Biophys. Struct. Mechanism 4, 27–36 (1978)

Biophysics of Structure and Mechanism
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Structural Analysis of Denaturant-Protein Interactions: Comparison Between the Effects of Bromoethanol and SDS on Denaturation and Renaturation of Triclinic Lysozyme

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Abstract. This paper summarizes our crystallographic studies of the interaction of denaturants with cross-linked triclinic lysozyme. Electron density maps of various bromoethanol-lysozyme complexes are analyzed and compared to those reported earlier for SDS-lysozyme complexes. Despite differences in the chemical nature and size of the two denaturants their mode of interaction with the protein is quite similar, suggesting the existence of a general mechanism for binding of hydrophobic-hydrophilic denaturants to proteins. Our results are consistent with the conclusion that lysozyme consists of two domains connected by a flexible segment and that this segment represents an internal degree of freedom of the protein.

Key words: Denaturation – Renaturation – Proteins (Lysozyme).

Introduction

The interactions of denaturants with proteins have been extensively investigated with a variety of physical techniques [1, 2]. We have recently reported crystallographic studies concerning the denaturation and refolding of cross-linked triclinic lysozyme [3, 4], and have shown that the denaturants can be classified in two main groups in terms of their effects on the crystals. For one denaturant, SDS, we explored the three-dimensional crystal structure after a cycle of denaturation and renaturation. We observed that after refolding there are several molecules bound to lysozyme, including one near the core of the molecule that separates the two wings of which the protein is composed. These results might be related to the later stages of protein folding. In order to investigate the generality of our previous study we undertook a three-dimensional X-ray study of triclinic lysozyme complexed with bromoethanol. It has previously been shown [3] that both SDS and bromoethanol have similar

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macroscopic effects on the lysozyme crystals. However, there are gross differences in their chemical nature and their size. From our comparison of the two denaturants it appears that the manner of protein folding is very possibly the same in both cases and that the interactions of bromoethanol with the protein molecule are very similar to those of SDS.

In the case of bromoethanol we have now extended our three dimensional studies to two points well before complete denaturation (as monitored by loss of the X-ray pattern), to enable us to investigate interactions of bromoethanol with the intact enzyme. We find that before any considerable denaturation occurs there is some binding of bromoethanol to the surface of the protein molecule without it causing any substantial conformational changes.

Experimental

Denaturation and renaturation experiments were carried out at room temperature with cross-linked triclinic lysozyme crystals [3]. Some crystals were partially denatured by soaking them in solutions of increasing concentrations of bromoethanol for long periods (at least three days in each solution). Others were soaked directly in 1.0 M bromoethanol and then transferred to denaturant solutions of lower concentrations for about four days to allow refolding. The crystals were then mounted in quartz capillaries together with the appropriate final concentration of denaturant solution. Complete sets of three dimensional data out to 2.5 Å resolution were collected for two bromoethanol concentrations (0.375 M and 0.75 M) before significant denaturation had occurred.

Two crystals were used for each concentration and the intensities were measured from precession photographs using an Optronics digital microdensitometer. The renatured crystals diffracted only to 3.2 Å and complete data sets out to this resolution were collected for two individual crystals, kept at final concentrations of 0.43 M and 0.0 M bromoethanol, using a Nonius Cad-3 diffractometer and the ω-scan procedure described in Yonath et al. [4].

The four sets of data were corrected for absorption [5] and scaled to data from a native cross-linked crystal [3], using an iterative Wilson Plot procedure [6]. Differences in structure amplitudes between denaturant treated and native crosslinked crystals were used to calculate electron density difference maps with phases calculated from the triclinic lysozyme structure [7] using an F^4 weighting system.

The maps obtained from crosslinked crystals before complete denaturation were searched for positive and negative features greater than 2 σ and the maps from renatured crystals were contoured at levels greater than 1.5 σ, and correlated with the difference maps from SDS-treated crystals [4].

Results

Figure 1 shows the changes in the minimum X-ray spacing observed for crosslinked triclinic crystals as a function of bromoethanol concentration for the entire cycle of denaturation and renaturation. The four three-dimensional data sets were collected

