Protein Science (1997), 6:2418–2425. Cambridge University Press. Printed in the USA.

Alteration of T4 lysozyme structure by second-site reversion of deleterious mutations

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(RECEIVED May 13, 1997; ACCEPTED July 23, 1997)

Abstract

Mutations that suppress the defects introduced into T4 lysozyme by single amino acid substitutions were isolated and characterized. Among 53 primary sites surveyed, 8 yielded second-site revertants; a total of 18 different mutants were obtained. Most of the restorative mutations exerted global effects, generally increasing lysozyme function in a number of primary mutant contexts. Six of them were more specific, suppressing only certain specific deleterious primary substitutions, or diminishing the function of lysozymes bearing otherwise nondeleterious primary substitutions. Some variants of proteins bearing primary substitutions at the positions of Asp 20 and Ala 98 are inferred to have significantly altered structures.

Keywords: bacteriophage; mutations; second-site revertants; T4 lysozyme

The lysozyme encoded by bacteriophage T4 is a small, globular, monomeric protein that has been the subject of extensive structural and genetic studies (for reviews, see Poteete & Hardy, 1994; Matthews, 1995). The native structure of the protein has been determined to 1.7 Å resolution by X-ray crystallography (Weaver & Matthews, 1987).

Systematic probing of the T4 lysozyme structure has shown that 74 of the 164 positions in its sequence are sensitive to single amino acid substitutions; that is, at least one single amino acid substitution at one of these positions results in at least a 50-fold reduction in function (Rennell et al., 1991). Not surprisingly, the most critical amino acid residues in the protein were found to be either buried, or else solvent-exposed, but in the active site cleft.

A second level of genetic probing of protein structure consists of isolating and characterizing intragenic second-site revertants of mutant proteins. A survey of second-site suppressors could be expected to reveal interactions between residues in the folded (or possibly, folding) structure. Some of the resulting multiply mutant proteins might be expected to have significantly altered structures, the solution of which could lead to insights on the protein folding problem.

An early study of second-site revertants of temperature-sensitive mutants of staphylococcal nuclease (Shortle & Lin, 1985) resulted mainly in the isolation of "global stabilizers." These were secondary mutations that, in themselves, increased the thermal stability of the protein. Other studies, involving the lysozymes of bacteriophages P22 (Rennell & Poteete, 1989) and T4 (Poteete et al., 1991; Bouvier & Poteete, 1996) uncovered structural variants in addition to global stabilizers.

Here we report the results of a systematic survey of second-site revertants in the T4 lysozyme gene. Presumptively significant structural variants are described.

Results

In a previous study (Rennell et al., 1991), we examined the effects of single amino acid substitutions in the T4 lysozyme gene by the use of amber suppressors. A bacteriophage P22 hybrid bearing the T4 lysozyme gene in place of its P22 counterpart was constructed, amber mutations were introduced into its lysozyme gene, and the mutant phages were tested for ability to form plaques on a collection of 13 *Salmonella* amber suppressor strains that each insert a different amino acid in response to the amber codon. Of 163 amber mutant phages tested in this way, 74 failed to form plaques, or formed tiny plaques at low efficiency, on at least one amber suppressor strain.

The method employed for finding second-site mutants was to select plaque-forming revertants of an amber mutant phage on a mis-suppressing host. Second-site revertants were distinguished from primary-site revertants by their host range: second-site revertants, unlike primary-site revertants, retain the original amber codon, and hence cannot form plaques on the nonsuppressing host. Further testing, backcrossing, and sequencing of candidates were as described in Materials and methods.

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Results of screening the collection of amber mutant phages for second-site revertants are summarized in Figure 1. Of the 74 phages tested, 55 were sufficiently defective in plating to permit the isolation of spontaneous revertants on at least one suppressor strain. In a few cases involving marginally tight mutants (which do not form plaques, but damage the bacterial lawn when plated in large numbers), 10–20 revertants were screened; more typically, numbers were 50–500. Phages with amber mutations in codons 20 and 26 yielded second-site revertants in this way. [Revertants of the Thr 26am mutant were described previously (Poteete et al., 1991).]

Intensive revertant screening was performed with 14 amber mutants. The mutants were chosen on the basis of phenotypic tightness, and on the likelihood, based on structural criteria such as solvent inaccessibility and low thermal factors, that an amino acid substitution at the corresponding site would lead to a significant structural disruption. In these cases, 2,000–60,000 UV radiation-induced revertants were screened by the use of a double-indicator plating technique, as described in Materials and methods. In this way, second-site revertants of mutants affecting codons 6, 58, 98, 99, 129, and 156 were obtained (see Fig. 1). In all, 18 different amber-secondary site combinations were found, including the two described previously (Poteete et al., 1991). Their sequence alterations are indicated in Table 1.

