Crystallographic Studies of Protein Denaturation and Renaturation. 1. Effects of Denaturants on Volume and X-ray Pattern of Cross-Linked Triclinic Lysozyme Crystals†

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ABSTRACT: Triclinic crystals of hen egg-white lysozyme cross-linked with glutaraldehyde have been treated with various denaturants and found to be susceptible to x-ray structure analysis even after major conformational changes in the protein. Cross-linked crystals were isomorphous with the native form, and electron density difference maps indicated the locations of intermolecular cross-links, but showed no appreciable differences in the protein conformation. Soaking of the cross-linked crystals in denaturant solutions of increasing concentrations caused corresponding increases in crystal volume and decreases in minimum observable x-ray spacings. These changes proved partly reversible on diluting the solutions, and measurements of crystal volume and minimum x-ray spacing were used to follow denaturation and renaturation as a function of concentration for several denaturants. Some of these, including bromoethanol and sodium dodecyl sulfate, had little effect on the crystals below critical concentrations at which there was a sharp volume increase and loss of x-ray pattern, which could, however, be regenerated to about 3.2-Å resolution. Others, including KCNS and urea, caused more gradual changes, but with a smaller degree of recovery. It is suggested that at least two different denaturation mechanisms are involved, with detergent-like reagents disrupting the hydrophobic interactions joining the two wings of the lysozyme molecule and hydrophilic denaturants interacting primarily with polar groups on the molecular surface.

X-ray crystallography has by now been used to determine the detailed three-dimensional conformations of more than 70 proteins, and this method of structure analysis has also served to elucidate modes of interaction between proteins and smaller molecules, such as enzyme inhibitors and haptens (Matthews, 1976). However, such x-ray structure determinations have been essentially confined to native conformations and have hardly touched on questions of protein flexibility involved in various interactions (Lakowicz and Weber, 1973; Honig et al., 1976) or larger conformational changes during protein folding (Anfinsen and Scheraga, 1975). Various denaturants have been used to study protein unfolding and refolding, but the sequence of conformational changes in these processes and the modes of interaction between denaturants and proteins have been studied only by spectroscopic and other methods, which may be well suited to rapidly changing situations, but are far less powerful than x-ray crystallography for providing detailed and comprehensive knowledge of molecular conformation.

We have sought to apply x-ray crystallography to the study of protein–denaturant interactions and intermediate conformations in the folding process by using intermolecular cross-linking to maintain the crystalline order, which is essential for this technique, even after substantial structural deformations. In particular, we have used glutaraldehyde cross-linking of the triclinic form of hen egg-white lysozyme to study the effects of various denaturing agents on this protein. In this paper we describe the effects of various denaturants on the x-ray pattern and volume of the cross-linked crystals, and in subsequent papers of this series we shall describe detailed x-ray structure analyses of partially unfolded lysozyme complexed with particular denaturants.

Lysozyme has been crystallized in several forms (Steinrauf, 1959), and the conformations of the tetragonal (Blake et al., 1965; Imoto et al., 1972) and recently of the triclinic form (Moult et al., 1976) have been determined. Despite some differences in surface regions of the molecules involved in intermolecular contacts in the crystals, the main structural features of lysozyme found in the two crystal forms are practically identical and can be conveniently described in terms of two wings or structural domains. One comprises residues 1 to 39 and 104 to 129 and is composed largely of helical regions, whereas the second wing, comprising residues 42 to 100, contains a region of antiparallel strands as well as a helical segment. The wings are held together largely by a number of hydrophobic interactions near the central core of the molecule.

Several crystal forms of lysozyme have been cross-linked with glutaraldehyde. In tetragonal crystals this leads to a loss of the fourfold screw-axis symmetry (D. J. Haas, unpublished results), but cross-linked triclinic crystals are isomorphous with the native form and, therefore, better suited for x-ray analysis (Yonath et al., 1973, 1975). Cross-linked monoclinic crystals have been reversibly denatured (Haas, 1968), but these have two lysozyme molecules in the asymmetric unit and the crystal structure has not yet been determined. Recently, x-ray diffraction data have been obtained from non-cross-linked tetragonal lysozyme crystals soaked in high concentrations of urea (Snape et al., 1974). However, this reagent evidently causes only limited conformational changes and does not disrupt the crystals, whereas some of the denaturants we have used dissolve non-cross-linked crystals even at very low concentrations.

