Unpaired Cysteine-54 Interferes with the Ability of an Engineered Disulfide To Stabilize T4 Lysozyme†

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ABSTRACT: We have introduced an intramolecular disulfide bond into T4 lysozyme and have shown this molecule to be significantly more stable than the wild-type molecule to irreversible thermal inactivation [Perry, L. J., & Wetzel, R. (1984) *Science (Washington, D.C.) 226*, 555–557]. Wild-type T4 lysozyme contains two free cysteines, at positions 54 and 97, and no disulfide bonds. By directed mutagenesis of the cloned T4 lysozyme gene, we replaced Ile-3 with Cys. Oxidation in vitro generated an intramolecular disulfide bond; proteolytic mapping showed this bond to connect Cys-3 to Cys-97. While this molecule exhibited substantially more stability against thermal inactivation than wild type, its stability was further enhanced by additional modification with thiol-specific reagents. This and other evidence suggest that at basic pH and elevated temperatures Cys-54 is involved in intermolecular thiol/disulfide interchange with the engineered disulfide, leading to inactive oligomers. Mutagenic replacement of Cys-54 with Thr or Val in the disulfide-cross-linked variant generated lysozymes exhibiting greatly enhanced stability toward irreversible thermal inactivation.

The disulfide bond is a unique structural feature of proteins which has characteristics of both primary and tertiary structure. The role of such bonds in protein structure has been studied previously by comparative analysis of chemical derivatives lacking one or more native disulfides. This approach suffers from the limitations common to all chemical modification approaches. A special limitation is that the only disulfides amenable to study by chemical methods are those which exist in naturally derived proteins.

Recently it has become feasible to use recombinant DNA/cloning techniques (Harris, 1983; Wetzel & Goeddel, 1983), combined with site-specific mutagenesis of cloned genes (Zoller & Smith, 1983), to produce large amounts of particular protein variants in *Escherichia coli* and other organisms. By combining these methods with computer graphics analysis (Langridge et al., 1981), it is possible to introduce new disulfide bonds into proteins (Villafranca et al., 1983; Perry & Wetzel, 1984). This approach can overcome many of the pitfalls of chemical modification. In addition, it allows specific placement of cross-links and construction of multiple variants for examination of the interaction of disulfides with other structural features.

Recently we reported the use of such techniques to insert a single disulfide between positions 3 and 97 of T4 lysozyme, and the ability of this bond to stabilize the protein toward irreversible thermal inactivation (Perry & Wetzel, 1984). Wild-type T4 lysozyme has two free cysteines, at positions 54 and 97, and no disulfides. Introduction of Cys-3, by mutagenesis, generated a molecule with three cysteines and three possible intramolecular disulfide arrangements. In this paper, we describe in detail the preparation and characterization of this disulfide-cross-linked T4 lysozyme [T4 lysozyme-(I3C^{-97C})].[1] In addition, we describe experiments which suggest that the unpaired Cys-54 of this derivative can compromise, via thiol/disulfide interchange, the ability of the disulfide to stabilize lysozyme. Finally, we report the construction and properties, including improved stability, of double mutants containing the disulfide but lacking Cys-54.

Experimental Procedures

Materials

E. coli strains D1210 (*lac*I^q, *lac*Y$^+$, *lac*O$^+$, *lac*Z$^+$, *gal*$^-$, *pro*$^-$, *leu*$^-$, *thi*$^-$, *end*$^-$, *hsm*$^-$, *hsr*$^-$, *rec*A$^-$, *rps*L$^-$) and AS19 (prototroph) were obtained from Drs. Hermann de Boer (Genentech) and Fred Young (Genentech). The phage M13mp10 was provided by Peter Seeburg (Genentech). Synthetic oligonucleotides were provided by the organic synthesis group at Genentech.

Methods

Lysozyme activity was assayed by the turbidimetric method as described by Tsugita et al. (1968), except that 1 mM ethylenediaminetetraacetic acid (EDTA)[2] was included in the assay buffer and the cellular substrate was lyophilized *E. coli*

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[1] In this paper, we designate chemical and genetic derivatives of T4 lysozyme by listing the changes in amino acid sequence in parentheses after the parent enzyme's name. Replacements are abbreviated, using the single-letter code for amino acids (Dayhoff, 1972); in this manner, replacement of Ile-3 with Cys is abbreviated "I3C". Multiple replacements and other residue descriptions are listed separated by diagonal slashes, e.g., I3C/C54T. The chemistry of cysteine residues is indicated by a superscript: covalent attachment of a Cys-3 replacement to Cys-97 in a disulfide bond is abbreviated I3C^{-97C}. Carboxymethylation is abbreviated 54C^{CM}. Thus, the name of the T4 lysozyme derivative containing a disulfide between Cys-97 and the newly introduced Cys-3, and with Cys-54 in the reduced state, is "T4 lysozyme(I3C^{-97C}/54C^{SH})". When the chemical state of cysteine is not indicated, it means it was not specifically established for that species or is not relevant to the discussion.

[2] Abbreviations: Cl_3CCOOH, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactoside; PMSF, phenylmethanesulfonyl fluoride; PEI, poly(ethylenimine); DEAE, diethylaminoethyl; CM, carboxymethyl; GSH, reduced glutathione; GSSG, oxidized glutathione; DTT, dithiothreitol; IAA, iodoacetic acid; IAM, iodoacetamide; RF, replicative form of M13 DNA; LB, Luria broth.

