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MINIREVIEW

Genetic Analysis of Bacteriophage T4 Lysozyme Structure and Function

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The functional properties of proteins are determined by their three-dimensional structures, which in turn are determined by the sequences of their polypeptide subunits. If we knew the rules by which sequences of amino acids fold into unique structures, we would realize three immediate benefits. (i) We could predict accurate three-dimensional structures of proteins from their amino acid sequences. At present, sequencing proteins (through their genes) is very much faster than crystallizing them and solving their structures; many important projects are hampered by the lack of a crystal structure. (ii) We could confidently design our own proteins, or modify existing ones, to serve as exquisitely specific catalysts, antigens, or therapeutic agents. (iii) We would advance by a major step towards the goal of understanding biological processes in terms of the chemistry and physics of the molecules that carry them out. We do not know these rules; the protein folding problem is one of the most basic intellectual challenges in molecular biology (4).

It has been known for three decades that purified, unfolded proteins usually can be induced to refold correctly in vitro, and that, therefore, sequence determines folded structure. The realization that some, perhaps many, proteins require the assistance of molecular chaperones for proper folding kinetics in vivo modifies this generalization without diminishing it. Research on the sequence determinants of protein structure has taken a number of different approaches, including the following.

(i) Solving the structures of hundreds of proteins by X-ray crystallography and examining the structures for clues in the intramolecular interactions thus delineated. The structural roles of hydrophobic interactions, hydrogen bonds, salt bridges, and disulfides in maintaining the folded structure are apparent in these structures, as well as the various suitabilities of different amino acid residues to fit into the characteristic secondary structures of proteins (α-helices, turns, etc.). An early (and, in this context, daunting) observation was that a great diversity of related sequences could assume essentially the same structure, i.e., the globin fold (20). Moreover, it later became clear that even apparently unrelated sequences could assume essentially similar structures (27). While definitive rules have not emerged from this approach, a useful generalization has, namely, that proteins which are related by sequence homology are likely to be even more closely related structurally. Thus, if a protein of unknown structure is found to be related by sequence homology to a protein of known structure, then the known structure is a good starting model for the unknown structure.

(ii) Development of computational formulas that predict folded structure. There are essentially two ways of doing this. The conceptually simpler way is to identify the conformation with the lowest calculated free energy. This method runs into two difficulties that have, so far, limited its applicability to smaller molecules: the astronomical number of conformations available to a macromolecule precludes systematic calculations of free energies, and the accuracy of the parameters for calculating energy as a function of interatomic displacement may not be sufficient for the task. The theoretically less well defined, but practically easier, way is to attempt to predict secondary structures from sequences by using algorithms that assess the statistical probability that a given stretch of sequence will be found in one or another element of secondary structure. Such algorithms make use of the existing database of hundreds of known protein structures. They are limited in both reach and accuracy: tertiary structure is not predicted, and the accuracy of secondary structure predictions turns out to be about 70 to 75% under the most favorable circumstances. On the other hand, an approach that combines this type of algorithm with sequence homology searching has recently proven fairly accurate in predicting the elements of secondary structure in the protein kinase catalytic domain before its structure was solved (discussed in reference 32).

(iii) De novo design of synthetic proteins. This approach is exemplified by the work of DeGrado and collaborators (7), who have synthesized polypeptides that fold into structures containing four α-helices. Recent work in this area has been directed towards designing variants of these polypeptides that have properties more like those of natural proteins (10).

(iv) Studies of mutant proteins. Studies of mutant proteins have of course played an important role in our understanding of basic life processes from the early days of molecular biology. However, until the recent advent of highly efficient and precise mutagenic methods based on recombinant DNA, their contributions to our understanding of protein structure (as opposed to function) were minimal. Significant advances have been made in this area in the last few years, stemming from three strategies. The first is what might be called the protein engineering approach, i.e., examining a protein structure, making informed guesses concerning the structures and stabilities of mutant variants, constructing the mutants, and testing them. This strategy has yielded a much improved quantitative understanding of the structural determinants of protein stability (9, 16, 28). The second is intensive, randomizing mutagenesis of an element of protein structure to sample "sequence