It is not clear to what extent crystallinity and cross-linking may constrain the natural denaturation and renaturation

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pathways of the protein. However, we have been able to produce major conformational changes in the crystals and elucidate detailed structures of intermediate conformations of the protein. Studies with a wide variety of chemical denaturant have shown two distinct kinds of denaturation, which are exemplified by detergent-like reagents and by salts. Our results appear to be consistent with studies of denaturation and renaturation in solution, and x-ray structure analyses of partly denatured or renatured lysozyme (to be reported in later papers of this series) have provided details of protein–denaturant interactions which indicate different protein unfolding mechanisms with detergents and salts.

Experimental Section

Preparation of Cross-Linked Crystals. Triclinic lysozyme crystals were grown in 1.8% sodium nitrate and 0.05 M acetate buffer at pH 4.5 (Steinrauf, 1959; Moult et al., 1976). Large (0.5–1 mm in length) and well-formed crystals were chosen. Cross-linking was carried out at room temperature, in the crystallization medium, by soaking the crystals in increasing amounts of glutaraldehyde. The initial concentration was 0.5%, and this was gradually increased to 6% by increments of about 1% per day. The crystals were soaked in the final concentration for at least 48 h, but sometimes for much longer periods up to 4 months. After cross-linking the crystals were washed in the original mother liquor for at least 24 h.

Denaturation and Renaturation. Denaturation experiments were carried out with individual cross-linked crystals, which were kept first in mother liquor and then in solutions of increasing concentrations of denaturants. The crystal was left in each solution for at least 5 h to reach equilibrium. After this period the volume of the crystal was measured with an optical microscope, and further measurements were taken at 30-min intervals until no further change in volume was observed. The crystal was then transferred to a solution of higher denaturant concentration and new volume measurements were made. This procedure was repeated until either no further volume change was observed in the new higher concentration, the maximum solubility of the denaturant was reached, or the crystal cracked, which happened very rarely. At this point the procedure was reversed, with the crystal being transferred to solutions containing decreasing concentrations of denaturant. Further volume measurements were made, and the experiment was terminated when the crystal was again in equilibrium with the mother liquor. All the experiments were performed at room temperature (20–25 °C) except for those with high concentrations of sodium dodecyl sulfate. Since the solubility of this compound is quite low at room temperature, these experiments were done at 35–40 °C.

These crystal soaking experiments were repeated, but instead of measuring volume changes we followed changes in the x-ray pattern by using still photographs taken on a precession camera. The denaturation was now characterized by the loss of resolution that followed soaking of the crystal in increasing concentrations of denaturant. In this the "complete denaturation" stage was determined when the whole diffraction pattern was lost. By soaking such denatured crystals in decreasing concentrations of denaturants it proved possible to recover the x-ray pattern to some extent. This gave an indication of the completeness of renaturation.

The denaturants used included bromoethanol, sodium dodecyl sulfate, urea, guanidine hydrochloride, guanidine thiocyanate, potassium thiocyanate (KCNS), lithium bromide (LiBr), and acetic acid.

Investigations were also made of the recovery of the x-ray patterns and volumes of crystals which had been heated to elevated temperatures and then returned to room temperature, at which the measurements were made. The crystals were heated in mother liquor for 4–6 h at various temperatures up to a maximum of 97 °C.

X-ray Data Collection and Electron Density Maps. A complete set of three-dimensional intensity data to 2.5 Å resolution was collected from two cross-linked lysozyme crystals using a Supper precession camera. One of the crystals was mounted about the a^* axis and the other about b^*. Altogether some 8675 reflections were recorded and their intensities measured using an Optronics digital microdensitometer. After discarding 43 reflections with intensities less than three times the standard deviation and scaling by the method of Rollett and Sparks (1960) we were left with 3217 reflections in the unique set. The agreement factor for zero-level Friedel pairs is 3%. This was calculated as $R_F = (\Sigma_i|\overline{F}^2 - F_i^2|)/\Sigma_i F_i^2$, where F_i is a measured structure amplitude and $\overline{F}$ is the average for a Friedel pair.

