



Folding and function of a T4 lysozyme containing 10 consecutive alanines illustrate the redundancy of information in an amino acid sequence

(α -helix/mutagenesis/protein folding/bacteriophage T4)

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ABSTRACT Single and multiple Xaa \rightarrow Ala substitutions were constructed in the α -helix comprising residues 39–50 in bacteriophage T4 lysozyme. The variant with alanines at 10 consecutive positions (A40–49) folds normally and has activity essentially the same as wild type, although it is less stable. The crystal structure of this polyalanine mutant displays no significant change in the main-chain atoms of the helix when compared with the wild-type structure. The individual substitutions of the solvent-exposed residues Asn-40, Ser-44, and Glu-45 with alanine tend to increase the thermostability of the protein, whereas replacements of the buried or partially buried residues Lys-43 and Leu-46 are destabilizing. The melting temperature of the lysozyme in which Lys-43 and Leu-46 are retained and positions 40, 44, 45, 47, and 48 are substituted with alanine (i.e., A40–42/44–45/47–49) is increased by 3.1°C relative to wild type at pH 3.0, but reduced by 1.6°C at pH 6.7. In the case of the charged amino acids Glu-45 and Lys-48, the changes in melting temperature indicate that the putative salt bridge between these two residues contributes essentially nothing to the stability of the protein. The results clearly demonstrate that there is considerable redundancy in the sequence information in the polypeptide chain; not every amino acid is essential for folding. Also, further evidence is provided that the replacement of fully solvent-exposed residues within α -helices with alanines may be a general way to increase protein stability. The general approach may permit a simplification of the protein folding problem by retaining only amino acids proven to be essential for folding and replacing the remainder with alanine.

The variability of the sequences of proteins observed in nature shows that many different amino acid sequences can code for essentially the same three-dimensional structure. From such naturally occurring sequences, however, it may not be apparent whether changes in a given amino acid are permitted independently or require concerted changes elsewhere in the molecule. Different types of saturation and directed mutagenesis (1–11) have shown that proteins can have a relatively large number of sites at which amino acid substitutions can be made relatively freely with little effect on folding and function. In most such studies one amino acid is changed at a time (1–9). In other cases a selected subset of amino acids is altered in combination and those variants that are functional are selected and identified (10, 11). While variants with a single amino acid substitution may permit protein folding and function, the question remains as to whether this is also the case for multiple replacements.

Here we describe experiments in which single and multiple alanine substitutions were made within an α -helix of T4 lysozyme, culminating in a protein with 10 consecutive alanines in sequence. Such experiments also test the role of

alanine as a “helix-favoring” amino acid and help identify those amino acids that are most important for the folding and stability of the protein (12).

MATERIALS AND METHODS

The α -helix comprising residues 39–50 is on the solvent-exposed side of the N-terminal domain of T4 lysozyme, 10–15 Å from the active-site cleft. The helix in the wild-type structure contains three alanines, at positions 41, 42, and 49 (Table 1). The almost completely buried amino acids Leu-39 and Ile-50 at the beginning and end of the helix were not mutated. Seven mutants with single substitutions—Asn-40 \rightarrow Ala (N40A), K43A, S44A, E45A, L46A, D47A, and K48A—as well as the polyalanine mutant N40A/K43A/S44A/E45A/L46A/D47A/K48A (A40–49) were constructed initially. The polyalanine mutant N40A/S44A/E45A/D47A/K48A (A40–42/44–45/47–49), where both Lys-43 and Leu-46 were left unchanged, was subsequently constructed, in an attempt to make a thermostable mutant containing as many alanines as possible in the helix. The double mutant E45A/K48A was also constructed to study the effect on stability caused by the replacement of the putative salt bridge between Glu-45 and Lys-48 (type $i, i + 3$) in wild-type T4 lysozyme.