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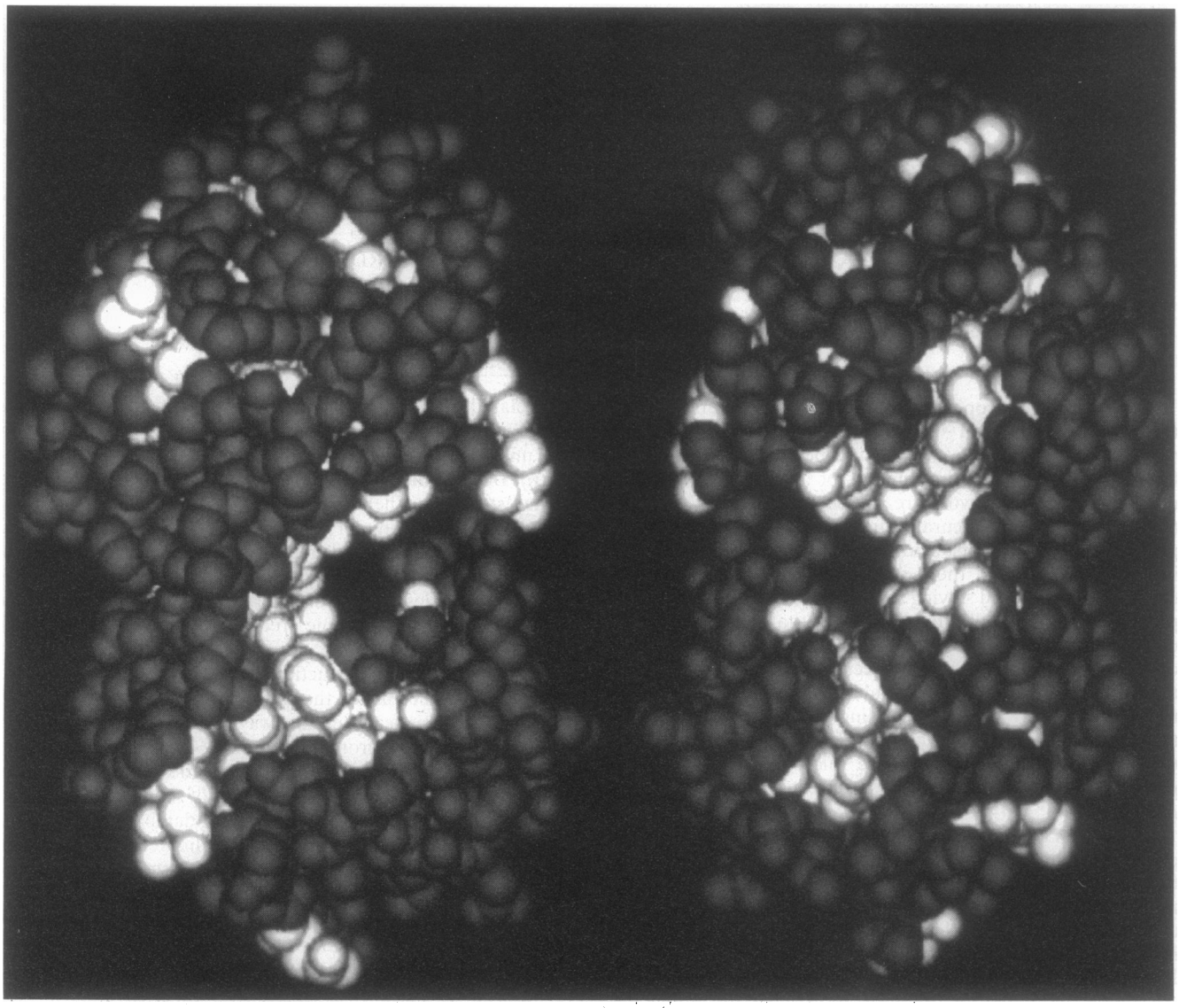

FIG. 1. Critical residues in T4 lysozyme. Atoms depicted in white are those of residues whose replacement (by at least two others) leads to loss of function. The figure was generated with Promodeler molecular graphics software (New England Biographics), using coordinates from the Brookhaven Protein Data Bank. Two views, rotated by 180°, are shown.

space" for functional combinations. This strategy, exemplified by studies of α-helices and the hydrophobic core of the phage λ repressor (13, 23), has revealed that, at least in some cases, a very large number of sequences can satisfy the structural and/or functional requirements of a particular piece of a protein. In an interesting variation of this approach, Davidson and Sauer (6) have found that a surprisingly large proportion (5%) of random sequences consisting of glutamine, leucine, and arginine residues fold into structures with protein-like properties. The third strategy is to perform systematic surveys of single amino acid substitutions, insertions, and deletions in a protein to identify the positions in the protein that are critical for function. The "feel" provided by these studies complements the "look" of crystallography.