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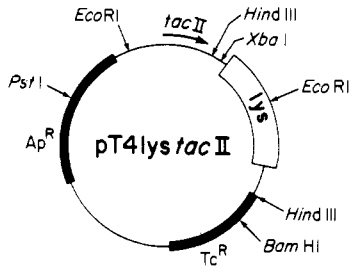

FIGURE 1: Plasmid vector for expression of T4 lysozyme and variants under control of the *tac*II promoter (de Boer et al., 1982).

B mutant AS19 (Sekiguchi & Iida, 1967). Lysozymes to be assayed were diluted and stored in a lysozyme dilution buffer composed of 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, and 2 mg/mL bovine serum albumin. HPLC was performed on a system composed of two M-6000 pumps and an M-660 solvent programmer (Waters), with monitoring of the eluent stream with a 788 dual-wavelength detector (Micromeritics) at 215 and 280 nm. SDS–PAGE was performed according to Laemmli (1970).

Site-Directed Mutagenesis. Restriction digests, isolation of DNA fragments, ligation reactions, and transformations were performed essentially as described by Maniatis et al. (1982). The single-stranded DNA template for the I3C mutagenesis was an M13mp10 derivative containing the *Xba*I/*Eco*RI small fragment of the T4 lysozyme gene of pT4lys*tac*II (Figure 1). The *Xba*I/*Eco*RI fragment for this construction was obtained from a plasmid precursor to pT4lys*tac*II, namely, pT4lysXR*trp*Δ5′ (Perry et al., 1985). The mutagenesis reaction was accomplished by using the "gapped duplex" method (Kramer et al., 1984). Mutants were identified by hybridization of DNA, on nitrocellulose blots of M13 plaques, with the ^{32}P-labeled mutagenesis primer. After washes of increasing stringency, the blots were autoradiographed (Wallace et al., 1981).

After verification by M13 DNA sequencing (Messing, 1983), DNA containing the mutated lysozyme gene fragment was excised from M13 with *Xba*I and *Eco*RI and purified by PAGE. This fragment was used to construct the T4 lysozyme(I3C) expression vector pT4lys3C*tac*II, in analogy to the strategy used to construct pT4lys*tac*II (Perry et al., 1985). The entire lysozyme gene of pT4lys3C*tac*II was sequenced by the M13 method to verify that only the desired mutation was introduced.

For the construction of other mutants, we used an M13mp8 derivative containing the entire lysozyme gene excised from pT4lys*tac*II by *Hin*dIII digestion. Construction of plasmid vectors for expression of variants employed an intermediate plasmid, pΔlys*tac*II, formed by excision of the *Hin*dIII fragment containing the T4 lysozyme gene from pT4lys*tac*II and reclosure. This plasmid was cleaved with *Hin*dIII and then ligated to the *Hin*dIII fragment from M13 genomes containing variant T4 lysozyme genes. Oligonucleotides used for introduction of mutations were

Ile-3 → Cys pAGAATTATGAATTGTTTTGAAATGTTA

Cys-54 → Thr pTACACCATTGGTATTGCGCCCAAT

Cys-54 → Val pTACACCATTGACATTGCGCCCAAT

Both fragments for mutations at position 54 were designed to introduce a new *Hha*I site in a silent mutation adjacent to the amino acid replacement site. This new restriction site was used for screening of M13 RFs and plasmids for the desired mutation.

Cell Growth and T4 Lysozyme Purification. A 4-L shake flask containing 1 L of M9 minimal medium (Miller, 1972) plus 10 μg/mL tetracycline and 50 μg/mL carbenicillin (Sigma) was inoculated with 25 mL of an overnight culture of *E. coli* D1210/pT4lys3C*tac*II in LB (Miller, 1972) plus 50 μg/mL carbenicillin. At late log phase (about OD_{650} = 2), IPTG was added to a final concentration of 1 mM. After 2 h, cells were harvested by centrifugation, and the pellet (4 g) was frozen. Thawing the pellet in 40 mL of 50 mM Tris-HCl, 1 mM EDTA, and 0.1 mM PMSF, pH 8, caused cell lysis. The lysate was treated, with stirring on ice, with 1.4 mL of 5% PEI-HCl, pH 7.5. The slurry was allowed to settle, then filtered through glass wool, and pelleted.

The supernate was loaded through tandem columns [(1) 10 mL of DEAE-cellulose; (2) 10 mL of CM-cellulose] equilibrated with 50 mM Tris-HCl, 3 mM 2-mercaptoethanol, and 1 mM EDTA, pH 8. After being washed with equilibration buffer, the DEAE column was removed and the CM column eluted with a linear gradient of 0–1.0 M NaCl in equilibration buffer (100 mL total volume). Lysozyme-containing fractions were pooled and chromatographed on a G-75 column equilibrated with 0.2 M sodium phosphate, pH 6.5, and 1 mM EDTA. Ion exchange and gel filtration were performed at 4 °C.

