γ 1	4.9
His 15-C γ	Val 92-C γ 2	4.7	Leu 15-C γ	Val 92-C γ 2	4.2
His 15-C δ 2	Val 92-C γ 1	4.6	Leu 15-C δ 2	Val 92-C γ 1	5.6
His 15-C δ 2	Val 92-C γ 2	5.2	Leu 15-C δ 2	Val 92-C γ 2	4.3
			Leu 15-Cδ1	Val 92-Cγ1	3.9
			Leu 15-Cδ1	Val 92-Cγ2	3.8
His 15-Nϵ2	Thr 89-Oγ1	2.7	Leu 15-C δ 1	Thr 89-O γ 1	4.2

^a Chicken enzyme structure from Wilson et al. (1992); turkey lysozyme structure from Harata (1993). PDB codes are 1HEL and 1LZ3, respectively. Unique interactions are presented in bold.

Ser 100. The negative charge of Asp 101 in WT may interact unfavorably with the partial negative charge of the α -helix dipole. However, two arguments can be raised against this explanation for the lower T_m of WT. First, replacement by the positively charged arginine or lysine yields proteins that are no more stable than those with neutral substitutions. Second, the pK_a of Asp 101 has a normal value of 4.0–4.25 (Bashford & Karplus, 1990), rather than the higher one expected from the hypothesized unfavorable helix dipole interaction.

An explanation for the relative thermostability for the position 101 substitution series is provided by a comparison of the WT and I55L/S91T/D101S structures. The O ^{δ 1} and O ^{δ 2} atoms of Asp 101 interact only with water molecules and are characterized by moderately high B -factors of 62 Å² and 53 Å², respectively. Polar residues might therefore be preferred at this position. The X-ray structure for the triple mutant I55L/S91T/D101S containing three of the six substitutions in the hs variant was solved at 1.7 Å resolution (Shih et al., 1995). Because it has been shown that the structural changes associated with conservative mutations in the 40–55–91 core are localized around the region of mutation (Wilson et al., 1992), the structural consequences of the D101S mutation can be analyzed separately. Figure 7A and B shows the region near residue 101 in the structures of WT and I55L/S91T/D101S, respectively. The D101S mutation has little effect on the overall structure of the protein. Only small main-chain movements limited to residues 100–105 are seen. C ^{β} of Asp 101 is in a close (2.93 Å) destabilizing interaction with Ile 98-O. The χ_1 torsion angle is different for Ser 101 in the triple mutant (101° versus –163° in WT), which results in a lengthening to 3.53 Å of the C ^{β} to 98-O distance, thus relieving the steric compression seen in WT. In addition, this rotation about the C ^{β} –C ^{γ} bond may allow the Ser-O ^{γ} to

form a hydrogen bond with the carbonyl oxygen of residue 98 ($r = 3.34$ Å). Thus, the D101S substitution may introduce a stabilizing interaction in place of a destabilizing one. The strain relief may similarly explain the other thermostabilizing substitutions at position 101, but the requisite structures are unsolved. The enhancement of protein thermostability by the relaxation of strain has been observed in a mutant of T4 lysozyme as well (Pjura & Matthews, 1993).

The specific bacteriolytic activity of the hs variant is 2.5-fold greater than that of WT

Some instances of decreased enzyme activity correlating with increased stability have been reported (Meiering et al., 1992; Varley & Pain, 1991). The hs construct of chicken lysozyme provides a counterexample. Its specific activity is 2.5-fold greater than that of WT under standard assay conditions (see the Materials and methods). This fortuitous property of hs can be attributed to the cumulative higher activities of several of its constituent substitutions. Their locations relative to the active site and the specific bacteriolytic activity of the mutants are summarized in Table 7. The product of the relative activities of the individual mutations is 3.6-fold, a value reasonably close to that of hs.