Bacteriophage T4 lysozyme is one of a small number of proteins that have been studied intensively by the use of a combination of genetic and structural techniques. The aim of this minireview is to summarize some insights that have been gained from this research, focusing on the genetic studies of our laboratory. The reader is referred to a recent review by Matthews (16) for a more structurally oriented treatment.

BIOLOGY AND HISTORY OF T4 LYSOZYME

T4 lysozyme is a monomeric protein of 164 amino acid residues that hydrolyzes peptidoglycan. Its role in the lytic cycle of the virus is to start the extracellular phase by liberating virions from the metabolically inert remains of the bacterial host. Although T4 lysozyme functions outside the cytoplasmic membrane, it is not a typical secreted protein. It is synthesized without a signal sequence, and its access to the bacterial cell wall from the cytoplasm is under the control of a number of phage-encoded regulatory proteins. (For an encyclopedic review of phage-induced bacterial lysis, see reference 36).

Mutant strains of phage T4 lacking lysozyme function are defective in lysis but can be rescued by externally added

lysozyme in the presence of agents that destabilize the outer membrane of the host. Early combined genetic and protein-sequencing studies of T4 lysozyme mutants by Streisinger and coworkers (30, 31) played a significant role in securing our present understanding of the genetic code. Determination of the crystal structure of the protein by Matthews and coworkers (24, 34) set the stage for combined genetic and structural studies. T4 lysozyme proved to be particularly amenable to such an approach. The wild-type protein, and many mutant variants, readily form crystals that diffract to high resolution. T4 lysozyme has also served as a useful model system in studies of protein folding/unfolding (5) and in the development of techniques for protein nuclear magnetic resonance spectroscopy (18).

In the original genetic studies of T4 lysozyme, the lysozyme gene (*e*) was, of course, borne by phage T4. As a genetic system for studying lysozyme by modern techniques, however, T4 has some disadvantages: it contains genes encoding other lysozymes, giving rise to a complicated spectrum of extragenic revertants of *e* mutants, and its DNA cannot be cut by most restriction enzymes. In more recent studies, gene *e* (or a synthetic variant) has typically been borne by either M13- or pBR322-based cloning vectors. A disadvantage of these latter systems is that lysozyme has no integral role in their life cycles and so is not subject to functional genetic selection. Accordingly, for some studies summarized below, the T4 lysozyme gene was inserted into phage P22, functionally replacing the (related) lysozyme gene of this phage. The resulting hybrid phage offers a number of experimental advantages over T4: it does not appear to encode other lysozyme genes, it can be propagated as a prophage (a useful property for some genetic construction steps), and its DNA can be cut with restriction enzymes. A related system, in which T4 lysozyme functionally replaces the endolysin of coliphage λ, has recently been described as well (2).

SYSTEMATIC MUTATION OF T4 LYSOZYME

Miller and coworkers (14, 19), working with the Lac repressor of *Escherichia coli*, developed the use of amber mutations and suppressors for systematic studies of amino acid substitutions in proteins. In principle, each amber mutation allows the testing of one substitution for each distinct amber suppressor amino acid insertion specificity. This approach was applied to T4 lysozyme. Amber mutations were introduced into each codon (except the first) of the lysozyme gene of the P22 hybrid. The 163 resulting amber mutant phages were tested for plaque formation on each of 13 efficient amber suppressor strains of *Salmonella typhimurium*, including 4 naturally-occurring mutants (35) and 9 strains bearing synthetic amber suppressor genes constructed in the laboratories of Miller, Abelson, and McClain (12, 17). These suppressors insert 13 different amino acids: Gly, Ala, Ser, Pro, Cys, Leu, Gln, Lys, Glu, His, Phe, Arg, and Tyr. This technique permitted assessment of the effects on function of 12 or 13 single amino acid substitutions at each position in the protein, for a total of 2015 (25).

In the construction of hybrid P22 phages bearing the T4 lysozyme gene, it was found that the level of lysozyme expression could be varied over a 1,000-fold range by the choice of sequences for transcriptional and translational regulation. The particular hybrid strain employed in systematic mutagenic studies was chosen for two properties: production of an amount of lysozyme activity comparable to that produced by wild-type P22 and lack of regulatory sequences that could mutate readily to yield a highly increased level of expression. In this background, it was found that, to score as defective, a mutation in lysozyme had to reduce the overall lysozyme activity to less than 3% of that produced by the hybrid phage bearing the wild-type lysozyme gene. Thus, only strong mutations were scored as deleterious.