Oxidation of T4 Lysozyme(I3C). A solution of 0.5 mg/mL T4 lysozyme(I3C) in 50 mM Tris-HCl, 0.2 M NaCl, and 1 mM EDTA was made 50 mM in GSH and the pH adjusted to 8.5 with a solution of unbuffered Tris base. After being stirred in an uncovered beaker at 25 °C for 2 h, the solution was made 10 mM in GSSG and stirred for 4 h. At this time, the reaction to form the disulfide cross-link was almost complete, as judged by HPLC analysis (Perry & Wetzel, 1984). The reaction mixture was dialyzed at 25 °C for 4 h against 2 L of 50 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, and 1 mM GSH, pH 8.5. HPLC analysis showed no reduced form. The pH was adjusted to 7.5 with HCl and the product stored at 4 °C.

Reduction of Lysozyme Derivatives. For thiol titration or spectroscopy of reduced protein, lysozyme samples were treated with 10 mM DTT in 0.1 M phosphate, pH 8.5, 7 M guanidine hydrochloride, and 1 mM EDTA. After 1 h at room temperature, the reaction mixture was dialyzed exhaustively at 4 °C against 0.1 M NaCl, 5 mM HOAc, and 1 mM EDTA. Stored in this buffer, reduced lysozymes are stable to oxidation for several days. For Ellman titrations, the reduction reaction mix was dialyzed at 4 °C against nitrogen-purged 10 MM NaOAc, pH 5.5, 8 M guanidine hydrochloride, and 1 mM EDTA.

Alkylation of T4 Lysozyme Sulfhydryl Groups. Solutions of T4 lysozymes (0.3 mg/mL) in 50 mM Tris-HCl, pH 8.5, 8 M guanidine hydrochloride, and 1 mM EDTA were reacted with IAA (10 mM) in the dark for 30 min and then dialyzed with two changes against 1 L of 50 mM sodium phosphate, 0.1 M NaCl, and 1 mM EDTA, pH 6.5.

RESULTS

Eleven percent of the M13 plaques generated in the Ile-3 → Cys mutagenesis procedure strongly hybridized to the mutagenesis primer under stringent conditions (Methods). Single-stranded template DNA was purified from four of these mutant M13 plaques; all of them contained the desired sequence. Other mutations were made with similar apparent efficiencies. *E. coli* D1210, transformed with pT4lys*tac*II plasmids containing mutant lysozyme genes, gave lysozyme

Table I: Thiol Contents of Various Lysozyme Derivatives[a]

T4 lysozyme derivative	mol of SH/mol of protein
wild type	1.7
wild type ($54C^{CM}/97C^{CM}$)	0.0
($I3C^{SH}/54C^{SH}/97C^{SH}$)	2.9
($I3C^{-97C}/54C^{SH}$)	1.1
($I3C^{-97C}/54C^{CM}$)	0.0

[a] Samples were prepared as described under Methods. Protein concentration was determined by the method of Bradford (1976). A 1-mL aliquot of each sample was adjusted to pH 9, and 20 μL of a 4 mg/mL solution of DTNB was added (Means & Feeney, 1971).

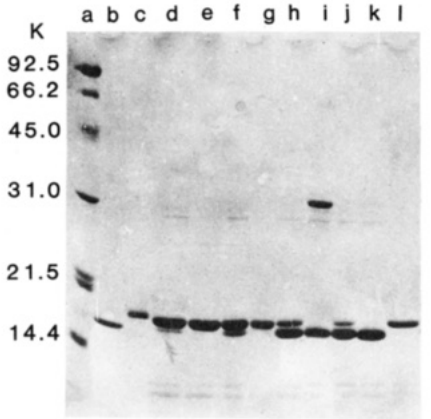

FIGURE 2: Nonreducing SDS–PAGE on various T4 lysozyme derivatives. Each sample was treated with 10 mM IAM before addition of SDS sample loading buffer to inhibit thiol/disulfide interchange in SDS. Samples were heated at 95 °C for 2 min and cooled before loading. Lane a, low molecular weight standards; lane b, native interferon αA; lane c, reduced, carboxymethylated interferon αA; lane e, wild-type T4 lysozyme; lane g, wild-type T4 lysozyme treated with sodium tetrathionate; lane d, T4 lysozyme(I3C) in pH 5.5 sodium acetate and 3 mM 2-mercaptoethanol; lane f, T4 lysozyme(I3C) dialyzed from pH 5.5 to pH 8 in 50 mM Tris-HCl and 1 mM EDTA, the solution used for the following reactions: lane h, 5 mM GSH and 5 mM GSSG (16 h, room temperature); lane i, 2.5 mM $Cu(OAc)_2$ (16 h, room temperature); lane j, 5 mM GSSG (16 h, room temperature); lane k, 10 mM sodium tetrathionate (16 h, room temperature); lane l, wild-type T4 lysozyme.

levels, after IPTG induction, of 5–10 mg L^{-1} OD_{650}^{-1}, similar to the levels of wild type in the same system (Perry et al., 1985).

The purification method described under Methods, which relies on the unusually high p*I* value of T4 lysozyme (Tsugita, 1971), resulted in lysozyme preparations greater than 95% pure, based on Coomassie staining of an SDS–PAGE gel. Three cysteines were found in purified, reduced T4 lysozyme(I3C) by titration of sulfhydryl groups with Ellman's reagent (Table I), and amino acid sequencing of the reduced, carboxymethylated protein revealed a carboxymethylcysteine residue in the third cycle of the Edman degradation (data not shown).