Although the specific reasons for the higher activity were not investigated in the present work, it could be noted that the side chain of Asp 101 participates in hydrogen bonding with the monosaccharide unit in subsite B (Cheetham et al., 1992). Most of the position 101 variants exhibit higher specific bacteriolytic activity than WT. Turkey lysozyme, which has a Gly at position 101, is twice as active as the chicken enzyme toward the bacterial cell wall substrate. The cleavage pattern of *p*-nitrophenyl-(GlcNAc)₅ shifts in the W62H/D101G double mutant in a man-

Table 6. Thermostability of position 101 variants of the chicken lysozyme^a

Residue at 101	Ser	Ala	Phe	Gly	Arg	Lys	Asn	Asp (WT)	Glu	Gln
T_m (°C)	76.3	76.0	75.9	75.2	74.7	74.5	74.1	74.0	74.0	73.8
$\Delta\Delta G$ (kcal mol ⁻¹)	0.87	0.76	0.72	0.45	0.27	0.19	0.04	—	0	–0.08

^a See Table 1, footnote a, for conditions.

Fig. 7. Partial structures of (A) WT (101 = Asp) and (B) I55L/S91T/D101S mutant lysozymes around the region of residue 101. PDB codes are 1HEL and 1LSM, respectively.

ner consistent with an increased occupancy of subsite B–F as opposed to A–E in WT (Kumagai et al., 1993). Occupancy of the former set of subsites is possibly accompanied by a larger hydrolytic constant.

Concluding remarks

Enhancement of protein stability is a major objective in protein engineering. The present study combined several approaches to achieve a substantial increment in the stability of chicken lysozyme. The increased stability from three replacements in the hs variant (A31V and I55L/S91T) can be attributed to denser and more nearly optimal packing. (See Richards and Lim [1993] and Matthews [1995] for recent reviews on hydrophobicity/packing.) Attempts to achieve higher stability by increasing packing density do not always succeed (e.g., Karpusas et al., 1989). However, the success of this approach is likely to be enhanced if a natural substitution found in a homologous protein is imported, as in the case of position 31 in chicken and human lysozymes.

Two thermostabilizing substitutions in the hs variant, (1) a polar to nonpolar replacement that allows a surface residue to engage in favorable nonpolar contacts upon side-chain rotation

toward the interior (H15L), and (2) a strain-relieving mutation (D101S) could only be understood within the framework of the X-ray structures that revealed the subtle changes associated with the mutations. The stabilizing effect of the R114H mutation is not understood, illustrating the incompleteness of present knowledge.

Materials and methods

Mutagenesis, expression, purification, and activity assay

Site-directed mutagenesis, expression, purification, and the specific bacteriolytic activity determination of lysozyme variants are described in Shih et al. (1995). Quail, pheasant, and turkey lysozymes were generous gifts from Dr. Ellen Prager and were purified from the egg whites of the respective birds according to Jollès et al. (1979) and Prager and Wilson (1971). Chicken egg-white lysozyme was purchased from Sigma and further purified by hydroxylapatite chromatography.

Thermal stability determination

Lysozyme undergoes reversible thermal denaturation transitions that obey a two-state process to a good approximation (O'Reilly & Karasz, 1970). The methods for sample preparation, T_m measurement, and analysis of denaturation data using a two-state model have been described previously (Shih et al., 1993, 1995). $\Delta\Delta G$, the change in the free energy of unfolding, ΔG_{mutant} minus ΔG_{wt} at the T_m of WT, was approximated from Equation 1, (Becktel & Schellman, 1987).

$$\Delta\Delta G \approx \Delta T_m * \Delta S_m. \quad (1)$$

ΔT_m is the difference in T_m of the variant from WT and ΔS_m is the entropy of unfolding of WT at T_m . The value of ΔS_m is 379 cal K⁻¹ mol⁻¹ for WT enzyme at its T_m of 74 °C (see Shih et al. [1995] for determination of ΔS_m and discussion). Authentic chicken lysozyme gave identical thermal denaturation profiles to those of the yeast-derived enzyme. The reported T_m value for each construct is the average of T_m 's obtained from at least three melting experiments.

Table 7. Specific bacteriolytic activity of thermostabilizing substitutions in hs variant

Mutant	Bacteriolytic activity (% WT) ^a	Location
hs	250	
H15L	100	Not near the active site
A31V	150	Helix B (25–36) containing active site residue Glu 35
TLT	110	In the internal core region beneath the active site (Malcolm et al., 1990)
D101S	180	At subsite B (Cheetham et al., 1992)
R114H	120	Near subsite F (Pincus & Scheraga, 1979)

^a Bacteriolytic activity was measured by the turbidity assay described in the Materials and methods. Standard deviations for multiple determinations were $\leq 10\%$.

