PRELIMINARY STUDIES ON THE DENATURATION OF CROSS-LINKED LYSOZYME CRYSTALS

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ABSTRACT When monoclinic lysozyme crystals are fully cross-linked with glutaraldehyde, and then the protein molecules are denatured while in the crystalline state, a single crystal-gel is formed which is a jelly-like crystal of a denatured protein molecule. It is highly disordered, but has crystalline optical and morphological properties and can be renatured to a cross-linked crystal resembling the original crystal as determined from the X-ray diffraction pattern. Experiments with the following denaturants are described: guanidinium chloride, bromoethanol, urea, and lithium chloride.

INTRODUCTION

During the course of studies on cross-linked lysozyme crystals (Haas, 1969, in preparation), it was found that in protein-denaturing solutions, the cross-linked crystals lost their birefringence and swelled to enormous sizes while keeping their original shape. After considerable study of this phenomena, it became clear that the protein molecules in fully cross-linked crystals can be denatured and renatured to a disordered cross-linked crystal that gives a similar X-ray diffraction pattern to the original crystals. Upon denaturation, the cross-linked crystal becomes a single-crystal gel which is a jelly-like single crystal of denatured protein molecules.

Incompletely cross-linked crystals have a surface of cross-linked protein molecules which contains the dissolved molecules of the interior when a denaturant is added that causes the crystal to swell. The cross-linked surface acts like a dialysis membrane, and the crystals swell to volumes of 100 times their original volume. Removing the denaturing agents produces renaturation of the molecules, but not recrystallization to a single crystal. The renatured crystal looks normal to the naked eye, but the interior molecules are not oriented as determined by optical and X-ray methods.

This paper describes preliminary studies of these crystals as well as their possible application for studying denaturation.

CROSS-LINKING PROCEDURE

Monoclinic lysozyme crystals (elongated prisms 1 mm long) were grown in 2% sodium nitrate at pH 4.5 (Steinrauf, 1959). The crystals were washed with 4% sodium nitrate and cross-linked as follows: fully cross-linked crystals were obtained by increasing the glutaraldehyde concentration by 2% a day up to 12%, keeping the pH about 8 with sodium bicarbonate. Fully cross-linked crystal were never obtained if the pH was much less than 8. The test for a fully cross-linked crystals was to denature the crystal in bromoethanol and obtain a diffraction pattern upon renaturation. Surface cross-linked crystals were produced by placing washed crystals in 1% glutaraldehyde (4% sodium nitrate) for only a few hours, then back into 4% sodium nitrate in order to prevent the glutaraldehyde from diffusing into the interior.

The denaturation experiments were restricted to monoclinic crystals since their shape greatly facilitated optical measurement of the retardation (see below).

DENATURATION

When surface cross-linked crystals are placed in strong denaturing solutions, they swell to enormous volumes (up to 100 times the original volume) which are a function of the degree of cross-linking. The fully cross-linked crystals represent the minimum swollen volume. Because the interior protein molecules dissolve and form a concentrated protein solution inside the surface membrane, the large volume increase results from the osmotic pressure of this solution. If left for several days in the denaturing solution, the swollen crystals eventually break up, and under the light microscope, one observes thin membranes in the shape of the crystal faces, corners, and edges. In this manner one can determine the extent of the surface cross-linking. By placing the swollen crystals in pure water, one obtains a crystal that looks normal and is about the same size as the original cross-linked crystal, but shows little birefringence and gives no diffraction pattern.

The denaturation procedure for the fully cross-linked crystals was as follows: crystals were fully cross-linked and put in pure water for 24 hr to remove the excess glutaraldehyde; then individual crystals were transferred to increasing concentrations of the denaturant (approximately 25°C). The crystals were left 6 hr or more in order to assure that equilibrium had been established. There are two optical tests for this: sharp extinction of the crystals between crossed nicols, and a uniform interference color on the flat upper surface. If equilibrium has not been reached, the center of the crystal shows stronger birefringence than the edges, as indicated by the lack of uniform extinction and color.