Overall, the suppression pattern indicated that T4 lysozyme was tolerant of single amino acid substitutions: 89 out of 163 positions (55%) accept all substitutions. Of 2015 substitutions tested, only 328 (16%) were scored as deleterious at 37°C. In general structural terms, positions on the surface were insensitive to substitutions, while positions on the inside (solvent inaccessible) or lining the active site cleft were sensitive (Fig. 1). Especially sensitive sites included the key catalytic residue Glu-11, residues involved in buried salt bridges near the catalytic site (Asp-10, Arg-145, and Arg-148), and a few others that may have critical structural roles: Gly-30, which lines the active site cleft and has a backbone conformation attainable only by a glycine residue, and Trp-138 and Tyr-161, which apparently anchor small α-helices on the surface of the molecule. These observations were consistent with earlier studies indicating that temperature-sensitive substitutions in T4 lysozyme occur in positions of low mobility and solvent accessibility (1).

FUNCTIONAL IMPORTANCE OF CONSERVED RESIDUES

Many proteins, including T4 lysozyme, are members of families that are related by sequence homology and function. The theory of evolution by natural selection suggests that amino acid residues that are conserved among family members are of greater functional importance than those that are not conserved. On the other hand, few residues are fully conserved in large families, and even highly conserved residues are clearly not irreplaceable. The relative functional importance of conserved amino acid residues has generally been assumed, but not tested.

The bacteriophage-encoded lysozyme family includes the lysozymes of phages T4, P22, ø29, and 21, as well as the more closely related baseplate lysozyme of phage T4. The sequences of these proteins, aligned to maximize amino acid identifies with a minimum of gaps, reveal that of the 164 residues in T4 lysozyme, 14 are fully conserved among the five lysozymes, 9 are found in three of the others, 15 are found in two of the others, and 125 are found in one or none of the others. The existence of data concerning the effects on biological function of a uniform set of amino acid residues at every position (but one) in lysozyme permitted a test of the hypothesis that conserved residues are more important than nonconserved residues (22). Since only 74 (45%) of the 163 tested residues in lysozyme are sensitive to one or more substitution, the null hypothesis, i.e., that conserved residues are no more sensitive to substitution than others, leads to the expectation that 6 or 7 of the 14 fully conserved residues should be sensitive to substitution. The probability that all 14 would be sensitive to substitution is approximately 0.000008, yet this was found to be the case. {This probability was calculated as the number of combinations of 14 of the 74 sensitive residues, (74!)/[(14!)(60!)], divided by the number of combinations of 14 of all 163 residues, (163!)/(14!)(149!). The previously reported probability of 0.000016 (27) was derived from a more approximate calculation.} The correlation of residue conservation and sensitivity to substitutions goes further. Of the nine T4 lysozyme residues that are conserved in three other bacteriophage lysozymes, six (67%) are sensitive to substitutions. Of the 15 residues that are found in two others, 5 (33%) are sensitive, as are 49 (39%) of the 125 nonconserved residues.

Another view supported by this analysis is that even highly conserved amino acid residues are generally replaceable. Among the fully conserved residues, 172 substitutions were tested. Only 81 were unacceptable; the remaining 91 did not eliminate lysozyme function. The 91 acceptable substitutions included many that are not "conservative" by any reckoning.

The substitution data indicate that not only are conserved residues generally more functionally important than nonconserved residues, but, conversely, residues that are especially sensitive to substitution tend to be conserved. Of the 10 positions in T4 lysozyme at which the fewest substitutions are allowed, 6 are fully conserved. Thus, the frequency of complete conservation among this group of residues is 60%, compared to 5% among the other residues in the protein (8 out of 153). On the other hand, it is worth noting that the other four members of this functionally critical group are not conserved. If this result is typical, then only about half of the most functionally critical amino acid residues in a protein can be picked out by homology searches.

CRITICAL ROLE OF C-TERMINAL HYDROPHOBIC STRIP RESIDUES

The research cited above identifies three groups of residues (buried, active site, and conserved) that are particularly sensitive to substitutions. An additional group of critical residues was identified by the use of a type of structure-predicting algorithm designed to identify a longitudinal hydrophobic strip of helix in a surface α-helix (33). Like some other schemes for predicting α-helices, it is based on the observation that α-helices on the surfaces of proteins tend to be amphipathic. Typically, one longitudinal sector of the helix will have predominantly hydrophobic residues, which pack into the hydrophobic core of the protein. It was found that the application of this algorithm to the α-helices of T4 lysozyme picks out a small set of residues that are more critical to function than buried residues and that are nearly as critical as the fully conserved residues (26).