The suppression patterns of amber mutant phages bearing second-site suppressor mutations are indicated in Table 1. Most of the second-site mutations enhance the amber mutants' plaque-forming ability on more than one suppressor. In six cases, while enhancing plaque formation on some hosts, the secondary mutations actually diminished plaque formation on other suppressor hosts. The implications of this behavior are discussed below.

Missense mutants corresponding to seven of the amber/second-site revertant pairs indicated in Table 1 were constructed as described in Materials and methods. As shown in Table 2, the plaque-formation phenotypes of these phages on the nonamber-suppressing host were generally consistent with those of the corresponding amber mutant phages on the relevant amber suppressor strain. In one case, the phage bearing the double missense allele Met 6-Ile/Ala 98-Pro formed tiny plaques at low efficiency (indicated as ± in the table), whereas the amber mutant phage bearing the allele am98/Met 6-Ile formed no plaques on the proline-inserting amber suppressor host. This small difference may be due to differences in level of synthesis of the mutant proteins. The synthetic amber suppressors employed in this study are less than 100% efficient. The proline suppressor, for example, was found to give 9–60% suppression of amber alleles in *lac*Z (Kleina et al., 1990).

The missense mutant lysozyme genes were introduced into the plasmid pDR739 for high-level expression and purification. The Ala 98-Gln mutant protein was too unstable for purification, being rapidly degraded in cell extracts. All others were purified to homogeneity in yields of 1–10 mg/L of culture (not shown).

Discussion

Types of intragenic second-site suppressors

The 18 amber-secondary combinations shown in Table 1 contain 15 different substitutions that increase the function of defective lysozyme variants. Of these 15, 10 are either remote from the sites of the primary mutations they suppress, or else have been found to suppress mutations at more than one primary site. The genetic data presented here, in combination with the wild-type lysozyme structure (Weaver & Matthews, 1987), suggest how some of these "global suppressors" exert their restorative effects.

Six of the second-site substitutions—Asn 68-Lys, Asn 68-Tyr, Asp 89-Glu, Asn 140-Lys, Thr 151-Ala, and Thr 151-Ser—are probably global stabilizers of the type identified by Shortle and Lin

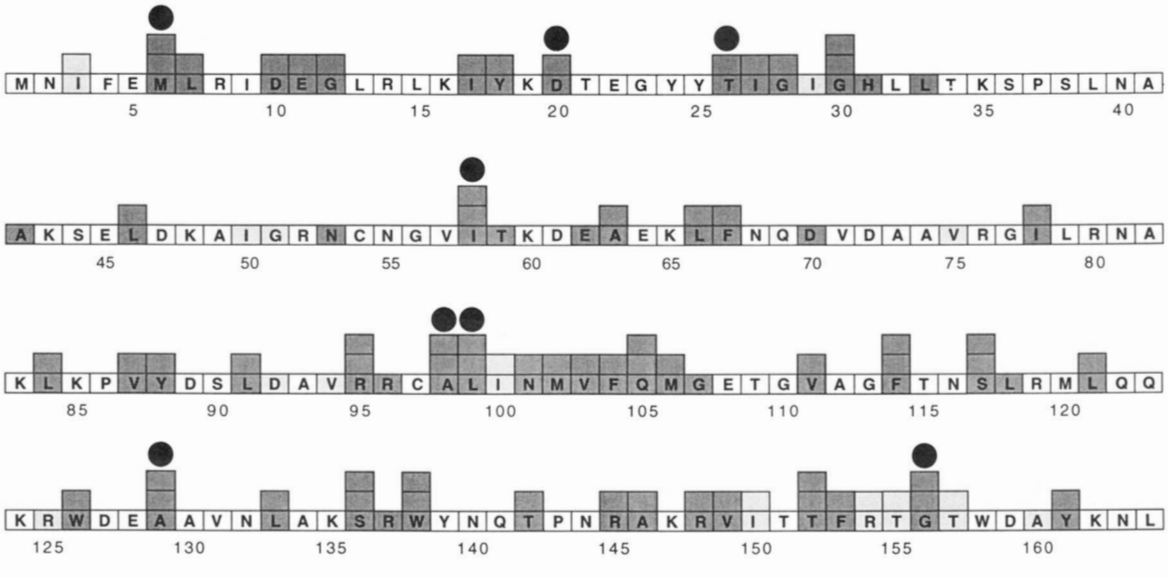

Fig. 1. Survey of substitution-sensitive sites in T4 lysozyme for isolation of second-site revertants. The primary structure of the protein is indicated, with positions sensitive to substitutions shaded gray (positions sensitive to two or more substitutions are darker gray). Boxes over the residues indicate positions at which at least one substitution led to a sufficient loss of lysozyme function to permit effective selection of revertants. Positions marked with two boxes on top are those for which intensive mutagenesis and screening for second-site revertants was performed. Black dots indicate positions of primary mutations for which second-site revertants were obtained. The second-site revertants of substitutions at position 26 have been described (Poteete et al., 1991). Revertants of substitutions at position 161 (not shown) were obtained by different methods (Bouvier & Poteete, 1996).