This set of observed structure amplitudes was scaled to the corresponding observed set for the native triclinic crystals (Moult et al., 1976) using an iterative Wilson plot (1942) and an overall difference temperature factor of 9.7, chosen as giving the best least-squares agreement for all shells of $\sin\theta/\lambda$. Weighted (Moult et al., 1976) electron density difference maps calculated with amplitudes $(F_{o,l} - F_{o_{nat}})$ and phases $\alpha_{c_{nat}}$ were constructed using 2.5-, 3-, and 5-Å data sets to help identify both gross and more detailed structural changes.

Results

Structure of the Cross-Linked Crystals. The cross-linked crystals differ from the native ones in their color, which is more yellow, in their greater mechanical strength, and in their stability over much wider ranges of pH and chemical environment. They are also resistant to proteolytic digestion by pepsin, chymotrypsin, and thermolysin, even after treatment with 9 M urea. Crystals cross-linked with glutaraldehyde for 3 days and for 4 months showed the same x-ray pattern, within experimental error, and essentially the same changes in volume and x-ray pattern with denaturant concentration.

As shown in Table I the cross-linked and native triclinic crystals are almost exactly isomorphous. The agreement factor (R) between observed structure factors for the two crystal types is 15%. Furthermore, the mode of binding of β-methyl Glc-NAc to the cross-linked crystals is the same as in the native crystals, although with somewhat lower occupancy (A. Sielecki and A. Yonath, in preparation). All the electron density difference maps between cross-linked and native crystals show very few features. In general, the protein region is clean but there are several peaks in the solvent region. One of these peaks can be interpreted as an intermolecular cross-link between Lys-13 and Lys-33 (Figure 1). Another with lower occupancy can be accounted for as an intermolecular cross-link between Lys-96 and Lys-33. One very low occupancy peak might represent an intramolecular cross-link between Lys-13 and Lys-33. The first two cross-links are probably derived from monomeric glutaraldehyde, whereas the third would require an extended polymer of glutaraldehyde.

Total amino acid analysis of the cross-linked crystals showed that $\frac{5}{6}$ of the total lysines in the crystal did react with glutaraldehyde (Table II).

Denaturation and Renaturation. As described above, measurements of crystal volume and minimum x-ray spacing were used to follow denaturation and renaturation as a function of denaturant concentration. In Figure 2 changes in crystal

TABLE I: Crystallographic Data for Native and Cross-Linked Triclinic Lysozyme.[a]

	Cell Dimensions						
	a (Å)	b (Å)	c (Å)	α (deg)	β (deg)	γ (deg)	Temp Factor (B)
Native	27.4	31.9	34.4	88 (0.7)	108 (0.6)	112 (0.0)	8.0
Cross-linked	27.4	31.6	34.1	88 (0.1)	108 (0.3)	112 (0.3)	17.2

[a] Both crystals have space group P_1 with one molecule (14 300 daltons) in the asymmetric unit:

$$R = \frac{\sum(|F_{o_{c.l.}}| - K|F_{o_{nat}}|)}{\sum|F_{o_{c.l.}}|} = 0.15$$

$$R = \frac{\sum(|F_{o_{c.l.}}| - K|F_{c_{nat}}|)}{\sum|F_{o_{c.l.}}|} = 0.37$$

$$R = \frac{\sum(|F_{o_{nat}}| - K|F_{c_{nat}}|)}{\sum|F_{o_{nat}}|} = 0.35$$

For R factor calculation we used 10–2.5 Å resolution data.

TABLE II: Amino Acid Analysis of Cross-Linked Crystals.