DSC

Thermodynamic stabilities of WT and hs chicken lysozymes were measured additionally by DSC employing a Microcal Inc. MC-2 instrument at a scan speed of 42°C h^{-1} . Two milliliters of a ca. 120 μM protein solution, extensively dialyzed against 20 mM sodium acetate, pH 5, and filtered through a 2- μm cut-off membrane (Millipore), were used for each experiment. Samples were thoroughly degassed. The excess specific heat of unfolding was monitored as a function of temperature. The data were collected, normalized for concentration, and analyzed with vendor-supplied software, using the two-state denaturation option. The ratio of the calorimetric to the van't Hoff ΔH values is approximately unity within 10% error. The free energies of denaturation for WT and hs lysozymes at 25°C were calculated from the Gibbs-Helmholtz equation

$$\Delta G_T = \Delta H_{cal}[1 - (T/T_m)] - \Delta C_p[(T_m - T) + T \ln(T/T_m)]. \quad (2)$$

It is generally difficult to obtain an accurate ΔC_p value due to the uncertainty in the baseline extrapolation in a calorimetric scan; hence, the WT ΔC_p value of $1.57 \text{ kcal mol}^{-1} \text{ K}^{-1}$ (Pfeil & Privalov, 1976) was used for both WT and hs lysozymes. This assumption is supported by work from Sturtevant's lab on a number of proteins. For example, Tanaka et al. (1993) found that ΔC_p varied only from 2.0 to $2.5 \pm 0.2 \text{ kcal mol}^{-1} \text{ K}^{-1}$ for a series of single amino acid replacements in staphylococcal nuclease. Incorporation of a 20% uncertainty in ΔC_p in the present data analysis leads only to a 3% error in ΔG_T for WT enzyme calculated at 74°C . The propagated error increases to 11% at 25°C . Thus, analysis at a temperature that is close to T_m reduces the contribution of the ΔC_p -dependent term (second term in Equation 2) to ΔG_T .

GdnHCl denaturation of lysozyme variants

Denaturation of lysozymes by GdnHCl was monitored by fluorescence intensity (Shih et al., 1995) or by CD. The decrease in ellipticity at 222 nm upon denaturation was followed with an AVIV 62DS CD spectrometer. All measurements were conducted in a temperature-controlled cell chamber maintained at 25°C . GdnHCl denaturation data were analyzed as described in Shih et al. (1995).

Determination of histidine pK_a values in WT, H15L/A31V/R114H, and F34L/R114H

Approximately 10 mg each of the purified yeast-derived lysozyme variants were dissolved in 1 mL of D_2O (99.96%, Cambridge Isotope Laboratories), dialyzed against $3 \times 1\text{-mL}$ changes of D_2O , and lyophilized. The exchangeable protons were deuterated at the respective T_m values of the variants ($72\text{--}79^\circ\text{C}$) for about 15 min. The exchanged protein solution was lyophilized, dissolved in D_2O to a concentration of ca. 1.2 mM, and the pD brought to ~ 3 with DCl (Sigma).

NMR measurements of the C2-H resonances of the histidine residues of lysozymes were performed at $25 \pm 1^\circ\text{C}$ using a GE Omega 500 MHz spectrometer, with 3-(trimethylsilyl)propionic acid as the internal standard. The titration was carried out at

pD values from ~ 3 to ~ 9 by adjusting the pD of the protein solutions with NaOD. The pD value was determined at the beginning and at the end of each NMR measurement using a Radiometer pH meter with a PHM80 probe (Ingold) calibrated against standard buffers in H_2O . The chemical shifts of the C2-H resonances at varying pD values (uncorrected meter readings) were fitted to the equation for a single ionization equilibrium.

Fluorescence and CD spectrometry

Fluorescence spectra were taken with a Perkin-Elmer LS50B luminescence spectrometer equipped with a cell chamber thermostatted at 25°C . Samples were excited at 280 nm and emission monitored from 300 to 400 nm. Excitation and emission band-passes were 2.5 nm and 5 nm, respectively. Approximately 2 μM lysozyme samples in 66 mM potassium phosphate, pH 6.4, were used. CD spectra were obtained as described in Shih et al. (1995).

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