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Disulfide Bond Engineered into T4 Lysozyme: Stabilization of the Protein Toward Thermal Inactivation

Abstract. *By recombinant DNA techniques, a disulfide bond was introduced at a specific site in T4 lysozyme, a disulfide-free enzyme. This derivative retained full enzymatic activity and was more stable toward thermal inactivation than the wild-type protein. The derivative, T4 lysozyme (Ile*^{3} *→ Cys), was prepared by substituting a Cys codon for an Ile codon at position 3 in the cloned lysozyme gene by means of oligonucleotide-dependent, site-directed mutagenesis. The new gene was expressed in* Escherichia coli *under control of the* (trp-lac) *hybrid* tac *promoter, and the protein was purified. Mild oxidation generated a disulfide bond between the new Cys*^{3} *and Cys*^{97}*, one of the two unpaired cysteines of the native molecule. Oxidized T4 lysozyme (Ile*^{3} *→ Cys) exhibited specific activity identical to that of the wild-type enzyme when measured at 20°C in a cell-clearing assay. The cross-linked protein was more stable than the wild type during incubation at elevated temperatures as determined by recovered enzymatic activity at 20°C.*

In globular proteins, disulfide bonds provide conformational stability (*1, 2*), although the detailed mechanisms by which these bonds influence not only the stability of native structures but also pathways of folding and unfolding have yet to be defined. Until recently, investigators have been limited to one basic approach—the study of protein derivatives generated through the chemical cleavage of disulfide bonds. Partly because of this limitation, most folding and stability studies on disulfide-containing proteins have been designed so that disulfide bond formation is a component of the folding process (*3*). Yet it is also of interest to investigate the differences in stability and folding pathways between two proteins that differ throughout an experiment in the number of cross-links (*4, 5*). For example, it has been shown that a disulfide-free derivative of α interferon irreversibly loses elements of native conformation when incubated under conditions tolerated by the native disulfide-bonded molecule (*5*).

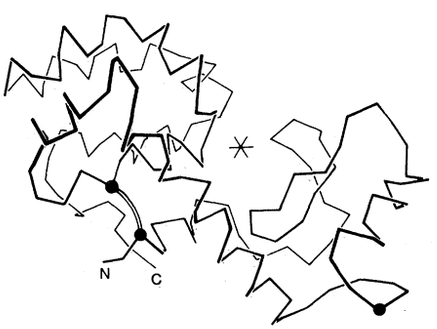

Fig. 1. Computer graphics simulation of T4 lysozyme (Ile^3 → Cys) α-carbon chain, showing the amino- and carboxyl-chain termini (N and C, respectively), the three cysteines (●), and the active site (star). Cys^3 and Cys^{97} are connected by a schematic disulfide.

Recent advances in techniques for specific mutagenesis (*6*) and efficient expression (*7*) of genes cloned into *Escherichia coli* make possible other experimental approaches. To study the modes by which disulfide bonds can stabilize proteins, we used these recombinant DNA methods to insert disulfide bonds into a normally disulfide-free protein. We chose as a model phage T4 lysozyme (*8*), a muramidase whose biological function is to break down the cell wall of an infected bacterium to facilitate the lytic release of replicated phage. The protein's x-ray crystal structure is known (*9*), and it has two unpaired cysteines and limited thermal stability (*8*). The thermodynamics of unfolding of T4 lysozyme have been studied through the thermal and solvent-effected reversible denaturation of the wild-type molecule and some temperature-sensitive mutants (*10*). T4 lysozyme also exhibits structural homology with egg-white lysozyme (*9*), a protein that contains four disulfide bonds. Recently we achieved efficient expression in *E. coli* of plasmid-encoded T4 lysozyme genes (*11*).

Theoretical calculations suggest that the degree of conformational stability conferred upon a protein by a cross-link increases with the number of amino acids of primary sequence that it spans (*1, 2*). We searched the T4 lysozyme crystal structure (*9*) for sets of amino acids widely separated in primary sequence but with side chain β carbons lying close (within 5.5 Å) in space. We further examined some of these by building simulations of disulfides with the use of computer graphics (*12*) (vector graphics PS300, Evans and Sutherland) and assessing them for the appropriate geometry (*1*). In no case was a simulated disulfide identical in structure to any protein disulfides characterized by x-ray diffraction (*1*). Several candidates, however, appeared to be particularly favorable by these criteria, and one of these was the disulfide produced between cysteines at positions 3 and 97. Examination of this simulated disulfide bond yielded the following values for its structural parameters (*1*): x_1, 117°; x_2, 25°; x_3, 134°; x'_2, 43°; x'_1, −162°; αC-αC distance, 5.71 Å; and βC-βC distance, 4.49 Å. Since residue 97 in wild-type T4 lysozyme is a cysteine, we chose to make a disulfide cross-link by introducing a cysteine at position 3 (Fig. 1).