Table 1. *Suppression patterns of revertant phages*[a]

	Gln	Tyr	Leu	Ser	Phe	Cys	Arg	Gly	Ala	His	Pro	Glu	Lys
M6am	++	±	++	++	±	+	−	++	++	+	+	±	±
M6amK16E	++	++	++	++	+	+	−	++	++	++	++	±	+
D20am	−	±	−	−	−	++	−	±	+	−	−	+	−
D20amG23D	+	+	+	±	±	++	+	±	+	−	±	+	±
T26am	−	+	−	++	±	+	−	+	++	±	−	−	−
T26amY18D	++	++	++	++	+	+	−	+	+	±	−	−	−
T26amY18H	++	++	++	++	+	±	−	±	+	−	−	−	−
I58am	±	±	±	+	++	+	−	±	++	±	+	±	−
I58amK16E	++	+	+	++	++	+	−	+	++	+	++	±	−
I58amN68K	++	+	++	++	++	++	−	+	++	±	±	±	−
I58amD89E	++	+	+	++	++	+	−	+	++	+	++	±	−
I58amN140K	++	+	+	++	++	+	−	+	++	+	++	±	−
I58amT151S	++	+	+	++	++	+	−	+	++	+	++	±	−
I58amT151A/E108V	++	++	++	++	++	+	−	+	++	+	++	±	−
A98am	±	−	±	++	−	++	−	++	++	−	++	−	−
A98amM6I	++	−	++	++	−	++	−	++	++	−	−	−	−
A98amK16E	++	−	++	+	−	+	−	++	++	−	++	−	−
A98amT152S	−	−	−	++	±	++	−	++	++	−	−	−	−
A98amF67L	++	−	+	++	−	+	−	++	++	−	++	−	−
L99am	++	++	++	++	+	+	−	±	++	++	±	±	+
L99amE108V	++	++	++	++	+	+	−	++	++	++	±	+	+
L99amN68Y	++	++	++	++	+	+	−	+	++	++	±	+	+
A129am	+	±	+	++	++	++	−	++	++	−	±	±	−
A129amT151A	++	+	++	++	++	++	−	++	++	±	+	+	−
G156am	+	−	+	++	−	+	±	++	++	−	−	±	±
G156amA93E	++	−	++	++	±	++	+	++	++	+	+	++	++

[a]Plaque-forming ability of P22 e416 sieA44 m44 bearing the indicated alleles of the T4 lysozyme gene, on an amber suppressor host that inserts the indicated amino acid in response to the amber codon. ++ designates large plaques; +, small plaques; ±, tiny plaques at low efficiency of plating; −, no plaques.

(1985). The Asn, Asp, and Thr residues affected are all solvent-exposed and alpha-helical in conformation. Asn, Asp, and Thr residues tend to destabilize alpha-helices because each has a side-chain group capable of forming a hydrogen bond to the peptide backbone, interfering with the hydrogen bonding pattern of the helix. Thr is additionally unfavorable for folding into an alpha-helix because of its beta-branched structure, which constrains the residue to essentially one conformation in an alpha-helix, at significant entropic cost. The five substitutions characterized here might be expected to increase the stability of the folded state generally, and, by so doing, partially compensate for many other mutations that decrease the stability of the folded state. Indeed, most of these mutations exhibit nonspecificity with regard to which primary substitutions they suppress. As shown in Table 1, the substitution Thr 151-Ala, for example, improves the function of lysozymes bearing six different substitutions at position 129, and impairs the function of none. The sole exception is Asn 68-Lys, which improves the function of lysozymes bearing Gln, Tyr, Leu, Ser, Cys, or Gly at position 58, but further damages a lysozyme-bearing Pro at the same position. A straightforward interpretation of this deleterious effect in terms of the folded structure is not obvious to us.

Two global suppressors—Lys 16-Glu and Ala 93-Glu—may exert their effects by altering the surface charge of the protein. T4 lysozyme has a strong net positive charge; diminishing it may facilitate its diffusion among the negatively charged surfaces of bacteria in the plaque (Dao-pin et al., 1991). Increased mobility may allow a marginally stable lysozyme to hydrolyze more peptidoglycan during its short lifetime, and thus to be scored as func-

Table 2. *Plaque-forming phenotypes of missense mutant phages*[a]

Mutant	Plaque formation on nonsuppressor host[b]	Predicted[c]
A93E	++	++
G156K	±	±
A93E/G156K	++	++
A98P	++	++
A98Q	±	±
M6I/A98P	±	−
M6I/A98Q	++	++

[a]Phages are P22 e416 sieA44 m44 bearing T4 lysozyme gene alleles encoding the indicated proteins.