Fig. 1 shows the results obtained for the denaturants bromoethanol, guanidinium chloride, urea, and lithium chloride. Each point on the graphs is the average value for two or more crystals in the same solution. Other experiments for which the data is not presented show that acetic acid and chloroethanol have curves similar to bromoethanol. The retardation (Wahlstrom, 1960):

$$\Delta = t(n_1 - n_2)$$

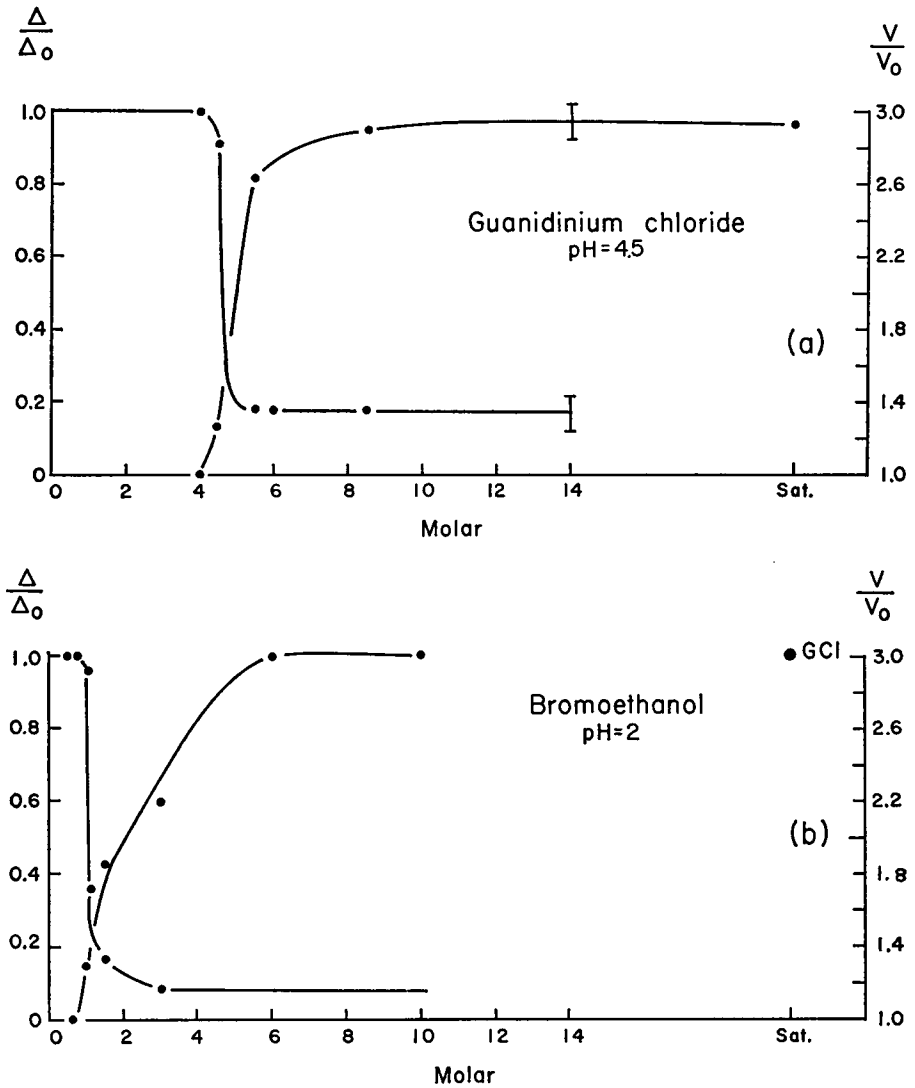

FIGURE 1 *a, b* (*a*) Normalized optical retardation and crystal volume for a fully cross-linked monoclinic lysozyme crystal with increasing concentrations of guanidinium chloride. Note the dramatic change at 4.5 M. Approximate errors are indicated by the vertical bars. (*b*) Same as (*a*) except bromoethanol used. Note the slow volume increase after 1 M which reaches the fully expanded form. The point is for the same crystal in saturated guanidinium chloride.

where t is the vertical thickness of the crystal, and n_1 and n_2 are the indices of refraction perpendicular to the microscope axis, is an optical measure of the asymmetry of the molecules in the crystal. For constant crystal thickness, a decrease in retardation indicates reduced asymmetry, i.e. denaturation. The crystal volume is obtained by measuring the three principle dimensions of the crystal optically.

