Control of Enzyme Activity by an Engineered Disulfide Bond

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A novel approach to the control of enzyme catalysis is presented in which a disulfide bond engineered into the active-site cleft of bacteriophage T4 lysozyme is capable of switching the activity on and off. Two cysteines ($Thr^{21} \rightarrow Cys$ and $Thr^{142} \rightarrow Cys$) were introduced by oligonucleotide-directed mutagenesis into the active-site cleft. These cysteines spontaneously formed a disulfide bond under oxidative conditions in vitro, and the catalytic activity of the oxidized (cross-linked) T4 lysozyme was completely lost. On exposure to reducing agent, however, the disulfide bond was rapidly broken, and the reduced (non–cross-linked) lysozyme was restored to full activity. Thus an enzyme has been engineered such that redox potential can be used to control catalytic activity.

PROTEIN ENGINEERING HAS BEEN successfully applied in order to increase our understanding of, and to modify, protein stability as well as function. Most of these studies of function have focused on residues thought to be important in substrate binding and catalysis [for examples, see (*1–5*)]. We report an approach to controlling catalytic activity by engineering a new disulfide bridge into the active-site cleft of an enzyme. The cross-link prevents substrate from binding to the active site, leading to complete inactivation. On exposure to reducing agent, however, the inactivated enzyme can recover its full activity by the breakage of the cross-link. In this way, one can turn the activity of the enzyme on and off by choosing conditions of suitable redox potential.

Bacteriophage T4 lysozyme (1,4-β-*N*-acetylmuramidase, E.C. 3.2.1.17) catalyzes the hydrolysis of the 1,4-β-glycoside linkage between the alternating units of *N*-acetylmuramic acid and *N*-acetylglucosamine in bacterial peptidoglycan (*6*). X-ray diffraction studies, together with model building, have indicated how the polysaccharide of *N*-acetylmuramic acid and *N*-acetylglucosamine binds within the active-site cleft (*7*), which is located between the amino- and carboxyl-terminal lobes of the molecule (see Fig. 1).

Theoretical calculation and model building, done in collaboration with M. Levitt, suggested a number of possible pairs of amino acids in T4 lysozyme that could be replaced with cysteine and so form disulfide bonds (*8*). Selection of preferred sites was based on reasonable adherence to the known geometry of disulfide bridges and on calculation of the strain energy associated with formation of the disulfide bond. Of these candidates, one pair of amino acids, at positions 21 and 142, was located on opposite sides of the active-site cleft. We anticipated that such a cross-linkage might prevent the cross-linked polysaccharide substrate from entering the active site and thereby inactivate the enzyme. Although the α-carbon distance (8.1 Å) between Thr^{21} and Thr^{142} in the refined wild-type structure (*9*) is slightly longer than those of naturally observed disulfides (ranging from 4.6 to 7.4 Å) (*10*), model building with the use of computer graphics suggested that the formation of a disulfide bond would be possible with slight, localized adjustments of the backbone conformation. Also, the structural flexibility of T4 lysozyme, which appears to allow interdomain hinge-bending motion (*9*), might facilitate formation of the disulfide bond.

To avoid possible thiol-disulfide interchange reaction with the newly engineered disulfide (*11*), we first synthesized a cysteine-free T4 lysozyme using oligonucleotide-directed mutagenesis (*12*). The cysteines at position 54 and 97 in the wild type were replaced with Thr (ACT) and Ala (GCT), respectively. The cysteine-free mutant [designated either C54T-C97A or pseudo–wild type (WT*)] had activity as well as stability essentially identical with those of the wild-type enzyme (see below). In addition, the WT* mutant crystallized isomorphously with wild type, and its structure at 1.7 Å resolution was very similar to that of wild-type lysozyme, except in the vicinity of the mutations. On the template of the WT* gene, oligonucleotide-directed mutagenesis was used again to replace both Thr^{21} (ACA) and Thr^{142} (ACA) by Cys (TGT). The resulting mutant, which contains four mutations relative to the wild-type gene, is designated 21C-142C-WT*. The two mutant genes, which were verified by DNA sequencing (*13*), were subcloned into the expression vector containing *tac* and *lacUV5* promoters (*14*). Expression was induced by the addition of isopropyl-β-thiogalactoside, and the mutant proteins were purified as described (*14, 15*).