Figure 2 shows the use of nonreducing SDS–PAGE to monitor the intramolecular cross-linking of T4 lysozyme(I3C). The relative amount of the more rapidly migrating, cross-linked form increases with the oxidizing strength of the reagent: tetrathionate and copper-catalyzed O_2 oxidation are the most efficient oxidants under these conditions. Subsequent to this experiment, we determined that even relatively mild redox buffers (containing a stoichiometric excess of reducing agent) can generate the cross-linked form (see Experimental Procedures). The lysozyme dimer in lane i presumably arises as a kinetic product of copper-catalyzed air oxidation; this cannot decay to the (presumably) thermodynamically favored cross-linked monomer because this oxidation system does not allow thiol/disulfide interchange.

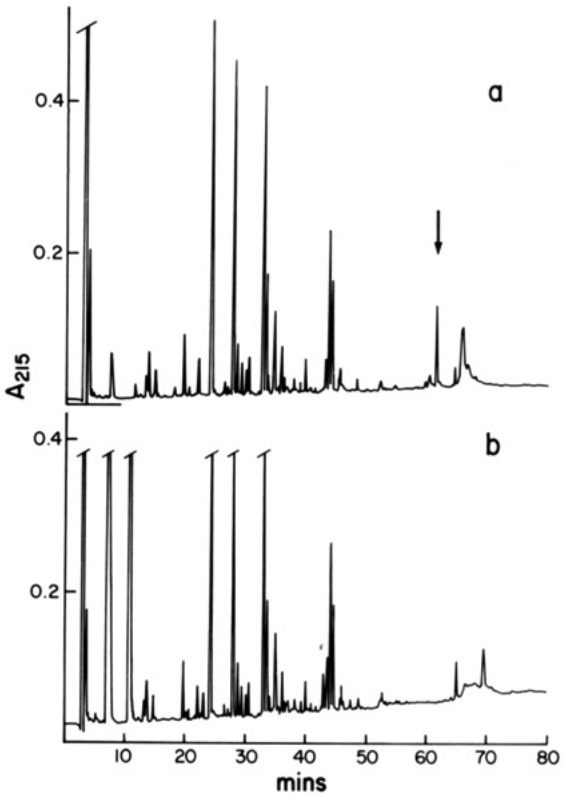

FIGURE 3: Analysis of trypsin digests of oxidized T4 lysozyme($I3C^{-97C}$) by HPLC. The sample was alkylated in urea with IAA and then dialyzed into 100 mM Tris-HCl, pH 8, 0.1 M NaCl, and 1 mM EDTA. The sample was heated to 95 °C for 5 min and then cooled to 37 °C, and trypsin (1:40 enzyme:substrate ratio) was added; after 3 h, an equivalent amount of trypsin was added; after 3 more h, the reaction was stopped by adjusting to pH 2. Samples were chromatographed in the system described in Figure 2 except the gradient was from 4% to 64% acetonitrile in 90 min. (a) 40-μg digest; (b) 40-μg digest after treatment at pH 9 with 10 mM DTT for 20 min at room temperature. The arrow indicates the only significant trypsin fragment peak whose mobility is affected by reduction.

Figure 3 shows the characterization of T4 lysozyme($I3C^{-97C}$) by HPLC of trypsin digests. Reduction with DTT of a portion of the trypsin digest of oxidized cross-linked mutant (Figure 3b) specifically eliminates a peak found in the unreduced digest (Figure 3a). Amino acid sequence analysis of this peptide is consistent with two tracks of sequence, Met-Asn-(Cys)-Phe-Glu-Met-Leu and (Cys)-Ala-Leu-Ile-Asn-Met-Val-Phe-Gln-Met-Gly, which are in turn consistent with a tryptic peptide containing a Cys-3 to Cys-97 cross-link (Perry & Wetzel, 1984).

Table I summarizes measurements of the thiol content of various lysozymes. It shows that the reduced single variant T4 lysozyme($I3C^{SH}/54C^{SH}/97C^{SH}$) possesses one more cysteine thiol than the wild-type protein and that, upon oxidation to form a disulfide, two cysteine thiols are masked from titration. It also shows that reaction of IAA with both wild-type T4 lysozyme and cross-linked T4 lysozyme($I3C^{-97C}/54C^{SH}$) eliminates titratable thiols.

Figure 4 shows the thermal inactivation of wild-type and cross-linked T4 lysozymes and their carboxymethylated derivatives at pH 8 and 67 °C. [Under these conditions, the wild type decays significantly faster than at pH 6.5 (Perry & Wetzel, 1984).] T4 lysozyme($I3C^{-97C}/54C^{SH}$) (Table I) decays over a period of 30 min to less than 10% of its starting activity. The figure shows that carboxymethylation stabilizes both the wild-type and the cross-linked variant; the mechanisms by which alkylation affects the stabilities of these two