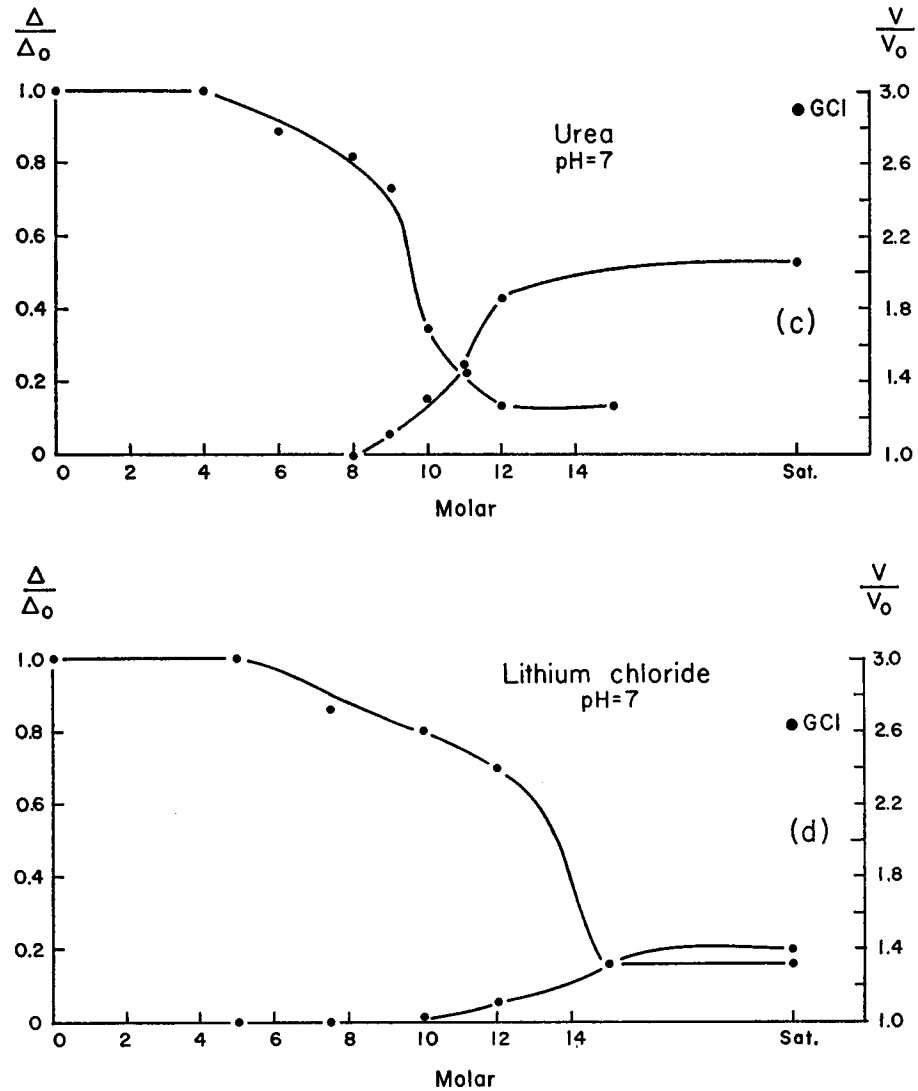

FIGURE 1 *c, d* (*c*) Same as (*a*) except urea used. Note that the crystal does not fully expand. (*d*) Same as (*a*) except lithium chloride used. Note that the crystal hardly expands.