[b]Plaque-forming ability scored as in Table 1.

[c]Plaque formation by an amber mutation-bearing variant of P22 e416 sieA44 m44 on an amber suppressor host in which the lysozyme protein should have the same primary structure as the missense mutant (data from Table 1).

tional. We have previously described a compensatory second-site mutation of this type, Lys 43-Ile (Bouvier & Poteete, 1996).

The two remaining remote suppressor mutations—Glu 108-Val and Phe 67-Leu—do not seem to fit into either the global stabilizer or reduced surface charge categories. Glu 108 is a surface residue located far from the buried residues Ile 58 and Leu 99, yet its replacement by Val apparently stabilizes lysozymes bearing substitutions at these positions. (In the case of Ile 58 substitutions, suppression is achieved by the combination of Glu 108-Val with Thr 151-Ala. We have not tested each mutation separately, but either independently suppresses other primary mutations, as shown in Table 1.) Phe 67 is alpha-helical and buried in the hydrophobic core of the protein. Its replacement by Leu apparently stabilizes lysozymes bearing substitutions of Gln or Leu at position 98, but destabilizes lysozyme bearing a Cys substitution at this position.

Five secondary substitutions are in close proximity to the altered residues in their corresponding primaries. In each case, there is direct or indirect evidence that they effect, in combination with the primaries, significant structural alterations of the lysozyme molecule. They are described below in a systematic discussion of each primary substitution.

Suppressible deleterious primary substitutions

Met 6 is in the middle of an alpha-helix. Its side chain is buried in the hydrophobic core of the protein. Substitutions of aromatic and charged residues at this position result in a loss of function, presumably due to structural destabilization. His and Pro substitutions lead to milder defects. The damage done by most of these substitutions can be reversed by the surface charge-reducing substitution Lys 16-Glu.

Matthews and coworkers have shown that some substitutions at position 6 result in a temperature-sensitive phenotype, and the encoded mutant polypeptides have lower melting temperatures than wild type (Alber et al., 1987). In general, substitutions resulting in such mild structural defects do not result in a loss of function as tested in the hybrid P22 system (Rennell et al., 1991).

Asp 20 abuts the solvent-filled active site cleft. The Asp 20 side-chain carboxylate, along with that of Glu 11, were originally proposed to be essential for conducting the catalytic chemistry of T4 lysozyme (Anderson at al., 1981; Matthews et al., 1981). The tolerance of the enzyme's function to several nonconservative substitutions (Asp 20-Cys and Asp 20-Ala), as well as the conservative substitution Asp 20-Glu (Anand et al., 1988; Hardy & Poteete, 1991; Rennell et al., 1991), indicated a less critical role for the Asp 20 side-chain carboxylate. A recent structure of a quasi-stable covalent adduct between a peptidoglycan substrate and several T4 lysozyme variants has led to a revision of the role for the Asp 20 carboxylate (Kuroki et al., 1993, 1995). This residue seems to optimize the enzyme's binding of the transition state, rather than playing a pivotal catalytic role.

The ability of lysozyme to tolerate substitutions at position 20 is greatly increased by the neighboring substitution Gly 23-Asp. Lysozyme bearing the Gly 23-Asp substitution, unlike wild type, retains function with Gln, Tyr, Leu, and even Arg residues at position 20. Complete flexibility is not attained, however: Ser, Phe, Gly, His, Pro, and Lys are not tolerated at position 20 in the Gly 23-Asp mutant background (Table 1).

Probably the most obvious explanation for the behavior of the Gly 23-Asp mutant is that the catalytic role of Asp 20 can be filled by an aspartate at position 23, which is nearby in the wild-type structure. This explanation is too simple, though. The spatial arrangement of Glu 11, Asp 20, and Gly 23 in the wild-type structure is shown in Figure 2. Without substantial structural rearrangement, it would not be possible to bring the side chain of the mutant protein's Asp 23 residue into the position occupied by Asp 20 in the wild type. There is an additional reason for thinking that functional mutant proteins bearing double substitutions at positions 20 and 23 might be structural variants: these two residues are in close contact as residues i and $i + 3$ in a type I beta turn (Rose et al., 1985). Such turns are stabilized by having a Gly at position $i + 3$. Moreover, Asp 20's side-chain carboxylate makes an extra, potentially stabilizing hydrogen bond with the amide nitrogen of residue 23. Both of these turn-stabilizing factors would be lost in the double mutant.