Mobility differences in nonreducing and reducing SDS–polyacrylamide gel electrophoresis (SDS-PAGE) were used to assay disulfide cross-linkage (*16*). The purified 21C-142C-WT* migrated with the same mobility as the wild type in the presence of dithiothreitol (DTT) or β-mercaptoethanol.

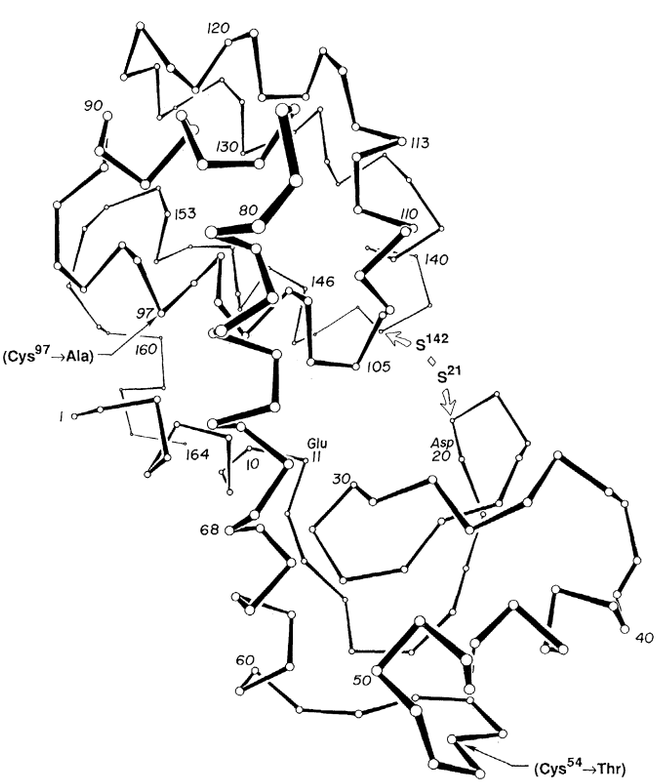

Fig. 1. Schematic view of the α-carbon backbone of bacteriophage T4 lysozyme, showing the locations of the mutations $Thr^{21} \rightarrow Cys$ and $Thr^{142} \rightarrow Cys$ that permit the formation of a disulfide bridge across the mouth of the active-site cleft. The active-site residues Glu^{11} and Asp^{20} are also shown, as are the locations of the ancillary mutations $Cys^{54} \rightarrow Thr$ and $Cys^{97} \rightarrow Ala$.

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However, in the absence of such reducing agents, 21C-142C-WT* migrated faster than wild type. Since disulfide bonds are rarely formed in *Escherichia coli* cell cytosol because of the high intracellular concentration of glutathione and since the mutant lysozyme was purified under nonreducing conditions, the Cys^{21}-Cys^{142} disulfide bond probably formed spontaneously during isolation. No free thiol groups could be detected for 21C-142C-WT* with 5,5′-dithiobis(2-nitrobenzoic acid) under denaturing conditions (*17*), which also indicated disulfide formation. Finally, reversed-phase high-performance liquid chromatography (HPLC) of 21C-142C-WT* showed the presence of non–cross-linked and cross-linked forms of the enzyme under reducing and nonreducing conditions (Fig. 2).