Various groups of residues of T4 lysozyme differ in their sensitivities to functional inactivation by substitutions. The protein as a whole scores 16; that is, 16% (328 of 2,015) of substitutions tested were found to be deleterious. Buried residues, as a group, are more sensitive to substitutions. Loss of function results from 42% of substitutions for residues with completely solvent-inaccessible side chains. The score of fully conserved residues is 47%. T4 lysozyme has nine standard α-helices, which account for 59% of its amino acid residues. In terms of sensitivity to substitutions, α-helical residues are typical of the protein as a whole: 16% of substitutions are deleterious.

Within the group of α-helical residues, though, those designated as belonging to the hydrophobic strip are more sensitive than average (26%), while nonhydrophobic strip residues are less sensitive (13%). Within the hydrophobic strips, the N-terminal residues score 26%, while the C-terminal residues score an extraordinarily high 44%. Statistical analysis indicated that the high degree of sensitivity of this group of residues was not attributable to a bias in its residue composition (such as, for instance, consisting predominantly of large hydrophobic residues, which are more sensitive to substitutions as a class) but rather had to be a matter of position in the protein's structure.

The hydrophobic-strip-of-helix algorithm thus identifies a set of critical amino acid residues in T4 lysozyme. A number of substitutions for any of the nine members of this group result in loss of function. The minimum number of deleterious substitutions (out of 12 or 13 tested at each position) in this group is four. The significance of this group of residues is apparent when one considers that only 74 of the 163 tested positions in T4 lysozyme are sensitive to any substitutions; only 43 are sensitive to four or more. Thus, the probability of picking, at random, a group of nine residues in which all are sensitive to four or more substitutions is approximately 0.000003. The apparent functional significance of residues identified in this manner exceeds that of residues identified on the basis of solvent inaccessibility and approaches that of the set of most highly conserved residues.

Examination of the T4 lysozyme molecule reveals a structural correlate of the observation of the relative importance of C-terminal hydrophobic strip residues. These residues are more tightly packed within the molecule than N-terminal strip residues; they are less solvent accessible and have lower temperature factors (B-values) in the crystal structure. This generalization is based on seven of the nine α-helices. The other two are omitted because one (residues 93 to 106) runs through the interior of the large domain of T4 lysozyme, and is thus not a surface α-helix, while the other (residues 137 to 141) has only one residue that fits the description of a hydrophobic strip residue (as defined by either the algorithm or structural criteria), and so its N-terminal and C-terminal hydrophobic strip residues are the same (Trp-138).

Rules for picking out critical residues are not completely reliable. Many substitutions are tolerated at buried and conserved positions, as well as at C-terminal hydrophobic strip positions. When examined in detail, proteins with unexpectedly tolerated substitutions turn out to have altered their structures in subtle ways, creating a context into which the altered residue fits (15). It is apparent that the acceptability of amino acid substitutions is governed by details of structural context which cannot be captured in a simple scalar quantity like solvent accessibility. The foregoing considerations may limit, but do not rule out, the utility of simple formulas in identifying key structural determinants of function. Even so, using a structure-predicting algorithm is perhaps not an obvious strategy for studying a protein for which a high-resolution, crystallographically determined structure is available. However, at least in this case, the algorithm was helpful in picking out a pattern in the effects of amino acid substitutions.

SECOND-SITE REVERTANTS

The contribution of studies of mutant proteins to our understanding of protein structure has been limited by a tendency of mutations, i.e., the great majority of amino acid substitutions do not significantly affect the protein's structure. Those that do tend to wreck the protein, making it unstable, insoluble, or sufficiently disordered to be intractable. Proteins with temperature sensitive (in other words, mild) structural defects tend to have structures essentially identical to the wild-type except in the space actually occupied by the altered amino acid residue (15).

One possible way to circumvent the limitations of single amino acid substitutions in generating informative structural variants of natural proteins is to look for mutants in which in a primary, structure-disrupting mutation is "fixed" by compensatory second-site mutations. Such multiply mutant proteins might contain significant structural alterations that could be attributed to a few changed residues. Second-site revertants have been characterized in a number of systems, most notably in staphylococcal nuclease. In this case, revertants of temperature-sensitive mutants were characterized as containing "global stabilizers"—second-site mutations that could suppress the

mutant phenotypes of many different primary mutations. It is thought that the global stabilizer mutations exert their effects by increasing the overall thermodynamic stability of the folded state, thus compensating for the destabilizing primary mutations (29).