Using the synthetic oligonucleotide

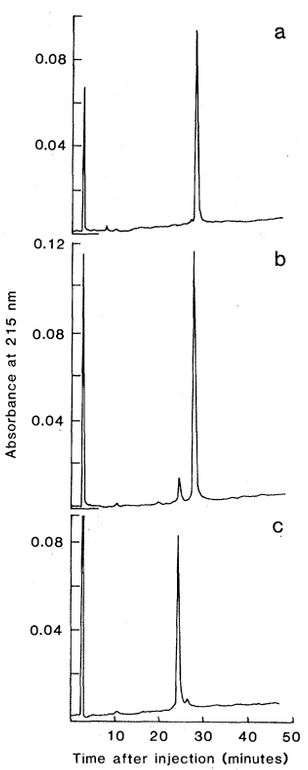

Fig. 2. Reversed-phase HPLC of lysozyme derivatives. Samples were loaded on a Vydak 218-TP546 C_{18} column (Rainin) and eluted at 1 ml/min with a linear gradient of 37 to 52 percent acetonitrile in 0.1 percent aqueous trifluoroacetic acid at 0.33 percent acetonitrile per minute. (a) Wild-type T4 lysozyme, (b) reduced T4 lysozyme (Ile^3 → Cys), and (c) oxidized T4 lysozyme (Ile^3 → Cys).

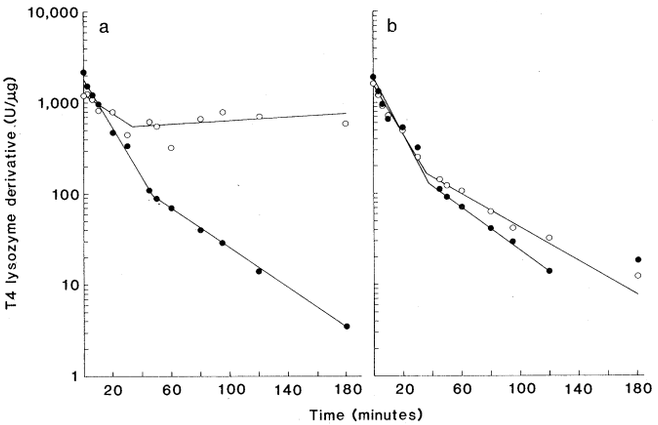

Fig. 3. Kinetics of inactivation of lysozyme derivatives. Lysozymes were dissolved at 3 μg/ml in 100 m*M* potassium phosphate (*p*H 6.5), 0.1*M* NaCl, and 1 m*M* EDTA; 100-μl portions were added to Pyrex tubes equilibrated at 67°C. At various times samples were plunged into ice and then diluted and subjected to the turbidity assay at 20°C (*8, 11*). (a) Wild-type T4 lysozyme (●) and T4 lysozyme ($Ile^3 \rightarrow Cys$) oxidized by incubation with sodium tetrathionate (○); (b) wild-type T4 lysozyme (●) and reduced T4 lysozyme ($Ile^3 \rightarrow Cys$) (○) in the presence of 10 m*M* β-mercaptoethanol.

pAGAATTATGAATTGTTTTGAAATGTTA and an M13mp10 derivative containing the 5′ half of the T4 lysozyme gene (*11*), we generated by standard methods (*6*) an M13 derivative containing the desired ATA to TGT mutation in the lysozyme gene (A, adenine; G, guanine; T, thymine). This was confirmed by M13 sequencing (*13*). The lysozyme sequence was excised from the double-stranded replicative form of M13 and used to reassemble an expression vector containing the full, mutated lysozyme gene under the control of the *tac* II promoter (*14*), as described for another lysozyme mutant (*11*). Transformants were screened by restriction analysis of isolated plasmid and for the ability of frozen cells to lyse upon thawing (*11*). Positive clones were checked on a sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) of crude cellular protein for the appearance of a new band after the addition to a growing culture of the *tac* (*lac*) inducer isopropyl-β-D-thiogalactoside. The plasmid isolated from one of these transformants was designated pT4Lys3C*tac*II. The entire lysozyme-coding sequence of this plasmid was confirmed by M13 sequencing.