Activities of mutant and wild-type lysozymes were determined by a turbidity assay (*18*). Single mutations at Cys^{54} → Thr or Cys^{97} → Ala did not alter enzyme activity. Also, the cysteine-free WT* mutant showed essentially the same activity as wild type (Table 1). In contrast, the addition of the mutations Thr^{21} → Cys and Thr^{142} → Cys to WT* did alter the activity. The reduced form of 21C-142C-WT* exhibited ~70% of the activity of the wild-type enzyme, whereas its oxidized form had no detectable activity (Table 1). The loss of activity of the oxidized, cross-linked 21C-142C-WT* variant was further confirmed by plate assay (*19*) with purified *E. coli* peptidoglycan as substrate, in which a concentration of the enzyme over 100 times greater than the minimal amount of wild-type lysozyme required to detect activity failed to make halos. The reduced form of 21C-142C-WT* lysozyme may not be quite as active as wild type for a number of reasons. Residue 21, the site of one of the Cys substitutions, is presumed to contact bound saccharides (*7*). Also, the adjacent residue, Asp^{20}, may participate in catalysis (*20*). It is not surprising that a structural perturbation in this vicinity reduces the activity of the enzyme by 30%.

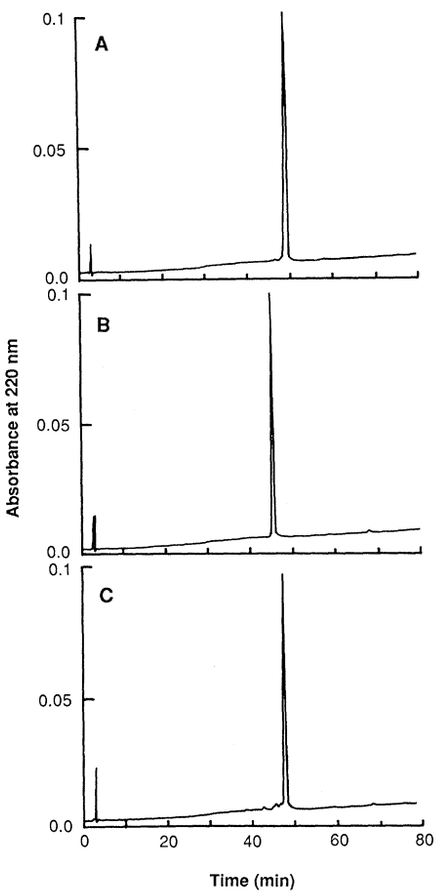

Fig. 2. Reversed-phase HPLC of T4 lysozyme derivatives. Purified proteins were injected on a Vydac C18 column (4.6 mm by 150 mm, Rainin) and eluted at 0.5 ml/min with a linear gradient of 30% to 54% acetonitrile in 0.1% aqueous trifluoracetic acid at 0.33% acetonitrile per minute. The oxidized form of the 21C-142C-WT* mutant forms spontaneously if the purified lysozyme is left under nonreducing conditions. The reduced form was prepared by exposure to 10 m*M* DTT in 0.1*M* tris-HCl buffer at *p*H 8.2 for 4 hours. (**A**) Wild-type T4 lysozyme. (**B**) Oxidized (cross-linked) form of 21C-142C-WT*. (**C**) Reduced (non–cross-linked) form of 21C-142C-WT*.

Because the disulfide bond is susceptible to reducing agents such as DTT, the activity of the 21C-142C-WT* cross-linked lysozyme can be restored by conditions of increasing reductive strength (Fig. 3). Here again, the disulfide cross-linked mutant has no ability to hydrolyze the *E. coli* cell wall. On exposure to 2 m*M* DTT, however, the disulfide bond is quickly broken, and the enzyme regains catalytic activity within a few minutes, resulting in the observed decrease of turbidity. This enzyme activation becomes more rapid at higher *p*H because the reducing agent then exhibits its full potential.

The introduction of a disulfide bridge into a protein can enhance its stability against thermal inactivation (*21*) or guanidine hydrochloride–urea denaturation (*22*). We also found (in collaboration with W. J. Becktel) that 21C-142C-WT* is more stable with respect to thermal unfolding than either the wild-type or the reduced mutant enzyme (*23*). This enhanced thermostability, in an inactive form, could be advantageous for the long-term storage of enzymes, especially proteases.