All experiments were carried out on a pseudo-wild-type lysozyme in which Cys-54 and Cys-97 in the native molecule were replaced with threonine and alanine, respectively (15). Methods for mutagenesis, protein purification, and thermodynamic and crystallographic analyses were essentially as described (12, 16–20). Rough estimates of combined catalytic activity and thermal stability were obtained by using the halo assay (21). The polyalanine mutant A40–49 was constructed by using the oligonucleotide 5'-ACGCCCAATAGCTGC-AGCTGCTGCAGCTGCAGCAGCAGCAAGTG-3' as the mutagenic primer and uracil-containing single-stranded DNA from the point mutant N40A as template (changes from this template are underlined). The mismatch of 9 bases in the oligonucleotide relative to the template led to a reduction in mutagenic efficiency to <10%, compared with \approx 75% obtained for other mutants. Sequencing of G+C-rich (polyalanine) regions, which frequently were prone to compressions in the sequencing gels, was facilitated by using Sequenase kits containing 7-deaza-GTP (United States Biochemical), which helps reduce internal G-C base pairing resulting in anomalous bands on standard sequencing gels.

Crystals isomorphous to wild-type lysozyme were obtained under similar conditions (22). X-ray data were collected for K43A, S44A, L46A, D47A, and A40–49 with a San Diego Multiwire Systems area detector, and the structures were refined at 1.7- to 1.9-Å resolution to R factors between 15.3% and 17.7% with good stereochemistry.*

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*Coordinates have been deposited in the Brookhaven Data Bank (accession numbers 1L64–1L68).

Table 1. Mutant lysozymes

Protein	Residue									
	40	41	42	43	44	45	46	47	48	49
Wild type	Asn	Ala	Ala	Lys	Ser	Glu	Leu	Asp	Lys	Ala
Mutant										
N40A	Ala	(Ala)	(Ala)							(Ala)
K43A		(Ala)	(Ala)	Ala						(Ala)
S44A		(Ala)	(Ala)		Ala					(Ala)
E45A		(Ala)	(Ala)			Ala				(Ala)
L46A		(Ala)	(Ala)				Ala			(Ala)
D47A		(Ala)	(Ala)					Ala		(Ala)
K48A		(Ala)	(Ala)						Ala	(Ala)
A40-49	Ala	(Ala)	(Ala)	Ala	Ala	Ala	Ala	Ala	Ala	(Ala)
A40-42/44-45/ 47-49	Ala	(Ala)	(Ala)	Lys	Ala	Ala	Leu	Ala	Ala	(Ala)
E45A/K48A		(Ala)	(Ala)			Ala			Ala	(Ala)
Solvent accessibility	0.78	0.32	0.00	0.23	0.57	0.42	0.00	0.24	0.60	0.15

Substitutions are at residues 40-49, within the helix comprising residues 39-50 in phage T4 lysozyme. The sites of the single and multiple alanine mutations are shown. Alanines in parentheses (Ala) in the mutant structures are already present in pseudo-wild type. The solvent accessibility was calculated (13) as a ratio of the side-chain area accessible to solvent in T4 lysozyme relative to the same area in an extended model peptide with identical amino acid sequence (14).

RESULTS

Thermodynamic data at pH 3.0 and pH 6.7 are summarized in Table 2. At pH 3.0 an increase in thermal stability compared with that of pseudo-wild-type lysozyme was observed for the single mutants N40A, S44A, and E45A and the multiple mutants E45A/K48A and A40-42/A44-45/A47-49. Destabilization occurred for the single mutants K43A, L46A, and K48A and for the polyaniline mutant A40-49. The most drastic decrease in stability at both pH values was caused by mutating either Lys-43 or Leu-46 to alanine (Fig. 1a). The mutant D47A exhibited essentially the same stability as pseudo-wild-type lysozyme at pH 3.0. At pH 6.7 a similar trend in thermal stability was observed except that E45A and D47A, two of the single mutants that replaced a charged

group, were significantly less stable at pH 6.7 than at pH 3.0 (Fig. 1a).

At both pH values the stabilities of the polyaniline mutants and of the double mutant E45A/K48A are slightly greater than the sum of the stabilities of their constituents (Fig. 1b).

Changes in catalytic activity associated with the respective mutations were minor, if present at all.