Second-site suppressor mutations that exert their effects via a structural alteration in the immediate vicinity of the primary site mutation were sought in the T4 lysozyme system. Phages with lysozyme amber mutations were mutagenized and plated on host strains bearing amber suppressors that generated nonfunctional lysozymes. Revertants were selected and screened for retention of the amber mutation by testing their ability to form plaques on an otherwise isogenic non-amber suppressor host. Reversion rates and proportions of secondary site revertants among revertants were found to vary greatly among different sites. Both global and allele-specific suppressors have been sequenced and characterized.

In one study, second-site revertants of an amber mutation in codon 26, which normally specifies Thr, were selected on a glutamine-inserting suppressor host and characterized (21). One secondary mutation resulted in a change from Tyr to His at position 18; another changed the same Tyr to Asp. Three mutant lysozymes, bearing the substitutions Thr-26→Gln, Thr-26→Gln/Tyr-18→His, and Thr-26→Gln/Tyr-18→Asp, were produced and purified following introduction of the corresponding genetic changes into a plasmid-based expression system. It was found that substitution of Thr-26 by Gln produced an enzyme with greatly reduced activity but essentially unaltered stability relative to the wild-type enzyme. The two revertant lysozymes exhibited enzymatic activities intermediate between those of the wild type and the primary mutant enzyme; both also exhibited melting temperatures approximately 3°C lower than that of either the wild type or the primary mutant enzyme. Crystals suitable for X-ray diffraction analysis were obtained from both revertant lysozymes but not from the primary mutant. Data were collected and analyzed, and structures of the double mutant lysozymes were refined at 1.8-Å resolution. Examination of the structures suggested that the side chain of Gln-26 in the primary mutant is forced to protrude into the active site cleft. In contrast, the crystal structures of the revertants revealed that the double substitutions (Gln-26 and His-18 or Gln-26 and Asp-18) fit into the same space that is occupied by Thr-26 and Tyr-18 in the wild-type enzyme; the effect is a restructuring of the surface of the active site cleft with essentially no perturbation of the polypeptide backbone. In one case (Tyr-18→Asp), the side chain of Arg-14, a nearby surface residue, is additionally rearranged. These observations suggest the following simple explanation for the effects of the amino acid substitutions on activity. In the primary mutant, the side chain of Gln-26 appears to protrude into the active site cleft, interfering with the binding of substrate. Substitution of Tyr-18 with His or Asp induces Gln-26 to rotate away from the active site cleft and to pack more toward the interior of the protein, partially alleviating the substrate-binding problem. The roles of His-18 and Asp-18 in this restructuring would appear to be active, in a sense, as these particular residues are both able to participate in novel hydrogen-bonding networks which stabilize Gln-26 in its out-of-the-way conformation.

The structures of Thr-26→Gln revertants do not shed much light on the general protein structure problem, because the substantial structural differences between them and the wild-type structure come about by alterations in the packing of side chains without any apparent movement of the polypeptide backbone. Another second-site revertant may be more informative in this regard (3). In this mutant, a primary defect brought about by replacement of Ala-98 with Leu is corrected by replacement of Met-6 with Ile. In this case, the primary mutation must cause a structural disruption in T4 lysozyme, because the β-carbon of Ala-98 is packed closely against the polypeptide backbone atoms of residue 152. The resulting steric clash cannot be repaired by a simple substitution at position 152, as no simple amino acid substitution would remove the backbone atoms. Accommodation of the larger residue at position 98 would appear, necessarily, to require movement of the polypeptide backbone. The second-site mutation, Met-6→Ile (M6I), has been extensively characterized by Matthews and coworkers. By itself, it confers a temperature-sensitive phenotype, although it reduces the measured melting temperature of T4 lysozyme by only a few degrees. However, its structural effects are interesting. The M6I protein crystallizes in two different forms: the familiar trigonal form of the wild-type enzyme and many mutant lysozymes and a new orthorhombic form. In the trigonal form, the structure of the M6I variant of T4 lysozyme is very similar to that of the wild type. In the new orthorhombic form, the molecule is arranged with four monomers in the asymmetric unit, and each one has a different conformation (8). The M6I mutation appears to lead, by an unknown mechanism, to an increased flexibility in T4 lysozyme. (This effect is probably not simply at the level of crystal packing; Met-6 is a buried residue that is not involved in intermolecular crystal contacts.) Thus, it might not be considered surprising that M6I could compensate for the structural distortion caused by replacements of Ala-98. However, such a facile explanation fails to account for an additional observation, derived from the suppression pattern of am98 M6I, which is that unlike its am98 parent, the double mutant fails to grow on a proline-inserting amber suppressor strain. Thus, M6I does not simply increase the range of permissible alterations at position 98 but rather does something more specific to accommodate the structural rearrangement of the primary mutation.