Amino Acid	Cross-Linked	Native
Lys	0.7	6
His	0.9	1
Arg[a]	11.0	11
Asp (and Asn)	20.4	21
Thr	7.2	7
Ser	9.7	10
Glu (and Gln)	5.3	5
Pro	2.5	2
Gly	11.9	12
Ala	12.3	12
Half-Cys	8.1	8
Met	1.8	2
Val	5.7	6
Leu	8.2	8
Ile	5.7	6
Phe	3.0	3
Tyr	2.2	3

[a] Was used for calibration.

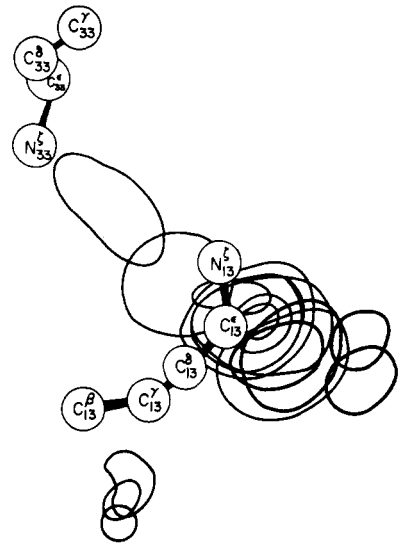

FIGURE 1: Superposition of 14 sections of the cross-linked native electron density difference map in the region indicating an intermolecular cross-link, between Lys-13 and Lys-33. Contours correspond to intervals of about 0.2 e/Å^3 beginning at about 0.3 e/Å^3 (2.2σ). There are no negative contours in this region.

volume are shown for a variety of reagents. The reagents differ appreciably in the sharpness of the transitions which they affect. The detergent-type reagents, having both hydrophilic and hydrophobic chemical groups, which include sodium dodecyl sulfate, bromoethanol, and acetic acid, cause very little expansion until critical molarities are reached, after which the crystal volume increases sharply to a maximum value. By contrast the hydrophilic reagents, LiBr, KCNS, and urea, cause very gradual increases in crystal volume, starting at low molarities. The total increase in volume for these hydrophilic reagents is also much less than that caused by detergents. Guanidine hydrochloride and guanidine thiocyanate show an intermediate behavior. The volume changes are caused more gradually than by the detergents, but the total volume increase is also large. Similar variations in volume expansion have been reported for cross-linked monoclinic crystals of lysozyme by Haas (1968).

Sharp and gradual loss of the x-ray pattern is caused by detergent-like and hydrophilic reagents, respectively. For detergents the x-ray pattern deteriorates very sharply and is lost completely at molarities corresponding to the onset of rapid volume expansion. An example of that is shown in Figures 3a and 3b, which show denaturation and renaturation pathways, based on measurements of crystal volume and x-ray resolution, respectively, for a detergent (bromoethanol) and a salt (KCNS).

Renaturation results in a shrinkage to within 5% of the original crystal volume and, generally, an appreciable recovery of the x-ray diffraction pattern. With detergent-like reagents renaturation follows a rather less sharp transition than denaturation. However, as is illustrated in Figure 3b, the transition is sharper and the degree of recovery more complete for crystals treated with detergents than with those treated with hydrophilic denaturants.

X-ray photographs of cross-linked crystals which had been heated and then cooled to room temperature showed only a slight deterioration in the pattern for maximum temperatures between 45 and 73 °C, but a complete loss of x-ray pattern for crystals heated to 78 °C and higher temperatures. This result agrees well with the denaturation temperature of lysozyme in solution as determined by magnetic resonance techniques (McDonald et al., 1971; Bradbury and Norton, 1974; Vincentelli and Leonis, 1973).