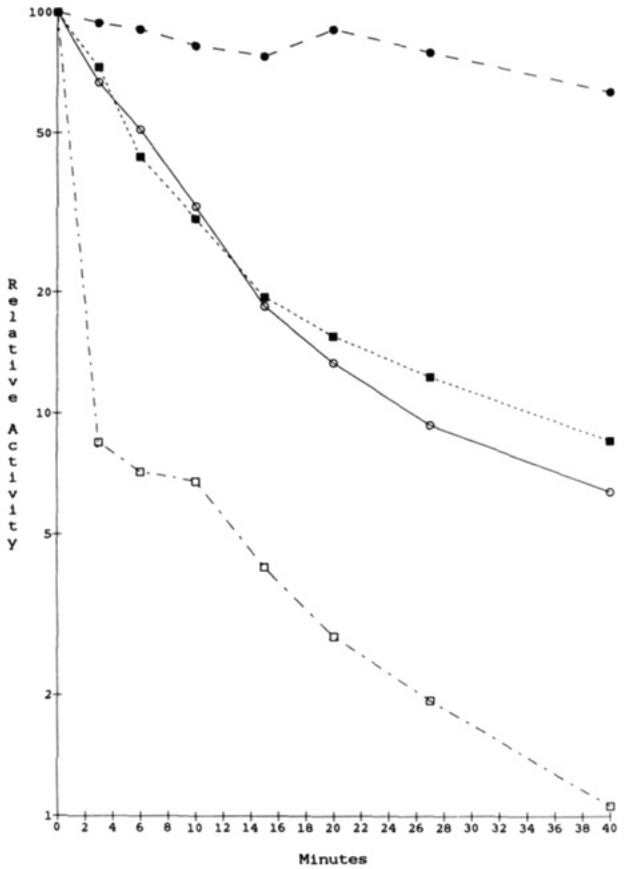

FIGURE 4: Thermal inactivation kinetics of various Cys-54 lysozyme derivatives. At time zero, 100-μL aliquots of lysozyme solutions were added to preequilibrated 6 × 50 mm Kimax culture tubes at 67 °C. At various times, tubes were removed to ice, diluted with lysozyme dilution buffer (Methods), and assayed for residual cell clearing activity at 20 °C. Heated samples contained 30 μg/mL lysozyme in buffer containing 100 mM potassium phosphate, pH 8, 100 mM KCl, and 1 mM EDTA. Lysozymes studied were wild type with (■) and without (□) IAA pretreatment (Methods) and disulfide-cross-linked T4 lysozyme(I3C^{-C97}/54C^{SH}) with (●) and without (○) IAA pretreatment. Values plotted are activities relative to unheated control for each variant.

Table II: Ability of Guanidine Hydrochloride Treatments To Restore Activity to Heat-Inactivated Lysozymes[a]

heat-inactivated lysozyme	% rel reactivation A	B	C
T4 lysozyme(54C^{SH}/97C^{SH}) (wild type)	89	83	46
T4 lysozyme(54C^{CM}/97C^{CM}) (wild type)	64	65	64
T4 lysozyme(I3C^{-97C}/54C^{SH})	0	59	1

[a] Samples were heated under the conditions described in Figure 4 and cooled on ice when residual activity was less than 10%. Ten-microliter aliquots were added to 500 μL of 8 M guanidine hydrochloride with no further additions (column A) or plus 10 mM DTT (column B) or 10 mM IAM (column C). After 10 min at room temperature, aliquots were diluted 25-fold into 0.1 M potassium phosphate, pH 4. After another 10 min at room temperature, this refolding reaction was diluted 1:2 with lysozyme dilution buffer (Methods). Activities were measured at 20 °C in the turbidimetric assay and are presented here relative to the unheated lysozyme derivative exposed to the same denaturing/renaturing conditions. Values are the averages of duplicate determinations.

molecules are different, however (see below). The carboxymethylated, cross-linked variant T4 lysozyme(I3C^{-97C}/54C^{CM}) exhibits only a slight loss of activity in this experiment.

Table II shows the different responses among various heat-inactivated lysozymes to a number of denaturation/renaturation treatment regimens. The majority of the activity

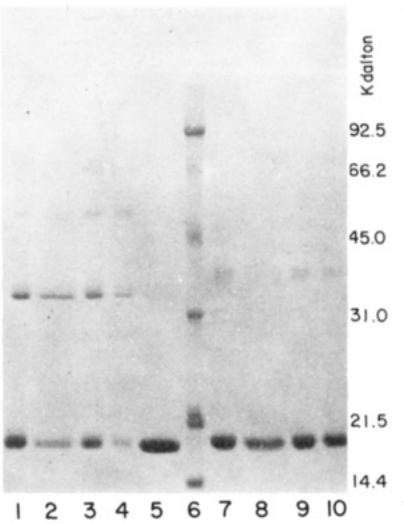

FIGURE 5: Products of thermal inactivation of disulfide-cross-linked T4 lysozyme(I3C^{-97C}/54C^{SH}). Two-milliliter samples were heated at 67 °C in 12 × 75 mm glass tubes in the pH 8 buffer described in Figure 4. Time points were cooled on ice, reacted 60 min with 5 mM IAM at room temperature, and then precipitated with 15% w/v Cl_3CCOOH at 0 °C overnight. Samples were spun in a clinical centrifuge and the pellets resuspended in about 200 μL of 2% SDS gel loading buffer containing 5 mM IAM. Unbuffered 2 M Tris was used to return the pH of the samples to a basic pH. Half of each solution was treated with 10 mM DTT before the gel was loaded. Lanes 6–10 were loaded in the presence of 10 mM DTT. Identity of samples: Cross-linked T4 lysozyme(I3C^{-97C}/54C^{SH}), the starting material before the pH was adjusted to 8 (lane 5); $t = 0$, immediately after adjustment to pH 8, 100% residual activity (lanes 1 and 7); 10 min, 67 °C, 87% residual activity (lanes 2 and 8); 20 min, 67 °C, 62% residual activity (lanes 3 and 9); 40 min, 67 °C, 26% residual activity (lanes 4 and 10); Bio-Rad low molecular weight standards (lane 6). A sample of the material loaded in lane 5 was also loaded in a lane remote from any reducing lanes (not shown); this showed that the apparent homogeneity of this sample was not an artifact of reducing agent diffusion [see Morehead et al. (1984)].