Second-site suppression of primary substitutions at position 26 (Thr in the wild type) has been described (Poteete et al., 1991). Substitution of Gln for the Thr at position 26 results in a protein that is stable, but enzymatically inactive because of structural occlusion of the active site cleft. The Tyr 18-Asp and Tyr 18-His substitutions, in combination with Thr 26-Gln, were shown to restructure the active site cleft, restoring partial enzymatic activity. The double mutants, though more functional, are less stable than the primary mutant. New data presented in Table 1, the suppression patterns of the amber-secondary combinations on the Phe, Cys, Arg, Gly, Ala, His, Pro, Glu, and Lys suppressors, are consistent with the concept that Tyr 18-Asp and Tyr 18-His are themselves destabilizing mutations. Lysozyme bearing the Tyr 18-Asp substitution is less tolerant than wild type of a substitution of Ala at position 26. The Tyr 18-His substitution has an even greater effect, sensitizing lysozyme to substitutions of Cys, Gly, Ala, and His at position 26.

The side chain of Ile 58 is buried in the hydrophobic core of the amino-terminal domain of lysozyme. Its atoms occupy a space that is bounded by atoms from 12 other residues. This position is sensitive to a large variety of substitutions, which presumably destabilize the molecule. Substitutions at six different positions, all remote in the wild-type folded structure, make the protein less

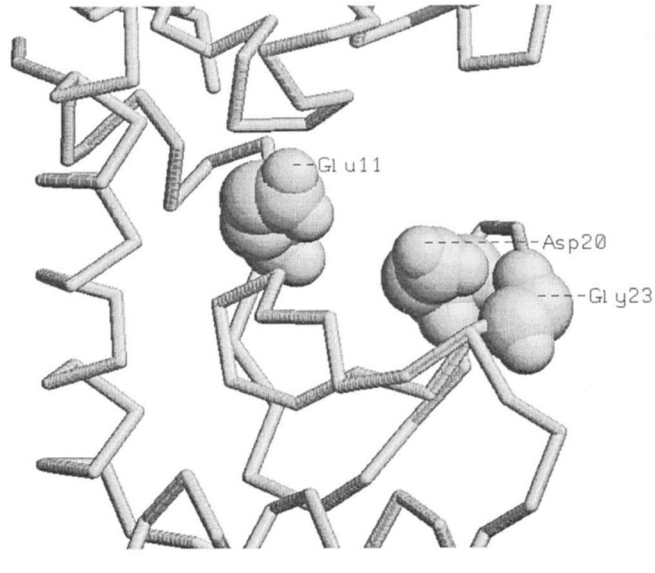

Fig. 2. Catalytic site of T4 lysozyme. Glu 11 and Asp 20 have been implicated in catalysis (see text). Replacing Gly 23 with Asp increases the range of residues that are tolerated at position 20.

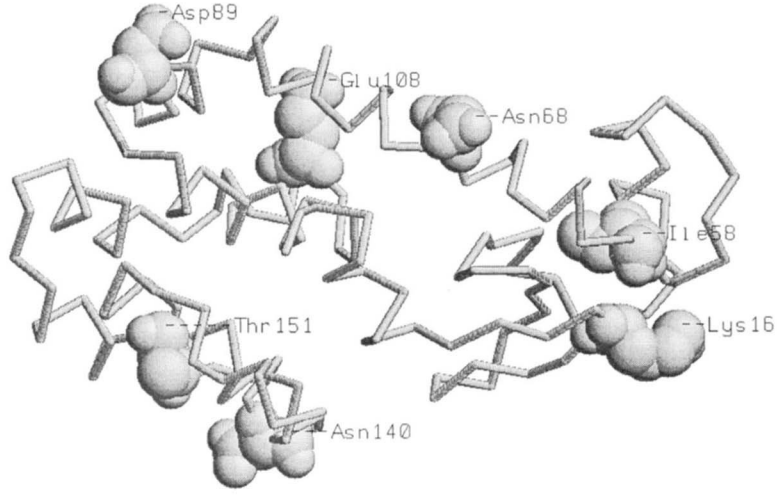

Fig. 3. Remote suppressors of Ile 58 mutations. Substitutions at positions 16, 68, 89, 108, 140, and 151 improve the function of lysozyme variants bearing alterations at position 58.

sensitive to substitutions at position 58 (Fig. 3). None of these suppressing substitutions makes the protein able to tolerate the charged residues Arg, Glu, or Lys at position 58, but all allow it to accept Gln, Tyr, Leu, and Gly. All but Asn 68-Lys improve the function of lysozyme variants bearing His or Pro at position 58. The Asn 68-Lys substitution is exceptional in that it actually makes the protein less tolerant of Pro at position 58, as mentioned above.

The beta carbon of Ala 98 is located centrally in the C-terminal domain of lysozyme, near the point of closest approach between two alpha-helices, including the one in which it is situated (see Fig. 4). No large residue can be put in the place of Ala 98 without a steric clash that could be accommodated only by movement of the polypeptide backbone. Indeed, as shown in Table 1, only the smallest residues (Gly, Ala, Ser, Cys, and Pro) are acceptable at position 98; moreover, the variant containing the substitution Ala 98-Gln was too unstable for purification. Four second-site mutations were found to suppress the functional defects of lysozyme variants with substitutions at position 98. Two, Lys 16-Glu and Phe 67-Leu, are remote (discussed above). The two others, Met 6-Ile and Thr 152-Ser, involve residues in close proximity to Ala 98 in the wild-type structure. Neither of these suppressor mutations seems to exert its effect by simply making the protein more generally tolerant of substitutions at position 98. As shown in Table 1, whereas wild-type lysozyme can tolerate Pro at position 98, the Met 6-Ile and Thr 152-Pro variants cannot.