The X-ray diffraction pattern of these crystals (except crystals soaked in lithium chloride) is lost as soon as a volume increase is observed. With lithium chloride this occurs between 6 and 8 M. The loss of the diffraction pattern in these experiments correlates well with molecular changes observed in viscosity and optical studies of lysozyme in solution (Leonis, 1956; Hamaguchi and Kurono, 1963; Bigelow, 1964).

With all the above denaturants except guanidinium chloride, dilution of the solution to pure water over a period of hours is accompanied by renaturation of the

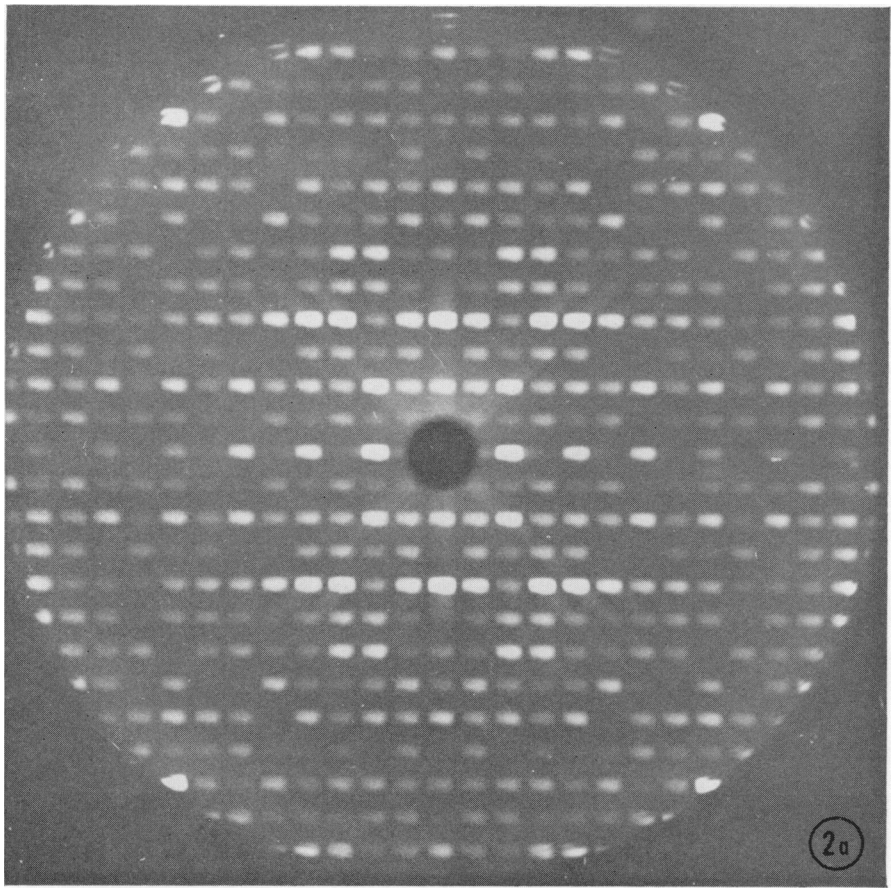

FIGURE 2*a* 9° precession photograph of native monoclinic lysozyme.

molecules, return of more than half the original retardation, and return of an X-ray diffraction pattern with a resolution of 6 A or less (Fig. 2). Guanidinium chloride is the exception, presumably because it reacts with the aldehyde group in a similar manner to the lysine amino groups or because it reduces the number of cross-links already present in the crystal.