The high-resolution crystal structures showed that the overall conformations of all of the mutants analyzed were similar to that of pseudo-wild-type lysozyme (refs. 22 and 25; A. E. Eriksson, W.A.B., and B.W.M., unpublished work). After superposition of the backbone atoms of the whole molecules, the root-mean-square difference in coordinates varied from 0.09 Å for mutant D47A to 0.20 Å for mutant A40-49.

Table 2. Thermal stabilities of mutant lysozymes

Protein	pH 3.0			pH 6.7		
	ΔT_m , °C	ΔH , kcal/mol	$\Delta \Delta G$, kcal/mol	ΔT_m , °C	ΔH , kcal/mol	$\Delta \Delta G$, kcal/mol
Wild type	—	118	—	—	127	—
N40A	1.2	117	0.43	0.88	125	0.32
K43A	-2.95	101	-0.96	-3.08	108	-1.03
S44A	1.2	122	0.44	0.98	119	0.34
E45A	1.47	125	0.55	0.04	124	0.01
L46A	-8.39	89	-2.62	-6.4	88	-1.86
D47A	-0.81	114	-0.28	-2.75	113	-0.95
K48A	-0.93	109	-0.32	-1.68	110	-0.56
A40-49	-8.47	70	-2.11	-10.70	64	-2.54
A40-42/44-45/47-49	3.09	117	1.06	-1.56	117	-0.56
E45A/K48A	1.04	121	0.38	0.03	127	0.01

The thermal stabilities of the mutants were determined by using a JASCO J-600 spectrophotometer to monitor the circular dichroism (CD) signal of the protein at 223 nm as a function of temperature (12, 19). Protein concentrations ranged from 0.01 to 0.03 mg/ml in 25 mM KCl/3 mM H_3PO_4 /17 mM KH_2PO_4 at pH 3.0 and in 0.15 M KCl/0.01 M $K_3/2H_3/2PO_4$ at pH 6.7. All mutant proteins were prepared and measured in parallel with the reference pseudo-wild-type protein. Full reversibility of thermal unfolding and folding of the mutants and pseudo-wild-type T4 lysozyme was achieved only at pH 3.0. At pH 6.7 the reversibility decreased to 80-90% for the mutants as well as pseudo-wild-type lysozyme. ΔT_m is the difference between the melting temperatures of mutant and pseudo-wild-type lysozymes measured under identical experimental conditions. Average values of T_m for pseudo-wild-type lysozyme (C54T/C97A) were 51.65°C at pH 3.0 and 62.2°C at pH 6.7. ΔH is the enthalpy of unfolding and $\Delta \Delta G$ is the difference between the free energy of unfolding of the mutant and that of pseudo-wild type determined from van't Hoff analysis using a constant ΔC_p model of 2.5 kcal/(mol·deg) (23, 24). The standard deviation of the mean for the melting temperatures is 0.15°C, and for the enthalpy is 4 kcal/mol. The estimated error in $\Delta \Delta G$ is ± 0.25 kcal/mol, which is dominated by random error for small values of ΔT_m and by the uncertainty due to the choice of a constant ΔC_p model and with the value assumed for ΔC_p itself for large values of ΔT_m . Positive ΔT_m and positive $\Delta \Delta G$ values denote a mutant protein more stable than pseudo-wild type.