Our results demonstrate the novel use of an engineered disulfide bond for the control of enzyme function. In the case of T4 lysozyme, a disulfide bridge was extended across the active-site cleft. An obvious extension of the method would be to engineer cysteines on opposite sides of the active site of an enzyme and to use a bifunctional mercaptan of appropriate size to bridge them. Another approach would be to engineer a single cysteine into the active-site region in a position such that the cysteine would not interfere with catalysis but, on reaction with a suitable mercaptan, would sterically block enzymatic activity [see (*24*)]. In these ways the need for a direct S–S bridge between the two cysteines would be avoided. Also, many enzymes undergo conformational changes on binding ligands or substrates. Such changes are especially important in allosteric enzymes for modulating activity. Introduction of an appropriate disulfide bridge, which either directly or indirectly prevents structural change, might be used to alter or to control the allosteric properties of enzymes.

Table 1. Relative activities of T4 lysozyme variants. Lysozyme activities were measured at 23°C by the turbidity assay (*18*). Protein concentrations were determined spectrophotometrically (molar absorption coefficient for wild-type T4 lysozyme, $\epsilon_{280\ nm}^{0.1\%} = 1.28$) (*18*). The oxidized and reduced forms of 21C-142C-WT* were prepared as described in the caption to Fig. 2. Exposure to oxidizing or reducing conditions makes no detectable change in the activity of the wild type or of WT*.

Variants	Relative activity (%)	
	Reduced	Oxidized
Wild type	100	(100)
C54T-C97A (WT*)	103	(103)
21C-142C-WT*	68	0

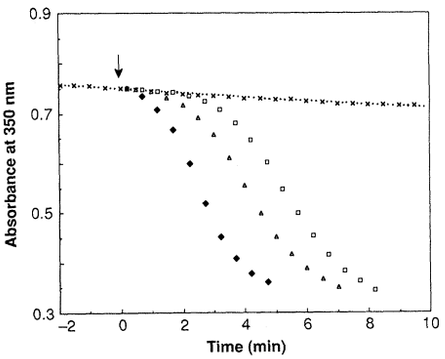

Fig. 3. Restoration of activity of oxidized 21C-142C-WT* by the addition of reducing agent. A suspension (0.85 ml) of lyophilized *E. coli* cells in 0.05*M* tris HCl buffer, *p*H 6.8 to 8.0, was mixed with 0.1 ml of the oxidized form of 21C-142C-WT* lysozyme (0.1 μg) in a cuvette in a spectrophotometer. The concentration of *E. coli* cells in the reaction mixture was adjusted to an absorbance at 350 nm of ~0.75. At time zero, indicated by an arrow in the figure, 0.05 ml of 40 m*M* DTT was added to the reaction mixture (lysozyme plus *E. coli* cells) and quickly mixed. The final concentration of DTT was 2 m*M*. The decrease in absorbance at 350 nm was monitored at 23°C. Absorbance of the reaction mixture without addition of 21C-142C-WT* (···). Absorbance in the presence of oxidized 21C-142C-WT* without the addition of DTT (x). Absorbance after the addition of DTT; *p*H 6.8 (□); *p*H 7.4 (△); *p*H 8.0 (◆).

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25. We thank J. Wozniak for technical assistance; D. C. Muchmore and F. W. Dahlquist for the lysozyme expression system; T. A. Kunkel for providing *Escherichia coli* strain CJ236 for site-directed mutagenesis; M. Lindorfer for facilities and advice concerning HPLC analysis, and G. Signor for measuring the activity by the plate assay method. We also thank L. H. Weaver and J. Bell for helpful discussion. Supported in part by grants from the NIH (GM21967) and the Lucille P. Markey Charitable Trust.