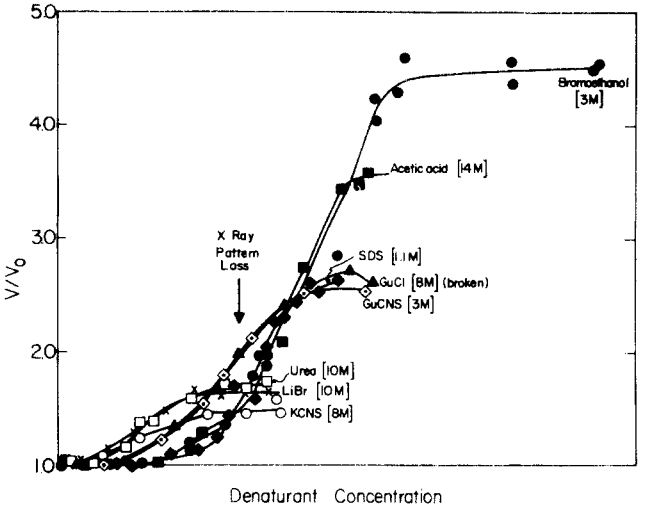

FIGURE 2: Volume changes for cross-linked triclinic lysozyme crystals as a function of denaturant concentration for several reagents. The curves were normalized so as to coincide at the point of total loss of x-ray pattern and the final concentration for each denaturant is shown at the end of the corresponding curve.

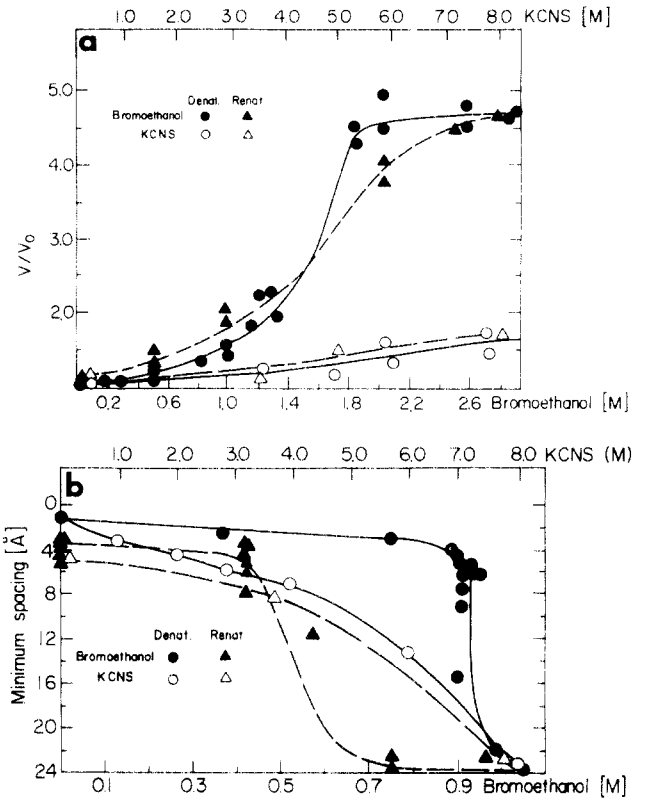

FIGURE 3: Denaturation and renaturation of cross-linked triclinic lysozyme crystals as a function of bromoethanol and KCNS concentration. The extent of denaturation and renaturation was determined by measurements of crystal volume (a) and of the minimum spacing observed in x-ray photographs (b). This value is defined as the spacing for which the decay is noticeable, i.e. where for a big crystal about 25% of the reflections appear in the x-ray photograph. The steepness of renaturation with bromoethanol is only approximately determined by the available data.

Discussion

Though we have been able to identify only two cross-links in the electron density difference maps, it seems quite likely that there may be more. The amino acid analysis shown in Table II indicates that five of the six lysine residues in the lysozyme molecule react with glutaraldehyde. A study of the triclinic crystal structure (Moult et al., 1976) indicates that Lys-116, which occurs in a region of intermolecular contact, is probably the unreacted residue. For two lysine residues to be cross-linked by a single glutaraldehyde their ϵ-amino nitrogen atoms should be 7.5 Å apart, which implies that their α-carbon atoms should be no more than 20 Å apart even allowing for substantial side-chain movements. N^{ξ}-Lys-13–N^{ξ}-Lys-33 (8.2 Å) and N^{ξ}-Lys-96–N^{ξ}-Lys-33 (8.8 Å) are the only two intermolecular separations which are close to the first requirement and they are apparently both cross-linked. Several additional pairs of lysines have their α carbons less than 20 Å apart, though we have found no clear indications of side-chain movements and cross-linking for them.