CATALYTIC ROLE OF Asp-20

A surprising observation that emerged from systematic genetic studies of T4 lysozyme concerned the role of Asp-20 in catalysis. According to the amber mutant suppression data, Cys can replace Asp-20 without loss of function. A mutant T4 lysozyme in which Asp-20 was replaced by cysteine was produced by the use of a gene containing a Cys sense codon at position 20 and then purified. The cysteine-substituted lysozyme was found to have a specific activity equal to 80% of that of the wild-type enzyme, a newly acquired sensitivity to thiol-modifying reagents, and a pH-activity profile that is very similar to that of the wild-type. These observations were inconsistent with a long-assumed electrostatic role of Asp-20 in catalysis and prompted reexamination of the mechanism of lysozyme catalysis (11).

CONCLUSION

Experiments with T4 lysozyme, as well as with other intensively studied proteins, serve to illustrate the importance of relatively unbiased genetic approaches to structure-function studies. An unbiased genetic approach has, in this case, been informative about the relationship between amino acid sequence and protein structure and has also yielded unexpected insights into the enzyme's catalytic machinery. Gratuitous findings of the latter sort are likely when a systematic genetic approach is applied to a protein with catalytic activity. The more goal-directed alternative to such approaches, i.e., the use of site-directed mutagenesis to test specific hypotheses derived from contemplation of a protein's structure (or even just its

sequence), is unquestionably powerful and fruitful. But it is at least arguable that the less model driven the experimental approach, the greater the likelihood of surprising results, and we may need surprising insights to solve the protein structure problem.