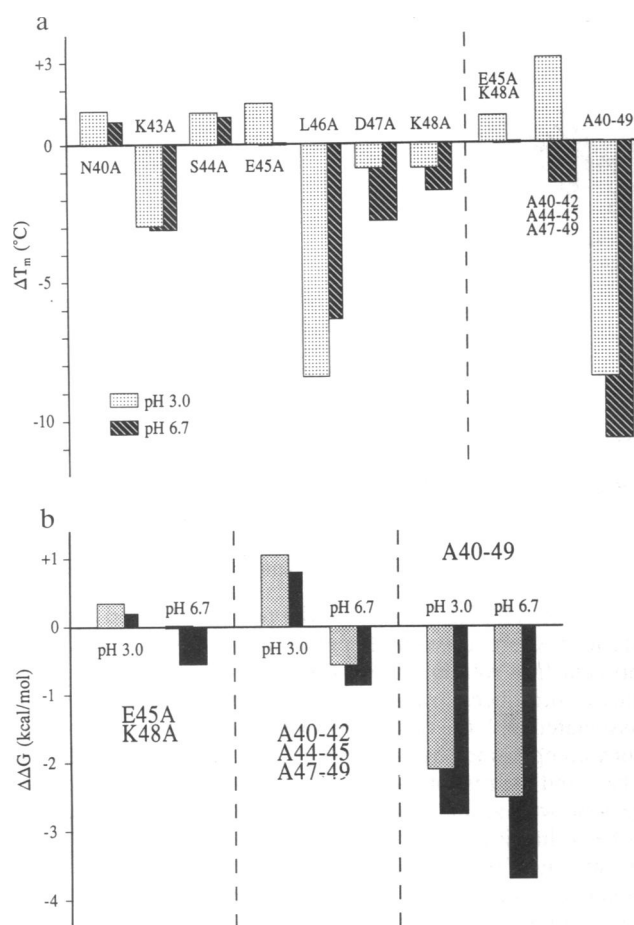


FIG. 1. (a) Differences in melting temperatures (ΔT_m) between pseudo-wild-type ($\Delta T_m = 0$) and mutant lysozymes at pH 3.0 (stippled bars) and pH 6.7 (striped bars). A positive value of ΔT_m indicates that the mutant is more stable than pseudo-wild type. (b) Differences in thermal stabilities between the polyaniline mutants E45A/K48A, A40-42/44-45/47-49, and A40-49 relative to pseudo-wild-type lysozyme at pH 3.0 and pH 6.7. The stippled bars show the change in free energy of unfolding ($\Delta\Delta G$) for the polyaniline mutants; the solid bars show the sum of the $\Delta\Delta G$ values of the constituent single mutations, as taken from Table 2 and *a*. Positive values of $\Delta\Delta G$ indicate variants more stable than pseudo-wild type.

Mutant K43A showed essentially no structural changes caused by the mutation other than the truncation of the lysine

side chain itself. The side chain of Lys-43 in pseudo-wild-type lysozyme contains partially buried atoms (C^β to C^δ) and solvent-exposed atoms (C^ϵ and N^ϵ). Atoms C^β to C^δ are in van der Waals contact with Leu-39, Asn-40, Asn-55, and Gly-56. Atom N^ϵ is in hydrogen-bonding distance of the side chain of Asn-55. Many of these interactions are lost in the mutant, leading to the formation of a small hydrophobic pocket on the surface of the protein. No new well-ordered solvent molecule could be detected within hydrogen-bonding distance of Asn-55.

Leu-46 in pseudo-wild-type lysozyme is completely buried and participates in a well-packed hydrophobic cluster that includes Ile-27, Leu-33, Ala-42, Thr-54, Gly-56, and Ile-58. The difference Fourier map for the mutant L46A (Fig. 2) has strong negative density indicating the deletion of atoms C^γ , C^δ , and C^ϵ of Leu-46. There are also additional features indicating that surrounding atoms tend to move somewhat toward the space vacated by the deletion of most of the leucine side chain. In the refined structure the largest main-chain movements were found for residues 27-30, 44-46, and 54-58 (up to 0.35 Å). The largest individual atoms shift occurred for atom C^δ of Ile-27 (0.55 Å). Notwithstanding these structural adjustments, the mutation caused the creation of an interior hydrophobic cavity. The size and interior surface area of the cavity were calculated by the method of Connolly (26), with a rolling water molecule of radius 1.2 Å as a probe. If one takes the coordinates of pseudo-wild-type lysozyme and truncates Leu-46 to an alanine, the calculated volume of the resultant cavity is 47.8 Å³. The actual cavity volume observed in the L46A mutant structure is 24.3 Å³. This means that in this instance the adjustments of the protein structure reduce the volume of the cavity by about 45% relative to the value if there were no relaxation of the structure whatsoever. No ordered solvent molecule that might occupy this cavity could be detected either in the final $2F_o - F_c$ map or the final $F_o - F_c$ map.