In fact, though cross-linking has been quite widely used in protein crystallography, we know of only one reported observation of cross-links in protein structure (Quiocho, 1974).

Other lysine pairs may be bridged by glutaraldehyde oligomers, which have been found in solution (Korn et al., 1972), and there may be various intramolecular cross-links as well as lysines bound to glutaraldehyde monomer or polymer without attachment to another residue. Unless the cross-links are quite taut or held in a well-defined position by hydrogen or hydrophobic bonds, all of these possibilities might lead to flexible chains with disordered structures which would not be readily observable in the difference maps.

It is of interest to consider to what extent our results may be related to protein denaturation and renaturation in solution. It is possible that the cross-links, S–S bonds, and intermolecular crystal forces may restrain the natural unfolding and folding of the protein. However, it has been shown that proteins with unreduced S–S bonds can still be converted to random coils by denaturant (Tanford, 1968), and the large increases in crystal volume we have observed indicate that appropriate reagents can cause major protein unfolding in spite of the cross-links.

Furthermore, there are parallels between the effects of the denaturants in solution and in the crystals, suggesting that the reagents use the same modes of attack on the protein even though some of the subsequent unfolding may be inhibited in the crystal environment. Thus, sodium dodecyl sulfate is a powerful denaturant of lysozyme in solution and urea is a very poor one (Hamaguchi and Kurono, 1963; Leonis, 1956). It was suggested that sodium dodecyl sulfate and several alcohols (Tanford, 1968) have similar modes of action in solution, whereas, in cross-linked crystals, sodium dodecyl sulfate and bromoethanol have been found attached to the same hydrophobic sites in lysozyme [see part 2 (Yonath et al., 1977a) and Yonath et al., 1977b]. This is so in spite of the fact that these reagents are very different in size and shape. Inorganic salts, which we have classified as hydrophilic reagents, have also been found to exhibit similar behavior in solutions (Von Hippel and Schleich, 1969) and in crystals (Bello et al., 1966). In both cases anions and cations have been found to attach to polar groups in peptide bonds.

It would appear from the results described above, as well as from the crystal-structure analyses of denaturant–lysozyme complexes described in part 2 (Yonath et al., 1977a) of this series, that there are at least two distinct modes of denaturation. Bifunctional detergent-like reagents might at low concentrations be absorbed on the protein surface by hydrogen bonds or salt bridges to their hydrophilic moieties without a major disruption of the protein conformation. At higher concentrations it appears likely then that the hydrophobic portion of the denaturant penetrates the interior of the lysozyme

molecule separating regions of the protein structure which were held together by hydrophobic interactions. The sharp loss of x-ray pattern we observed should occur at the onset of denaturant penetration of the lysozyme core, which leads to major protein unfolding accompanied by an increase in crystal volume. Hydrophilic denaturants, on the other hand, cannot readily penetrate the hydrophobic core of the lysozyme molecule. Their effects are apparently largely confined to polar groups on the surface of the protein, where they can disrupt the hydrogen bonding pattern and the solvent structure, but do not cause major changes in the shape of the protein, as was found for the complex of urea and tetragonal lysozyme (Snape et al., 1974). However, it is possible that very high concentrations of hydrophilic reagents and the powerful guanidine denaturants can eventually disrupt some of the backbone hydrogen bonding inside the molecule and cause an appreciable degree of unfolding (Bradbury and King, 1969).

Renaturation involves a reversal, in the case of detergents, of major but fairly simple movements of structural domains (Yonath et al., 1977a,b) and, presumably for hydrophilic reagents, of an accumulation of small local deformations. It is not surprising that renaturation should be sharper and more complete in the former case. In studying renaturation after treatment with sodium dodecyl sulfate and with bromoethanol, we have found denaturant molecules trapped inside the refolded lysozyme molecule (Yonath et al., 1977a,b), which can account for the incomplete recovery of the x-ray diffraction pattern. However, for hydrophilic reagents there is as yet no detailed crystallographic analysis describing the renaturation pathway. Further x-ray structure analyses of proteins affected by hydrophilic denaturant and by heat denaturation, now in progress, may help to clarify the mechanisms in these cases.

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