Lysozyme purified (*11*) from cells transformed with pT4Lys3C*tac*II had an additional cysteine residue, as shown by titration of protein thiols with Ellman's reagent (data not shown). In addition, automated Edman degradation of the carboxymethylated protein's amino terminus gave the amino acid sequence Met-Asn-CMCys-Phe-Glu-Met-Leu-Arg-Ile-Asp-Glu, consistent with the desired replacement at position 3.

Using mobility in a nonreducing SDS-PAGE as an assay for cross-linking (*5, 15*), we investigated a number of oxidizing conditions for their ability to drive formation of a disulfide bond in the mutant lysozyme (data not shown). We found that T4 lysozyme ($Ile^3 \rightarrow Cys$) purified in the presence of β-mercaptoethanol migrated with the same mobility as wild-type T4 lysozyme. Exposure of this lysozyme mutant to conditions of increasing oxidative strength produced increasing amounts of a faster migrating, presumably cross-linked, species in the gel assay. The same conditions produced no effect on the wild-type enzyme. Reaction of reduced T4 lysozyme ($Ile^3 \rightarrow Cys$) with either sodium tetrathionate or with a glutathione redox buffer generated the cross-link. The results of reversed-phase high-performance liquid chromatography (HPLC) of this product (Fig. 2) suggested the presence of a homogeneous, cross-linked form (*5*).

The added Cys at position 3 brought the total number of cysteines in the protein to three. To determine which of the three possible pairings of cysteines we had obtained in the oxidation, we analyzed trypsin fragments of the oxidized protein. T4 lysozyme ($Ile^3 \rightarrow Cys$) oxidized with sodium tetrathionate was treated with iodoacetic acid to eliminate disulfide scrambling and then digested with trypsin. By comparing the reversed-phase HPLC profiles of the digest with and without reducing agent (*16*), we identified the peptide containing the disulfide bond (not shown). Amino acid compositional analysis (not shown) was in good agreement with one of the three possible disulfide peptides, and the amino acid sequence of this peptide contained, as expected, two tracks. These were consistent with the sequences Met-Asn-(Cys)–Phe-Glu-Met-Leu- and (Cys)-Ala-Leu-Ile-Asn-Met-Val-Phe-Gln-Met-Gly arising from a peptide generated from a disulfide bond between Cys^3 and Cys^{97} of T4 lysozyme ($Ile^3 \rightarrow Cys$).

Neither the Ile → Cys mutation at position 3 nor the formation of the disulfide bond between Cys^3 and Cys^{97} produced any significant effect on enzyme activity. In the turbidity assay (*8, 11*) at 20°C, the relative activities of various T4 lysozyme derivatives were as follows: wild type, 100; reduced ($Ile^3 \rightarrow Cys$), 98 ± 5; oxidized ($Ile^3 \rightarrow Cys$), 97 ± 5.

As an initial investigation of the effect of the added cross-link on protein stability, we heated wild-type and ($Ile^3 \rightarrow Cys$) T4 lysozymes at 67°C and removed portions at various times to determine residual activity (Fig. 3). The initial decay in activity in the wild type had a half-life of 11 minutes. The disulfide-linked mutant appeared to decay more slowly, with an initial half-life of 28 minutes (*17*). More dramatically, the activity of the disulfide form did not fall below 50 percent of the starting activity. In contrast, the wild-type enzyme exhibited only about 0.2 percent of its starting activity after 180 minutes.

Since the disulfide mutant was derived from the wild type by two sequential changes (that is, a primary sequence substitution and a cross-linking of that substituted residue), we assessed the relative contributions of these changes to the difference in stability (Fig. 3a). In the presence of reducing agent, the $Ile^3 \rightarrow Cys$ mutant behaved essentially identically to wild-type T4 lysozyme, suggesting that it was the cross-link and not the amino acid substitution alone that provided the additional stability observed (Fig. 3b).