lost in the thermal inactivation of wild-type T4 lysozyme can be recovered by addition of guanidine hydrochloride followed by dilution to native conditions. This suggests that under these conditions most of the activity is lost via conformational transitions followed by aggregation or precipitation. The presence of reducing or alkylating agents does not greatly influence recovery after guanidine treatment which suggests that thiol/disulfide chemistry does not play a significant role in inactivation or reactivation of the wild-type protein. This is true for carboxymethylated wild type as well but is in contrast to the result with heat-inactivated T4 lysozyme(I3C^{-97C}/54C^{SH}). Activity can only be recovered from this preparation if DTT is included with the guanidine treatment. This suggests that a disulfide bond restricts the ability of this molecule to renature.

Figure 5 shows that the heat inactivation of T4 lysozyme(I3C^{-97C}/54C^{SH}) results from the formation of disulfide-linked oligomers of lysozyme. While the starting material is monomeric (lane 5), adjustment to pH 8 at room temperature generates some dimeric material (lane 1), and heating at 67 °C generates higher oligomers, some incapable of passing through the stacking gel [lanes 3 and 4 contain Coomassie-stained material at the top of the stacking gel, which does not show up well in the photograph (lanes 2–4)]. The disulfide-bonded nature of these oligomers is revealed by the regeneration of monomeric material when samples are loaded in reducing agent (lanes 7–10). Similar gels (data not shown) suggest that most of the inactivated wild type formed under these conditions is monomeric and does not contain disulfide bonds.

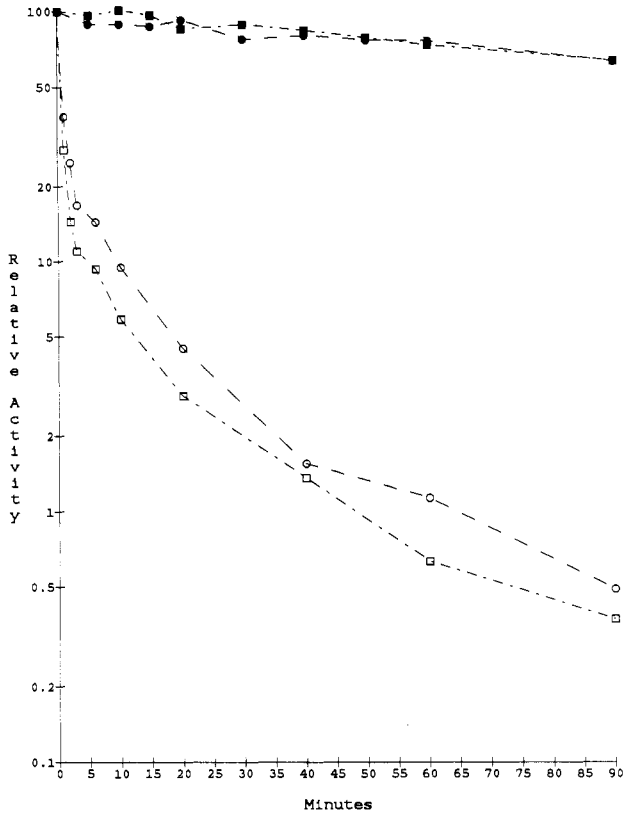

FIGURE 6: Thermal inactivation kinetics of T4 lysozyme derivatives containing replacements at position 54. Conditions were the same as described in the legend to Figure 4. Samples were T4 lysozymes (C54T) (○) and (C54V) (□) and disulfide-cross-linked lysozymes ($I3C^{-97C}$/C54T) (●) and ($I3C^{-97C}$/C54V) (■).

Figure 6 shows that replacement of Cys-54 with Thr or Val generates cross-linked derivatives that are highly stable to incubation at pH 8 and 67 °C. These stabilities are similar to that of T4 lysozyme($I3C^{-97C}$/$54C^{CM}$) (Figure 4).

Figure 6 also shows that (non-cross-linked) single variants C54T and C54V exhibit about the same low stability as wild-type T4 lysozyme and are less stable than carboxymethylated wild type (Figure 4). Carboxymethylation of C54T and C54V produced derivatives [T4 lysozymes ($C54T/97C^{CM}$) and ($C54V/97C^{CM}$)] about as stable as carboxymethylated wild type (data not shown). It is not clear how reaction with IAA stabilizes non-cross-linked lysozymes, since it has been shown (see above) that inactivation of wild type does not involve significant disulfide formation. Cys-97 may be destabilizing in some other way; CM-Cys-97 may provide stability through noncovalent effects, or IAA may be modifying other residues to provide the stabilization.

Table III presents specific activities of the lysozyme genetic variants described in this paper. While replacements tend to lower the activity, cross-linking between residues 3 and 97 has no detectable effect on activity.