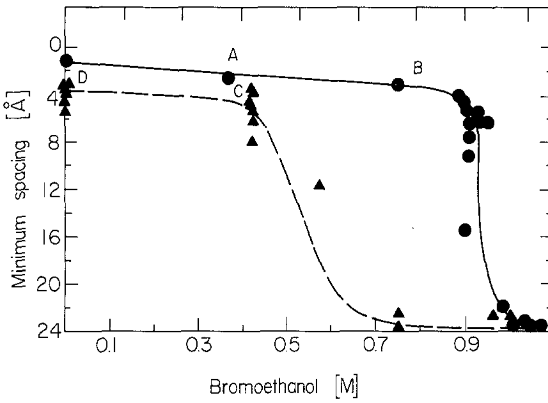

Fig. 1. Minimum X-ray spacing observed for crosslinked triclinic lysozyme as a function of bromoethanol concentration. Complete three-dimensional data sets were collected for conditions indicated by points *A*, *B*, *C* and *D*

Fig. 2. Superposition of 13 sections of the electron-density difference map between 0.75 M bromoethanol treated crystal (point *B* in Fig. 1) and the native crosslinked one. The superimposed model is of the native molecule, and the map shows part of the active site

Table 1. Details of data collection and cell constants for the bromoethanol-treated crosslinked triclinic

Case	Conditions	Final bromo-ethanol concen-tration	Cell dimensions					
			a Å	b Å	c Å	$\alpha°$	$\beta°$	$\gamma°$
O	Native crosslinked	0.0 M	27.4	31.6	34.1	88.0 (1)	108.0 (3)	112.0 (3)
A	before complete denaturation	0.37 M	27.6	32.0	34.3	88.0 (1)	108.0 (3)	113.0 (0)
B	before complete denaturation	0.75 M	27.6	32.2	34.3	89.0 (1)	108.0 (4)	112.0 (7)
C	denatured and renatured	0.43 M	28.0	32.2	34.6	89.0 (7)	108.0 (6)	113.0 (0)
D	denatured and renatured	0.0 M	28.0	32.1	34.3	89.0 (7)	108.0 (4)	112.0 (7)

[a] $\sigma = \left[\frac{1}{N}\sum_{j}^{N} \varrho(j)^2\right]^{1/2}$, where N is the number of grid points in the map and ϱ is density of each grid point

for conditions indicated by points *A*, *B*, *C*, and *D* and compared with data for point 0 which corresponds to crosslinked native triclinic lysozyme. Details of the data collection and cell constants are given in Table 1.

Crystals Before Complete Denaturation

Point *A* refers to crosslinked crystals that were exposed to 0.37 M bromoethanol and point *B* to those soaked in 0.75 M bromoethanol. The crystals were isomorphous with the native crosslinked ones, and diffracted to ~ 1.1 Å. Three dimensional data were collected photographically out to 2.5 Å resolution. The conventional agreement factor between native crosslinked and 0.75 M bromoethanol treated crystals $\left(R = \frac{\sum ||F_{\mathrm{Bret}}| - |F_{\mathrm{C.L.}}||}{\sum |F_{\mathrm{C.L.}}|}\right)$ is 0.13. The electron density difference maps corresponding to 0.37 M and 0.75 M bromoethanol show much the same few features (Table 2). There are more positive regions than negative ones, and the highest peak lies in the active site of the enzyme [8], between Trp 108, Glu 35 and Asp 52. This peak appears in both maps at about the same occupancy, suggesting that a 0.37 M concentration of bromethanol is sufficient to entirely saturate this site.

It has been shown [9] that alcohols are inhibitors of lysozyme and indeed the mode of binding of bromoethanol is very similar to that found for propanol [10]. It is clear that one bromoethanol molecule is attached with high occupancy to the active

lysozyme crystals

Resolution Å	Number of crystals used	Comparison to crosslinked				
		Number of reflections measured	Number of merged reflections after 3 σ cut off	Difference in temperature factor from C.L. crystals	R factor[b]	σ^a of difference map
2.5	2	8675	3217	–	–	–
2.5	2	8695	3198	17.5	0.15	6
2.5	2	8738	3202	18.5	0.13	8
3.25	1	1600	957	66	0.26	17
3.2	1	1600	753	54	0.20	12

[b] $R = \frac{\sum(|Fobs| - |Fobs\ (C.L.)|)}{\sum |Fobs|}$

site (Table 2 and Fig. 2) whereas all the others are distributed on the surface and are weakly bound to the protein.

Renatured Crystals

Crystals that have been fully denatured were soaked in 0.43 M bromoethanol (point *C*) and in mother liquor (point *D*). In the first case the average of monitored intensities decreased by 15% during the course of data collection, but for the second the average decrease was less than 5%. The second crystal also has a lower temperature factor (Table 1) and provided rather cleaner electron density maps.