DISCUSSION

The behavior of the denaturants falls into two categories: those which fully swell the crystal, and those which do not. Thus, in the former group we can assume that the protein molecules are fully unfolded (Tanford et al., 1966; Tanford et al., 1967). The reason for the behavior of the latter group is not clear. It may be due either to

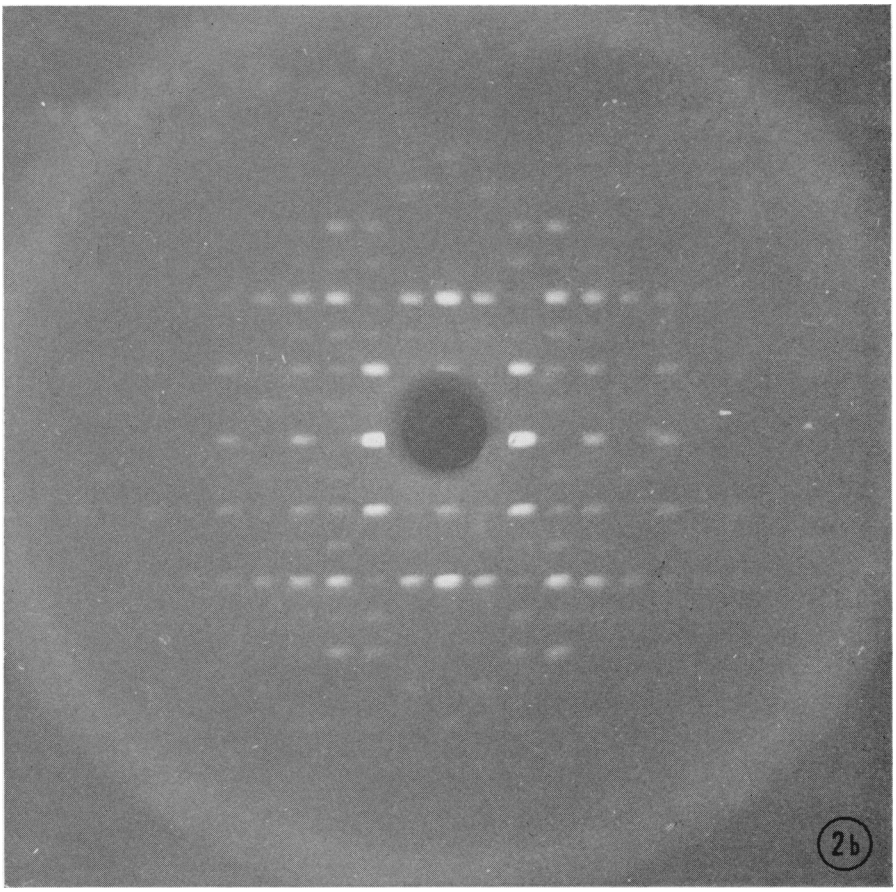

FIGURE 2*b* Same as 2*a* except photograph of renatured bromoethanol swelled crystal. The crystal recovered 60% of the original retardation value.

their inability to unfold the protein molecules or to their ability to complex the various groups in the protein molecules.

From the above graphs several observations can be made: (*a*) since the volume of the crystals does not increase until most birefringence is lost, the protein molecules must not unfold until substantially rearranged by the denaturants; (*b*) guanidinium chloride completely opens the protein molecules once unfolding begins, i.e. no apparent intermediate stages seem to exist; (*c*) bromoethanol, chloroethanol, acetic acid, and urea have obvious intermediate states of unfolding; (*d*) urea and lithium chloride do not completely swell the crystals.

The measured volume increase of the denatured form will be a function of the

crystal space group since the cross-linking pattern in the crystal will depend on the relative orientation of the molecules to one another. In any case, the swollen crystal can be pictured as resulting from outward forces on the cross-linked lysines in the protein molecule. An equilibrium arrangement is obtained that may not necessarily be the same as would be obtained if the molecule were free in solution.

Nevertheless, one can observe an end point in unfolding of the protein molecules as well as measure the same end point for various denaturants. Because the X-ray diffraction pattern shows that the renatured molecule is so similar to the native form, it seems likely that the exact same crystal can be regenerated provided the renaturation and recrystallization are performed slowly enough. This reduces to a form of annealing the crystal by slow dilution of the denaturant.

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