Table III: Relative Activities of Lysozyme Variants[a]

variant	rel activity: reduced	rel activity: oxidized (cross-linked)
wild type	100	
I3C	78	77
C54T	85	
C54V	89	
I3C/C54T		53
I3C/C54V		54

[a] Activities were measured in glass cuvettes at 23 ± 0.5 °C in the turbidity assay. To minimize artifacts from possible nonlinearity in the assay, aliquots of each stock solution were chosen to produce about the same observed rate. Protein concentration was determined by the Bradford (1976) assay using wild-type T4 lysozyme as a standard. The values obtained were in good agreement with concentrations calculated by using a value of 1.22 OD_{280} units/mg of lysozyme (Tsugita, 1971). Measurement of reduced T4 lysozyme(I3C) was made in buffer containing 10 mM DTT, which produced no effect on wild-type activity.

Discussion

Examination of disulfide bonds in proteins whose refined X-ray crystal structures are available suggests that there may be some constraint on one's ability to introduce such bonds by mutagenesis methods. One such constraint is the α-carbon distance of the residues to be bridged; these distances in characterized protein disulfides vary from 4.5 to 6.5 Å (Richardson, 1981; Thornton, 1981). In addition, the five dihedral angles generated by the disulfide cross-link are also found to be confined to certain values because of electronic and steric considerations (Richardson, 1981). For example, χ_3, the sulfur–sulfur dihedral angle, is constrained to values near ±90°.

In an oxidizing environment, there is a considerable driving force for the formation of even a somewhat disfavored disulfide bond. This suggests that any pair of cysteines inserted into a protein at sites within a few angstroms of the disulfide range might be induced to form a disulfide under appropriate conditions. Thus, formation of a disulfide is in itself not sufficient evidence for the compatibility of the disulfide with the native structure.

After examination of the T4 lysozyme X-ray crystal structure (Remington et al., 1978), the 3–97 locus was chosen on the basis of the perceived steric requirements of disulfides and the fact that residue 97 in the wild-type enzyme is a cysteine (Perry & Wetzel, 1984). The results shown in Figure 2 suggest that the 3–97 disulfide forms even in thiol/disulfide exchange buffers of significant reducing strength, a characteristic of native disulfide bonds in globular proteins (Creighton, 1983). There is no evidence for the formation of incorrect intramolecular disulfides involving Cys-54, a residue remote from positions 3 and 97 in the three-dimensional structure (Remington et al., 1978). The retention of good specific activity in the cross-linked derivative shows that the active-site geometry is intact. Circular dichroism spectra provide no evidence for any major secondary or tertiary structural changes caused by the formation of the disulfide (Mulkerrin et al., 1986). Only X-ray crystallographic studies, however, will reveal the extent to which adjustment by the polypeptide chain was required for accommodation of the disulfide bond.

In our initial experiments (Perry & Wetzel, 1984; Figure 2), we formed the 3–97 disulfide using sodium tetrathionate (Means & Feeney, 1971). The disulfide-cross-linked product was clearly more thermally stable than the wild-type protein, by activity retained after incubation at 67 °C. At the same time, the kinetics of inactivation were difficult to interpret; after an initial decay phase with a rate only slightly lower than wild type, activity plateaued at about 50% the starting value. Subsequent studies suggested that the biphasic response was in part due to the presence of two forms of disulfide-cross-linked material and that the forms differed in the chemical status of Cys-54.

Using the more controllable oxidation chemistry of a glutathione redox buffer (Saxena & Wetlaufer, 1970), we prepared a disulfide-cross-linked derivative with Cys-54 in the reduced state (Methods and Table I). A portion of this material, as well as some wild-type lysozyme, was alkylated with

IAA to modify Cys-54 (and Cys-97 of the wild type) (Table I).

The kinetics of thermal inactivation of these alkylated derivatives (Figure 4) suggests a role for an unpaired thiol in the inactivation of T4 lysozyme(I3C^{-97C}). The nature of this effect is revealed by experiments summarized in Table II and Figure 5; when the thiol of Cys-54 is heated, it apparently attacks the 3–97 disulfide of another molecule, forming a disulfide-linked dimer which can go on to oligomerize by further thiol/disulfide interchange. Thiol/disulfide interchange is known to be a problem in proteins possessing both disulfide bonds and unpaired cysteines (Sogami et al., 1969; McKenzie et al., 1972; Nikkel & Foster, 1971; Mark et al., 1984; Wang et al., 1984). Formation of disulfide-cross-linked oligomers by rearrangement of disulfides has been documented for human leukocyte interferon (Morehead et al., 1984) and other proteins.

Figure 4 shows that alkylation with IAA also stabilizes wild type. This implies a role for Cys-97 and/or Cys-54 in the inactivation of this molecule. However, the guanidine renaturation experiments (Table II) and gels on inactivated product (not shown) indicate that inactivation of wild-type T4 lysozyme cannot be accounted for by intermolecular disulfide bond formation. It is not known how alkylation produces the observed stabilization.

Having implicated the free thiol of Cys-54 in the destabilization of disulfide-cross-linked lysozyme (see above), we chose to replace that residue genetically. Computer graphics analysis suggested that Thr and Val would be good choices for the substitution. In fact, Table III shows that neither substitution greatly affects the enzymatic activity of lysozyme in the wild-type background, although either, together with the I3C replacement, drops activity to about 50% that of the wild type.