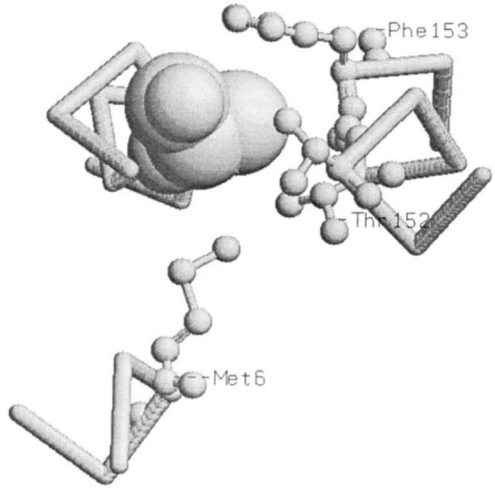

Fig. 4. Environment of Ala 98 in the wild-type T4 lysozyme structure. Ala 98 is shown in space-filling mode. Its beta carbon is located near the point of closest approach of two alpha-helices. Residues with side-chain atoms within 5 Å are shown in ball-and-stick mode. Substitutions at position 6 and, to a lesser extent, at 152, alter the spectrum of acceptable substitutions at position 98.

Much is known about the structures and properties of the Met 6-Ile lysozyme variant, and of variants at position 98, from the studies of Matthews and coworkers. The Met 6-Ile lysozyme is particularly interesting. This substitution itself is not stabilizing; rather, it has a temperature-sensitive phenotype. The wild-type lysozyme structure is minimally perturbed by the Met 6-Ile substitution, but the mutant protein has an unexplained tendency to crystallize in a form ($P2_12_12_1$) different from that of wild type and many other mutant variants of T4 lysozyme ($P3_221$). In the $P2_12_12_1$ form, the asymmetric unit is composed of four monomers, each in a slightly different conformation; solution of the crystal structure provided a sort of stop-motion series illustrating hinge movement of the N- and C-terminal domains relative to each other. In none of these conformations, however, is there room to accommodate a large residue at position 98 without movement of the polypeptide backbone (Faber & Matthews, 1990). The crystal structures, and thermal stability properties, of a number of mutant lysozymes bearing the substitution Ala 98-Val have been described (Daopin et al., 1991). This substitution reduces the thermal stability of the molecule, and causes a local structural distortion of the protein backbone, pushing the two closely approaching alpha-helices apart by as much as 0.7 Å. It is likely that the larger, hydrophilic Gln side chain causes still more severe structural consequences in this position; the instability of the Ala 98-Gln mutant lysozyme (above) is consistent with this view.

Leu 99 and Ala 129 are both alpha-helical residues with side chains buried in the hydrophobic core of the C-terminal domain. Both positions are sensitive to a number of substitutions. Both positions are made nonspecifically more tolerant of substitutions by remote suppressor mutations, described above. Eriksson et al. (1993) found that substitutions of Phe, Met, Ile, Val, or Ala for Leu 99 were destabilizing. In other studies, substitutions of Val, Leu, and Met for Ala 129 were shown to be destabilizing (Karpusas et al., 1989; Baldwin et al., 1996).

Gly 156 is part of a surface turn. Its conformation (phi = 76°, psi = 18°) is energetically unfavorable for any residue other than glycine. Position 156 is sensitive to a number of substitutions, but it accepts substitutions of small residues (Ala and Ser) particularly well (Table 1). Larger residues in this position create the additional problem of unfavorable contacts with Arg 95, which is partially buried and itself located in a position sensitive to substitutions. The substitution Ala 93-Glu, a surface charge-reducing change discussed above, nonspecifically increases the tolerance of lysozyme for substitutions at position 156. Gray and Matthews (1987) described the crystal structure of T4 lysozyme bearing the randomly generated temperature-sensitive Gly 156-Asp substitution. They found that the backbone conformation of the Asp 156 residue was close to that of the original Gly residue in the wild type, and hence under strain; the loop containing the mutant residue is forced outward, away from unfavorable contacts with Arg 95 and Asp 92.

Other stabilizing mutants of T4 lysozyme

A number of studies by Matthews and coworkers involved the generation, by random mutagenesis or by design, of T4 lysozyme variants bearing amino acid substitutions that stabilize the molecule. In the course of this study, we re-isolated two of these substitutions, Thr 152-Ser and Thr 151-Ser.