The superposition of mutant D47A and pseudo-wild-type lysozyme reveals movement of Thr-54 away from the mutation site D47A (0.4 Å) and the movement of solvent (no. 176) closer to the mutation site, now forming a hydrogen bond with O^{71} of Thr-54. The amide of Thr-54, however, has lost its hydrogen-bonding partner.

The initial difference Fourier map for the polyaniline mutant A40-49 clearly shows negative density features due to the truncation of the seven side chains (N40A, K43A, S44A, E45A, L46A, D47A, and K48A; Fig. 3a). In addition, paired positive and negative density peaks are found throughout the entire structure due to some rigid-body movements

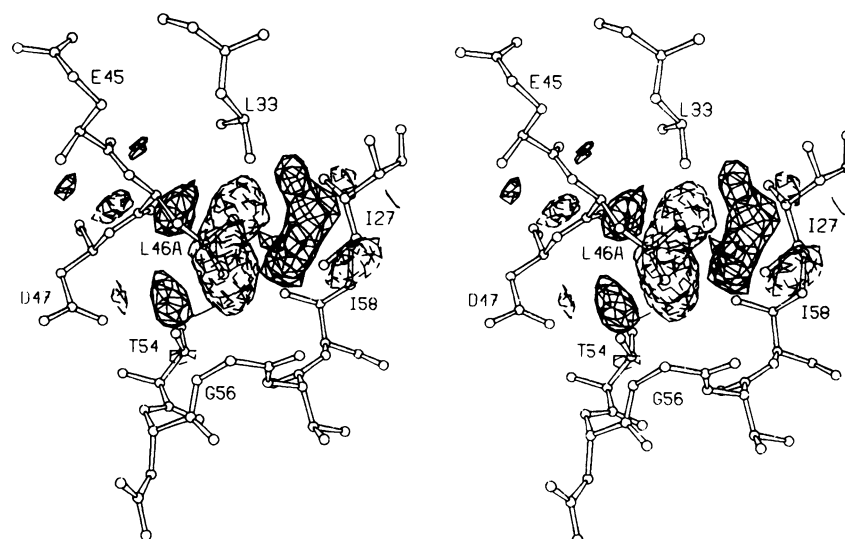


FIG. 2. Stereodrawing showing the structure of T4 lysozyme in the vicinity of Leu-46. Superimposed is the difference in electron density between mutant L46A and pseudo-wild-type lysozyme. Coefficients are $F_{mut} - F_{wt}$, where structure amplitudes for both mutant (mut) and pseudo-wild-type (wt) lysozyme were observed quantities. Phases were calculated from the refined crystal structure of pseudo-wild-type lysozyme (22, 25). Positive density is shown by continuous lines, and negative density by broken lines. The contour level is $\pm 4\sigma$, where σ is the root-mean-square density through the unit cell. The resolution is 1.9 Å.