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REFERENCES

1. **Alber, T., S. Dao-Pin, J. A. Nye, D. C. Muchmore, and B. W. Matthews.** 1987. Temperature-sensitive mutations of bacteriophage T4 lysozyme occur at sites with low mobility and low solvent accessibility in the folded protein. Biochemistry **26:**3754–3758.
2. **Baldwin, E., J. Xu, and O. Hajiseyedjavadi.** 1994. Construction and functional selection of a T4 lysozyme gene library randomly mutagenized at five specific sites. *In* Advances in Protein Chemistry IV, in press. Academic Press, Inc., New York.
3. **Bouvier, S. E., and A. R. Poteete.** Unpublished data.
4. **Branden, C., and J. Tooze.** 1991. Introduction to protein structure. Garland Publishing, Inc., New York.
5. **Chen, B. L., W. A. Baase, H. Nicholson, and J. A. Schellman.** 1992. Folding kinetics of T4 lysozyme and nine mutants at 12°C. Biochemistry **31:**1464–1476.
6. **Davidson, A. R., and R. T. Sauer.** 1994. Folded proteins occur frequently in libraries of random amino acid sequences. Proc. Natl. Acad. Sci. USA **91:**2146–2150.
7. **DeGrado, W. F., D. P. Raleigh, and T. Handel.** 1991. *De novo* protein design: what are we learning? Curr. Opin. Struct. Biol. **1:**984–993.
8. **Faber, H. R., and B. W. Matthews.** 1990. A mutant T4 lysozyme displays five different crystal conformations. Nature (London) **348:** 263–266.
9. **Green, S. M., A. K. Meeker, and D. Shortle.** 1992. Contributions of the polar, uncharged amino acids to the stability of staphylococcal nuclease: evidence for mutational effects on the free energy of the denatured state. Biochemistry **31:**5717–5728.
10. **Handel, T. M., S. A. Williams, and W. F. DeGrado.** 1993. Metal ion-dependent modulation of the dynamics of a designed protein. Science **261:**879–885.
11. **Hardy, L. W., and A. R. Poteete.** 1991. Re-examination of the role of Asp^{20} in catalysis by bacteriophage T4 lysozyme. Biochemistry **30:**9457–9463.
12. **Kleina, L. G., J. Masson, J. Normanly, J. Abelson, and J. H. Miller.** 1990. Construction of *Escherichia coli* amber suppressor tRNA genes II: synthesis of additional tRNA genes and improvement of suppressor efficiency. J. Mol. Biol. **213:**705–717.
13. **Lim, W. A., and R. T. Sauer.** 1989. Alternative packing arrangements in the hydrophobic core of λ repressor. Nature (London) **339:**31–36.
14. **Markiewicz, P., L. Kleina, C. Cruz, S. Ehret, and J. H. Miller.** Analysis of 4,000 altered *Escherichia coli lac* repressors resulting from suppression of nonsense mutations at 328 positions in the *lacI* gene. J. Mol. Biol., in press.
15. **Matthews, B. W.** 1987. Genetic and structural analysis of the protein stability problem. Biochemistry **26:**6885–6888.
16. **Matthews, B. W.** 1993. Structural and genetic analysis of protein stability. Annu. Rev. Biochem. **62:**139–160.
17. **McClain, W. H., and K. Foss.** 1988. Changing the acceptor identity of a transfer RNA by altering nucleotides in a "variable pocket". Science **241:**1804–1807.
18. **McIntosh, L. P., A. J. Wand, D. F. Lowry, A. G. Redfield, and F. W. Dahlquist.** 1990. Assignment of the backbone ^{1}H and ^{15}N resonances of bacteriophage T4 lysozyme. Biochemistry **29:**6341–6362.
19. **Miller, J. H.** 1991. Use of nonsense suppression to generate altered proteins. Methods Enzymol. **208:**543–563.
20. **Perutz, M. F., J. C. Kendrew, and H. C. Watson.** 1965. Structure and function of haemoglobin II: some relations between polypeptide chain configuration and amino acid sequence. J. Mol. Biol. **13:**669–678.
21. **Poteete, A. R., S. Dao-Pin, H. Nicholson, and B. W. Matthews.** 1991. Second-site revertants of an inactive T4 lysozyme mutant restore activity by restructuring the active site cleft. Biochemistry **30:**1425–1432.
22. **Poteete, A. R., D. Rennell, and S. E. Bouvier.** 1992. Functional significance of conserved amino acid residues. Proteins Struct. Funct. Genet. **13:**38–40.
23. **Reidhaar-Olson, J. F., and R. T. Sauer.** 1988. Combinatorial cassette mutagenesis as a probe of the informational content of protein sequences. Science **241:**53–57.
24. **Remington, S. J., W. F. Anderson, J. Owen, L. F. Ten-Eyck, C. T. Grainger, and B. W. Matthews.** 1978. Structure of the lysozyme from bacteriophage T4: an electron density map at 2.4 Å resolution. J. Mol. Biol. **118:**81–98.
25. **Rennell, D., S. E. Bouvier, L. W. Hardy, and A. R. Poteete.** 1991. Systematic mutation of bacteriophage T4 lysozyme. J. Mol. Biol. **222:**67–88.
26. **Rennell, D., A. R. Poteete, M. Beaulieu, D. Z. Kuo, R. A. Lew, and R. E. Humphreys.** 1992. Critical functional role of the COOH-terminal ends of longitudinal hydrophobic strips in α-helices of T4 lysozyme. J. Biol. Chem. **267:**17748–17752.
27. **Rossman, M. G., and P. Argos.** 1976. Exploring structural homology of proteins. J. Mol. Biol. **105:**75–95.
28. **Sali, D., M. Bycroft, and A. R. Fersht.** 1991. Surface electrostatic interactions contribute little of stability of barnase. J. Mol. Biol. **220:**779–788.
29. **Shortle, D., and B. Lin.** 1985. Genetic analysis of staphylococcal nuclease: identification of three intragenic "global" suppressors of nuclease-minus mutations. Genetics **110:**539–555.
30. **Streisinger, G., F. Mukai, W. J. Dreyer, B. Miller, and S. Horiuchi.** 1961. Mutations affecting the lysozyme of phage T4. Cold Spring Harbor Symp. Quant. Biol. **26:**25–30.
31. **Streisinger, G., Y. Okada, J. Emrich, J. Newton, A. Tsugita, E. Terzaghi, and M. Inouye.** 1966. Frameshift mutations and the genetic code. Cold Spring Harbor Symp. Quant. Biol. **31:**77–84.
32. **Thornton, J. M., T. P. Flores, D. T. Jones, and M. B. Swindells.** 1991. Prediction of progress at last. Nature (London) **354:**105–106.
33. **Torgerson, R. R., R. A. Lew, V. E. Reyes, L. Hardy, and R. E. Humphreys.** 1991. Highly restricted distributions of hydrophobic and charged amino acids in longitudinal quadrants of α-helices. J. Biol. Chem. **266:**5521–5524.
34. **Weaver, L. H., and B. W. Matthews.** 1987. Structure of bacteriophage T4 lysozyme refined at 1.7 Å resolution. J. Mol. Biol. **193:** 189–199.
35. **Winston, F., D. Botstein, and J. H. Miller.** 1979. Characterization of amber and ochre suppressors in *Salmonella typhimurium*. J. Bacteriol. **137:**433–439.
36. **Young, R.** 1992. Bacteriophage lysis: mechanism and regulation. Microbiol. Rev. **56:**430–481.