The Thr 152-Ser substitution was designed to compensate for the destabilizing Ala 98-Val substitution, by creating a cavity to make room for the larger side chain of Val (Daopin et al., 1991). It was found that the Thr 152-Ser substitution, itself a destabilizing mutation, partially stabilized the Ala 98-Val mutant lysozyme. This small effect is consistent with our finding that the Thr 152-Ser substitution exerts a suppressing effect on a Phe substitution at position 98. Structurally, the Thr 152-Ser substitution allowed a slight relaxation of the strained Ala 98-Val mutant structure, permitting Val 98 to move 0.3 Å closer to atoms at position 152 than it could when position 152 was occupied by Ser. However, the major structural effect of the Ala 98-Val substitution, the forcing apart of two alpha-helices in the C-terminal domain, was unaffected by the Thr 152-Ser substitution, and the measured thermal stabilization conferred was small in magnitude, consistent with our finding that the Thr 152-Ser substitution is the weakest suppressor of the four we found by random mutagenesis and selection.

By the use of a screen for randomly generated stabilizing mutations in T4 lysozyme, 11 variants with increased thermal stability were isolated and characterized (Pjura et al., 1993; Pjura & Matthews, 1993). One of them was Thr 151-Ser, which we also found, as a suppressor of presumptively destabilizing substitutions at position 58. The Thr 151-Ser substitution increased the melting temperature of lysozyme at near-neutral pH by 1.0°, under conditions in which denaturation was reversible. The structural effect of the mutation was to place the Ser 151 hydroxyl at the position occupied by the gamma-methyl group of Thr 151 in the wild-type molecule, creating a favorable hydrogen bonding network with Thr 155 and a bound water molecule.

In a review, Matthews (1995) summarizes the structural and thermodynamic characterization of more than 200 mutant T4 lysozymes; 61 of these mutants have thermal stabilities greater than wild-type ($\Delta T_m > 0.1°$). Several of these mutant proteins have properties particularly relevant to this study. The substitutions Ala 129-Leu and Ala 129-Met both destabilize T4 lysozyme, but thermal stability can be partially restored by the engineered substitution Leu 121-Ala (Baldwin et al., 1996). Leu 121-Ala is destabilizing by itself, but compensates for the other mutations by permitting repacking of the hydrophobic core of the molecule. In one study, Ser 44, a solvent-exposed residue in the middle of an alpha-helix in T4 lysozyme, was chosen as a relatively context-free test case for a systematic assessment of the contribution of general residue helical propensity to protein stability (Blaber et al., 1994). By measuring the thermal stabilities of substitution variants, the investigators could rank the 20 standard amino acid residues. The mutations we describe above as probable global stabilizers all involve alpha-helical residues, which change from a relatively helix-destabilizing residue to a relatively helix-stabilizing residue as determined for substitutions at position 44 (Blaber et al., 1994).

Comparison of approaches

The experimental approach to isolating structural variants of a protein employed in this study was to seek, in as model-free a manner as possible, intragenic second-site revertants of highly deleterious mutations. In principle, such an approach offers two advantages over protein engineering. (1) It allows for the isolation of mutants that might not be predicted by theories based on our current incomplete understanding of the protein folding question. Structural studies of some such variants might be expected to be especially informative. (2) It permits isolation and characterization of proteins bearing substitutions that, by themselves, destabilize the protein to the point of making it biochemically intractable.

Both of these advantages were realized. The substitution Gly 23-Asp increases the functional flexibility of position 20 in a way that was not readily predictable. Similarly, Met 6-Ile alters the pattern of acceptable substitutions at position 98 in a way that we would not have predicted and is still not obviously explained by contemplation of the wild-type structure. The Met 6-Ile substitution, moreover, permitted purification of a mutant lysozyme bearing the substitution Ala 98-Gln, which by itself made the protein too unstable to work with. Structural studies of the missense mutant variants described in Table 2 are in progress.

Materials and methods

Bacteria and phage

Salmonella typhimurium strain MS2310 and a series of 13 isogenic derivatives bearing amber suppressors have been described (Youderian et al., 1982; Rennell et al., 1991). P22 Kn321 sieA44 m44, P22 e416 sieA44 m44, and variants of the latter bearing amber mutations in the T4 lysozyme gene also have been described (Rennell & Poteete, 1989; Rennell et al., 1991). Derivatives of P22 e416 sieA44 m44 bearing other mutant alleles of the T4 lysozyme gene were constructed by crossing P22 Kn321 sieA44 m44 with variants of plasmid pLH416, as described previously (Rennell et al., 1991). Plating phenotypes of P22 e416 sieA44 m44 bearing

mutant alleles of the T4 lysozyme gene were determined as described (Rennell et al., 1991).

Plasmids

Methods for plasmid construction and propagation were standard. Specific methods for oligonucleotide-directed mutagenesis and screening of T4 lysozyme-bearing plasmids were described previously (Rennell et al., 1991).