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Physiological Constraint on Feeding Behavior: Intestinal Membrane Disaccharidases of the Starling

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Animals clearly choose what they eat and can even choose among chemically different sugars. The physiological and biochemical mechanisms that constrain feeding choices are largely unknown. In this study, European starlings (*Sturnus vulgaris*) preferred mixture solutions of D-glucose plus D-fructose to equimolar (double molar caloric value) solutions of sucrose. Intubation feeding of sucrose did not increase blood glucose levels. Sucrose is a useless energy source for these birds because they lack a single digestive enzyme (sucrase) on the small intestinal brush border membrane. However, the membranes possessed separate maltase and isomaltase disaccharidases. This expression pattern and expression patterns of membrane disaccharidases among mammals suggest a role for intestinal enzymes in the coevolutionary interactions between vertebrates and their plant food sources.

ANIMALS CAN CHOOSE AMONG foodstuffs that differ only subtly in chemical composition (*1–3*). Feeding choices in some birds have been shown to reflect morphological traits (*4*). However, the physiological and biochemical mechanisms that influence animal feeding choices are largely unknown. In the present study, the European starling provided an example of preferences for simple sugars as directly influenced by physiological factors. Our results illustrate how a digestive constraint, the lack of a single enzyme (sucrase), can profoundly influence food preference.

We offered starlings a choice between two paired tubes containing either sucrose or an iso-osmotic mixture (1:1) of glucose plus fructose at three concentrations (*2, 5*). The offerings were alike in all respects except for the type of sugar. At all concentrations, starlings preferred the mixture of glucose plus fructose to sucrose (Fig. 1). When presented with a choice between a sucrose solution and water, starlings preferred water and appeared to develop a conditioned aversion for sucrose (*2*). Starlings fed sucrose solutions developed signs of discomfort and osmotic diarrhea, corroborating earlier reports (*1, 2*).

To test whether starlings are unable to digest sucrose, we measured the concentration of blood glucose after force-feeding starlings with either sucrose (3 g per kilogram of body weight administered in 0.87*M* solutions) or a 1:1 mixture of glucose plus fructose (3 g per kilogram of body weight administered in 1.66*M* solutions). There was no increase in blood glucose concentration after starlings were given a sucrose meal, but substantial increases were found after they were fed the mixture of monosaccharides (Fig. 2).

Next, we investigated the intestinal mechanism responsible for these observations. Using standard techniques (*2, 6, 7*), we measured the activities of maltase (E.C. 3.2.1.20), sucrase (E.C. 3.2.1.48), trehalase (E.C. 3.2.1.28), lactase (E.C. 3.2.1.23), and isomaltase (E.C. 3.2.1.10) in preparations of small intestinal mucosa and brush border membranes (*8*) from 18 starlings. The substrates (28 m*M* each) were maltose, sucrose, trehalose, lactose, and isomaltose (*9*). As predicted, we observed a complete absence of sucrase activity in starlings (Table 1). We were also unable to detect any lactase or trehalase activity. However, the intestinal preparations exhibited both maltase and isomaltase activities (Table 1).

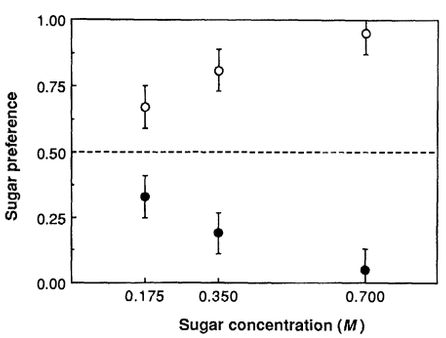

Fig. 1. Starling preferences for sugars. Equimolar solutions of sucrose and a mixture of glucose plus fructose (1:1) were offered paired to starlings (16 birds per sugar concentration test group). Sugar preference is defined as the volume of each sugar solution consumed divided by the total volume of solutions consumed during a 1.5-hour trial. The mixture of glucose plus fructose was preferred to sucrose at all test concentrations. The 95% confidence intervals are shown with mean preferences. A value of 0 would represent complete rejection, a value of 1.0 would represent complete preference, and the dashed line at 0.5 represents no preference. ○, glucose + fructose; ●, sucrose.

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