The stabilities against irreversible thermal inactivation of these cross-linked double variants are greatly enhanced, compared to the wild-type and position 54 single replacements (Figure 6). This confirms the destabilizing role of Cys-54 in the disulfide variants. Cross-linked T4 lysozyme(I3C^{-97C}/C54T) at pH 6.5 and 75 °C has a rate of inactivation about 15% that of wild-type T4 lysozyme; under these conditions, this double variant exhibits essentially the same stability as hen egg white lysozyme (Mulkerrin et al., 1986; Wetzel, 1986), a structurally related protein possessing four disulfide bonds.

We are currently investigating the mechanism(s) by which the disulfide bond, in the absence of complicating thiols, stabilizes T4 lysozyme to both reversible thermal unfolding and irreversible thermal inactivation. It has been shown that the disulfide cross-link increases the T_m of T4 lysozyme in reversible melting experiments (W. Becktel and W. Baase, personal communication). Stability toward irreversible inactivation seems to be achieved by the ability of the disulfide to close down the conformational route of inactivation observed for wild-type T4 lysozyme in the 65–85 °C range (Wetzel, 1986). While we do not know at the molecular level how the disulfide achieves this effect, it is interesting to note that the 3–97 bond provides a covalent link between the N- and C-terminal lobes. In fact, the α helices containing residues 3 and 97 have been proposed to be part of a stable core of wild-type T4 lysozyme [B. Maigret, unpublished results; cited in Desmadril & Yon (1984)].

The introduction of disulfide bonds to generate proteins of altered conformational properties has important commercial potential. In addition, new disulfides might be utilized to increase our knowledge of protein folding, stability, and structure/function relationships (Wetzel, 1986). In better understanding the mechanism(s) by which the 3–97 bond and other disulfides provide(s) structural stability to T4 lysozyme, we hope to elucidate some general structural roles of disulfides in globular proteins (Wetzel, 1986). In addition, such cross-linked T4 lysozymes may prove useful in extending earlier studies of lysozyme stability (Hawkes et al., 1984) by allowing reversible melting studies to be extended into the neutral and basic pH range.

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Bis(8-anilinonaphthalene-1-sulfonate) as a Probe for Tubulin Decay†

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ABSTRACT: The fluorescent apolar probe bis(8-anilinonaphthalene-1-sulfonate) (Bis-ANS) has been used to detect structural correlates of the well-known but poorly understood decay of tubulin function, by which tubulin loses its ability to polymerize and bind drugs in a complex, time-dependent way. The present results indicate that the decay of tubulin is accompanied by the appearance of hydrophobic areas, which bind a total of six Bis-ANS molecules with a dissociation constant of 19 μM. This binding seems to be a result of localized structural changes that are taking place in the tubulin molecule and can be used as a probe for these changes. In particular, circular dichroism measurements revealed no significant changes in the average secondary structure of the protein during the time required for complete binding of the Bis-ANS molecules. Preincubation of tubulin with the antimitotic drugs colchicine, podophyllotoxin, and vinblastine slows the rate of appearance of the hydrophobic region. Vinblastine has the maximal effect followed by colchicine and podophyllotoxin. In contrast, preincubation with maytansine has no effect. In addition, lowering the temperature decreases the rate of appearance of this region. These results correlate with the effect of drugs on the alkylation of tubulin sulfhydryl groups by iodoacetamide [Luduena, R. F., & Roach, M. C. (1981) *Biochemistry 20*, 4444–4450] and with the ability of inhibitors of microtubule assembly to permit the polymerization of tubulin into nonmicrotubule structures.

Horowitz et al. (1984) have recently shown that the hydrophobic probe bis(8-anilinonaphthalene-1-sulfonate) (Bis-ANS)[1] binds to tubulin, inhibiting the formation of microtubules. They have shown this to be due to the strong binding at a single site on tubulin with a K_d of 2 μM. The monomer of Bis-ANS, 1,8-ANS, also binds to tubulin at a single site but with no effect on microtubule assembly and a K_d of 25 μM (Horowitz et al., 1984; Bhattacharyya & Wolff, 1975). Most of these studies were done at a fixed time and temperature. It is well-known that tubulin shows time- and temperature-dependent structural changes and is known to have multiple binding sites for several drugs (Luduena, 1979). Bis-ANS has been shown to bind at more than one site in several proteins (Daniel & Weber, 1966; Anderson, 1971; Takashi et al., 1977; Bohnert et al., 1982). The experiments described herein demonstrate the presence of more than one site for the binding of Bis-ANS to tubulin and that additional binding sites appear as a function of time and temperature. Maximum binding of Bis-ANS is attained in 2 h at 37 °C. Colchicine, podophyllotoxin, and vinblastine prevent changes that are taking place in the tubulin molecule. These results may indicate that the decay of tubulin involves the appearance of hydrophobic areas on the surface of the molecule and that low temperature and certain drugs can stabilize the tubulin molecule and slow down decay.

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[1] Abbreviations: MAPs, microtubule-associated proteins; 1,8-ANS, 8-anilinonaphthalene-1-sulfonate; Bis-ANS, bis(8-anilinonaphthalene-1-sulfonate); EDTA, ethylenediaminetetraacetic acid.

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