Plasmid pLH506 was constructed by replacing the *Nde* 1–*Bgl* 2 T4 lysozyme-bearing segment of pLH416 (Hardy & Poteete, 1991) with DNA sequences bearing the tetracycline resistance-conferring (tet^R) gene of pBR322. The tet^R gene was flanked by *Nde* 1 and *Bgl* 2 linkers, which were ligated to the filled-in *Eco*R 1 and *Ava* 1 sites, respectively, of pBR322.

Construction of a plasmid for high-level production of T4 lysozyme involved several steps. (1) The $P_{A1/04/03}$ promoter (Lanzer & Bujard, 1988) from pUHE21-2 (generous gift of M. Berlin and H. Bujard) was removed as an *Ssp* 1–*Eco*R 1 fragment, and placed between the corresponding sites in pBR322. The effect of this construction was to create a derivative of pBR322 with a 90-base pair insertion, between bases 4357 and 4358, containing $P_{A1/04/03}$. Sequences between the *Eco*R 1 and *Cla* 1 sites of this derivative were removed by digestion with the appropriate restriction enzymes, removal of the resulting 5′ overhanging single-stranded ends with mung bean nuclease (used as recommended by the supplier, New England Biolabs), and ligation. The resulting plasmid is designated pDR709. The structure of its *Eco*R 1–*Cla* 1 junction was determined to be as expected by DNA sequencing (data not shown). (2) The T4 lysozyme gene in pLH415 (Hardy & Poteete, 1991) was altered by mismatched oligonucleotide-directed mutagenesis to introduce a silent *Sac* 1 restriction site at codons 45–46 (GAATTA changed to GAGCTC), and a silent *Apa*L 1 site at codons 97–99 (TGTGCATTG changed to TGTGCACTG). The resulting plasmid is designated pDR710. (3) The $P_{A1/04/03}$-bearing *Pst* 1–*Hin*d 3 fragment of pDR709 was joined to the modified T4 lysozyme-bearing *Hin*d 3–*Pst* 1 fragment of pDR710. The T4 lysozyme gene in the resulting recombinant was further altered by oligonucleotide-directed mutagenesis to introduce a Cys-to-Thr substitution at position 54 (TGC-ACC) and a Cys-to-Ala substitution at position 97 (TGT-GCT; note that this change eliminated the *Apa*L 1 site introduced previously). The resulting plasmid, designated pDR739, expresses the "cysteine-less wild-type" lysozyme (Matsumura & Matthews, 1989) from the strong $P_{A1/04/03}$ promoter under tight control of *lac* repressor in a host bearing F′ $lacI^q$.

Revertant isolation

Spontaneous revertants of amber mutation-bearing phages on nonpermissive amber suppressor strains were selected and screened to distinguish primary and secondary site revertants as described (Poteete et al., 1991). UV-induced revertants were produced by irradiating phage stocks and passaging them through permissive host strains in which SOS error-prone repair was induced, as described (Rennell & Poteete, 1989). A double-indicator plating procedure was employed to permit the screening of large numbers of revertants. UV-mutagenized phages were mixed with a nonpermissive amber suppressor host, allowed to adsorb, then plated over or under a lawn of the nonsuppressor host. Primary revertants, lacking the original amber codon in the lysozyme gene, lyse the layer of nonsuppressor host cells; second-site revertants, retaining the original amber codon, fail to do so, and can be distinguished visually.

Candidate second-site revertants that, after two or three cycles of plaque-purification, retained the phenotype of plating on the previously nonpermissive amber suppressor, but not on the nonsuppressor host strain, were tested by reconstruction. This procedure was designed to rule out the possibility that extragenic suppressor mutations were responsible for the altered plating phenotype of the revertant. The T4 lysozyme gene from the revertant phage was excised as an *Nde* 1–*Bgl* 2 fragment from phage DNA, and inserted between the *Nde* 1 and *Bgl* 2 sites of pLH506, then re-introduced into P22 e416 sieA44 m44 as described above. If the revertant phage thus reconstructed exhibited the appropriate plating phenotype, its lysozyme gene was sequenced as described (Rennell et al., 1991; Bouvier & Poteete, 1996).

Missense mutant construction

Missense mutations were introduced into the T4 lysozyme gene of pDR739. *Escherichia coli* strain W3110 F′ $lacI^Q$ bearing the mutant plasmids was cultured, induced to produce lysozyme with isopropylthiogalactopyranoside, and mutant lysozymes were purified, as described (Poteete et al., 1991).

Acknowledgments

This research was supported by grant AI24083 from the National Institutes of Health. We thank Chris Schmidt for technical assistance, Milan Jucovic for helpful discussions, and Drs. M. Berlin and H. Bujard for the gift of plasmid pUHE21-2.

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