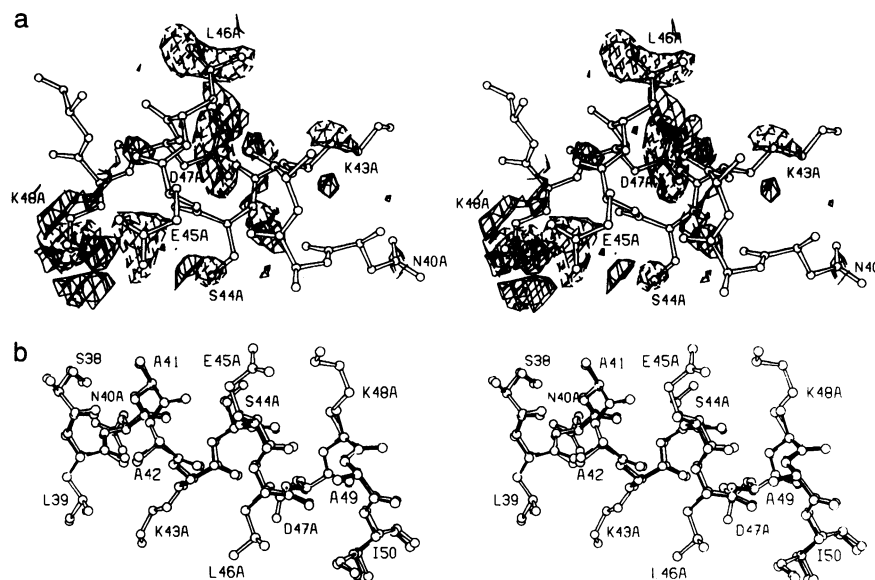


FIG. 3. (a) Stereodrawing showing the structure of residues 40–49 of the α -helix comprising residues 39–50 in T4 lysozyme. Superimposed is the difference in electron density, resolution 2.0 Å, between the polyalanine mutant A40–49 and pseudo-wild-type lysozyme. Other conventions are as for Fig. 2. (b) Superposition of the α -helix 39–50 of the refined structure of mutant A40–49 (filled bonds) and pseudo-wild-type lysozyme (open bonds). The root-mean-square deviation for main-chain atoms is 0.2 Å.

(or change in unit cell dimension). The superposition of the polyalanine and the pseudo-wild-type helix reveals very little change in geometry of the helix associated with the mutations (root-mean-square deviation of main-chain atoms of residues 40–49 is 0.25 Å; Fig. 3b). Essentially all structural changes found for the single mutants described above were present in the polyalanine mutant.

DISCUSSION

One of the most striking results of the present study is the freedom with which alanines can be substituted in α -helix 39–50 of T4 lysozyme with relatively little effect on stability, folding, and function. In three cases (N40A, S44A, and E45A) alanine substitution results in a slight increase in stability; in two cases (D47A and K48A) there is a slight decrease in stability; and in two other cases (K43A and L46A) the destabilization is more pronounced. Even in the most severe case (L46A), the stability of the mutant protein is comparable with the stability of temperature-sensitive mutants of T4 lysozyme isolated by random genetic screens (e.g., ref. 27). In the extreme case in which there are 10 consecutive alanines (A40–49), the mutant protein still folded correctly and could be crystallized. The results clearly demonstrate the redundancy that is present in the amino acid sequence of a protein (7, 9, 10, 12, 28, 29). The present results are consistent with the notion that a relatively small fraction (less than half) of the amino acids in the protein are critical for folding. Also, in agreement with Zhang *et al.* (12), and others (19, 30–32), alanine is shown to be a helix-stabilizing residue.

For the mutants N40A and S44A the thermal stability of the protein increased by 0.3–0.4 kcal/mol at both pH 3.0 and pH 6.7. In both cases the side chains are completely solvent-exposed, are noncharged, and do not interact with the rest of the molecule. The crystal structure of mutant S44A reveals no structural changes except the deletion of the hydroxyl of Ser-44. At low pH, mutant E45A shows the greatest increase in thermostability of the single Xaa \rightarrow Ala mutants. This is consistent with the higher helical propensity of alanine compared with glutamate, as found for model peptides (31, 32) and in the replacement E128A (12).

What is the physical basis for alanine having a higher helical propensity than any of the other amino acids? In the case of the β -branched amino acids it is likely that steric interference between the side chain and atoms within the preceding turn of the α -helix contributes to reduced helix

propensity (19). In the case of the non- β -branched residues, steric interference with the preceding turn of the α -helix will prevent free rotation about the C $^{\alpha}$ –C $^{\beta}$ bond but not necessarily introduce strain (33). Apparently the entropy cost associated with the restriction of the side-chain motion is not fully compensated by favorable interactions between the side chain and the rest of the α -helix (12, 34). For this reason any residue with atoms beyond the β -carbon is less stable in an α -helix than is alanine (12). In the case of glycine the increase in entropy of the unfolded state is expected to destabilize the α -helical conformation of this residue relative to any residue that contains a β -carbon, including alanine (34–36).