Further characterization of this mutant is required to unravel the mechanism or mechanisms by which the added disulfide modulates both the rate and extent of inactivation. Preliminary data points to a major role for the unpaired Cys at position 54 in the thermal decay kinetics of the disulfide mutant (*23*). We also have preliminary evidence suggesting that the inactivation of wild-type T4 lysozyme under these conditions is largely, but not completely, due to conformational denaturation. There are several possible ways in which an added disulfide cross-link might provide stability against irreversible conformational decay. There may also be some irreversible chemical damage to T4 lysozyme under the conditions of our experiments. If so, a cross-link could inhibit such chemical effects by restricting the reversible unfolding or breathing of the protein, limiting access to chemically sensitive sites.

Recombinant DNA methods of polypeptide synthesis (*7*) recently have been used to produce engineered proteins (*18*) that incorporate changes in the native

structure ranging from a deletion of about 30 amino acids in proinsulin (*19*) to single amino acid changes in tyrosyl–transfer RNA synthetase (*20*) and dihydrofolate reductase (*21*). In each of these cases examination of x-ray crystal structure data has contributed to the design of the alteration. In modeling disulfide bonds, computer graphics analysis of crystal structure data may be especially important, since there appear to be important configurational as well as steric constraints on protein disulfide bonds (*1*).

Our results show that introduction of disulfides into proteins that have evolved without them is possible and that such derivatives can retain activity and also have enhanced stability. This has implications not only for studies of protein folding, structure, and function but also for commercial applications of engineered enzymes and other proteins.

While this work was in progress, Villafranca *et al.* (*21*) described a disulfide-bonded *E. coli* dihydrofolate reductase prepared by methods similar to those we have described. This derivative was fully active in the reduced state but had "significantly diminished" activity in the oxidized state. No data on the stability of this derivative was reported.

Note added in proof: T4 lysozyme ($Ile^3 \rightarrow Cys$) with a 3 to 97 disulfide link and a free thiol at Cys^{54} shows a thermal stability at 67°C, identical to the wild-type enzyme. The disulfide derivative with Cys^{54} blocked by reaction with iodoacetate has an activity half-life of about 6 hours under these conditions; in its reduced state, it loses stability in the same way as the material described in Fig. 3b. The two phases of activity decay for the mutant in Fig. 3a are thus due to two molecular forms, differing at Cys^{54} by the presence or absence of a blocking group (probably thiosulfate from the oxidant).

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Prophenoloxidase Activation: Nonself Recognition and Cell Cooperation in Insect Immunity

Abstract. *The mechanism of nonself recognition by the immune system of insects is unknown. In this report the activation of the prophenoloxidase system in the wax moth* Galleria mellonella *by a microbial product is shown to enhance the recognition of nonself material. These results explain previous observations of the interaction of two different blood cell populations in the cellular defense reactions of insects.*

An understanding of the interrelations between the insect host and parasite is important because numerous insects act as vectors of the most devastating diseases. Many of the disease organisms, such as those causing malaria and also possibly trypanosomiasis, have one or more stages in the hemocoel of their hosts, where they apparently develop and migrate unmolested (*1*). This is unexpected considering the speed and effectiveness of the insect cellular defense reactions in response to various parasites and foreign material (*2*).

Despite the efficacy of arthropod immune systems, insects and other invertebrates—in contrast to vertebrates—do not use immunoglobulins as recognition molecules and yet exhibit considerable selectivity and activity in their nonself reactions (*2, 3*). The body fluids of many noninsectan invertebrates, however, contain factors capable of acting as opsonins and stimulating the uptake of foreign particles by the blood cells (*3*). The most likely candidates for the invertebrate recognition molecules are the agglutinins, which are present in a wide range of species (*3–5*). However, in only two species, *Crassostrea gigas* and *Mytilus edulis*, have agglutinins been purified and shown unequivocally to have opsonic properties (*6, 7*). Insect hemolymph also contains agglutinins, but in the few species tested these did not enhance phagocytosis (*8*) either because the experimental systems used were suboptimal or because other factors may play a role in nonself recognition.

Melanin deposition around parasites is commonly associated with the cellular and humoral defenses of arthropods (*9–11*); in consequence, this substance and precursor enzymes used in its production, such as phenoloxidase, have been regarded as important components of these immune reactions. The prophenoloxidase-activating system has been reported to be triggered by microbial products (*10, 12, 13*), possibly through limited