It might be expected that at pH 6.7 there would be a reduction in stability in E45A due to disruption of the putative salt bridge between E45 and K48. The present results can best be rationalized by assuming that in solution Lys-48 interacts preferentially with the α -helix dipole rather than with Glu-45, consistent with the respective entropy cost of localizing the interacting partners (37). The loss in stability observed for K48A at both pH 3.0 and pH 6.7 can then be attributed to the loss of the helix–dipole interaction, which will occur at both pH values. At pH 3.0 the double mutant E45A/K48A has stability similar to that obtained by summing the two single mutants E45A and K48A. This indicates that at this pH value there is no interaction between residues Glu-45 and Lys-48 (or the interaction between these residues is the same as that between Ala-45 and Ala-48). At pH 6.7, however, the double mutant E45A/K48A is slightly more stable (0.55 kcal/mol) than the sum of the single mutants. This is perhaps the opposite to what might have been expected; i.e., the removal of the putative Glu-45...Lys-48 salt bridge decreases protein stability. It provides further evidence that such surface salt bridges contribute little to stability (30, 37).

The largest decreases in stability were observed for the single mutants K43A and L46A and, consequently, for the polyalanine mutant A40–49. The truncation of both side chains, which are located on the buried side of the helix, leads to the loss of hydrophobic interactions for mutant K43A and the formation of an internal hydrophobic cavity for mutant L46A. To some extent the movements of residues towards the center of the Leu-46 cavity compensate for the loss in stabilization (38). The situation for mutant K43A is similar. Most of the loss of stability appears to be due to the loss of the hydrophobic contribution of two partially buried side-chain methylene groups.

The polyalanine mutant A40–49, which includes 10 consecutive alanines, is relatively thermolabile because it includes the two destabilizing replacements K43A and L46A. The overall stability of A40–49 is slightly greater than the sum of the stabilities of the single-mutant constituents at pH 3. In general terms, the results of Zhang *et al.* (12), together with those presented here, suggest that the changes in stability associated with successive alanine substitutions within an α -helix are independent, unless cooperative effects are mediated through contacts of the helix with the remainder of the protein structure. In the cases examined to date such cooperative effects are relatively small.

Within α -helix 40–49, Leu-46 and, to a lesser degree, Lys-43 are important for folding in that they clearly contribute to the overall stability of the protein. For the fully buried residue Leu-46 this result is not surprising, but the importance of the partially buried residue Lys-43 might not have been anticipated. In fact, Lys-43 is seen to contribute hydrophobic interactions to the folded protein via the hydrocarbon part of its side chain. Asn-40, Ser-44, Glu-45, Asp-47, and Lys-48 are unimportant in the sense that they can be replaced with alanine either individually or in concert and have relatively little effect on folding and stability. The roles of the residues that are already alanine in pseudo-wild-type lysozyme, Ala-41, Ala-42, and Ala-49, are not addressed in the present study.

The finding that solvent-exposed residues on the surface of α -helix 40–49 can be exchanged with alanine with little effect on stability is consistent with other studies showing a correlation between solvent accessibility and allowed amino acid substitutions (1, 9, 12, 28, 39). It is also consistent with the ideas that protein structures are tolerant of change (40) and that it is the rigid (or buried) parts of proteins that contribute most to stability (14).

The question whether the successive incorporation of more and more alanines in a protein will change its solubility or interfere with the partitioning between the polar and nonpolar amino acids during folding (28) remains unanswered. In the present case up to seven alanine substitutions have been made, with six of these involving the loss of a polar residue. Five alanines have also been substituted in α -helix 126–134 of T4 lysozyme (12, 41), and the mutant with the two polyalanine helices included in the same protein has also been constructed (X.-j. Zhang, unpublished results). In none of these cases is there an obvious change in protein solubility or aggregation.

In summary, it does seem possible to stabilize a protein by multiple Xaa \rightarrow Ala substitutions of solvent-exposed amino acids within α -helices. The use of multiple alanine substitutions also may provide a method of simplifying the protein folding problem by focusing on amino acids essential for folding and stability.

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