Comparison of SDS and Bromoethanol Difference Maps

From the difference maps one can identify bromoethanol molecules located at various sites near the protein as was found in the case of SDS binding [4]. Moreover, careful inspection of the maps shows that many residues remote from the bromoethanol sites are located in new positions, and that the gross features of the bromoethanol difference maps are very similar to those found in the SDS difference maps [4]. For this reason the maps corresponding to points *C* and *D* were correlated with the difference maps of refolded lysozyme measured in 0.35 M and 0.0 M SDS. The correlation factors between the various maps are given in Table 3. There is considerable similarity between point *D* and the two SDS maps whereas the correlation of point *C* is somewhat lower. The locations common to bromoethanol and SDS molecules are given in Table 4.

Table 2. Possible locations of bromoethanol molecules before the denaturation of the triclinic lysozyme crystals

Bromo-ethanol molecule No.	Coordinates[b] in the 0.75 M bromoethanol map (Å)			Coordinates[b] in the 0.37 M bromoethanol map (Å)			Possible contacts with the protein[c]	Relative occupancy[d]	
	x	y	z	x	y	z		0.75 M bromoethanol	0.37 M bromoethanol
1*D*[a]	− 3.8	28.4	3.8				Arg 5, Trp 123	0.10	
2*D*	3.2	23.5	22.8	3.8	22.7	22.4	Glu 35, Asp 52, Lue 56, Glu 57[e], Ilu 58, Asn 59[e], Trp 63[e], Ilu 98, Ala 107, Trp 108	1.00	1.00
3*D*	−10.3	22.5	27.6				Tyr 20, Arg 21, Lys 96, Val 99, Ser 100	0.15	
4*D*	9.6	23.2	34.3	10.7	22.5	35.1	Asp 48[e], Arg 61, Trp 62, Gly 71, Ser 72[e], Arg 14[g]	0.10	0.10
5*D*	13.5	12.9	34.6	13.9	12.9	36.2	Asn 65[f], Gly 67, Arg 68, Thr 69, Pro 70, Gly 71, Ser 72[e], Gln 121[e, g] Arg 125[g], Gly 126[g]	0.30	0.10

[a] All the molecules are labeled *D* to indicate possibly denatured states, as apposed to *R* that indicates the renatured refolded states
[b] The coordinate system is that used for native triclinic lysozyme (Moult et al., 1976)
[c] Distances up to 6 Å were considered for both cases
[d] Molecule No. 2*D* was assumed to have occupancy = 1, based on the integrated peak heights
[e] Only for 0.37 M case
[f] Only for 0.75 M case
[g] From a neighbouring molecule

Table 3. Correlation[a] between bromoethanol and SDS electron density difference maps. (All maps are of the renatured state)

	Bromoethanol 0.43 M	Bromoethanol 0.0 M	SDS 0.35 M	SDS 0.0 M
Bromoethanol 0.43 M	1.0	0.59	0.42	0.46
Bromoethanol 0.0 M		1.0	0.62	0.65
SDS 0.35 M			1.0	0.79
SDS 0.0 M				1.0

[a] The correlation (C), between two difference maps is:

$$C(\varrho_1, \varrho_2) = (\sigma(\varrho_1)\,\sigma(\varrho_2))^{-1} \int_{\text{unit cell}} (\varrho_1(r) - \bar{\varrho}_1)\,(\varrho_2(r) - \bar{\varrho}_2)\, d^3r$$

where ϱ_1 and ϱ_2 are the electron densities of the two maps $\bar{\varrho}_1$ and $\bar{\varrho}_2$ are the mean value of these densities, and $\sigma(\varrho_2)^2$ are the mean square densities

Table 4. Locations of bromoethanol and SDS molecules in the renatured crystals

Molecule No.	Coordinates[a] of SDS (Å)			Coordinates[a] of bromoethanol (Å)			Nearest neighbours
	x	y	z	x	y	z	
1*R*	− 5.9	11.6	16.9	− 6.0	12.0	15.9	His 15, Ilu 88, Thr 89
2*R*	− 2.1	24.7	8.6	− 3.0	24.9	7.5	Trp 123, Lys 33, Phe 38
3*R*	—	—	—	6.7	22.5	23.1	Asp 52, Gln 57
4*R*	− 1.0	18.2	34.2	—	—	—	Thr 47, Lys 97

[a] The coordinates given are for the point of highest electron density in the peak

It is clear that both denaturants are found at almost the same site (No. 1*R* of Table 4) in the interior of the protein between His 15 and Ilu 88 (Fig. 3). An additional site nearer the surface (No. 2*R*) is also common to both denaturants and appears to involve a hydrophobic pocket created by a displacement of the side chains of Phe 38 and Trp 123 (Fig. 4). It appears from model building that the bromoethanol molecule in this site might make a hydrogen bond to the guanidinium group of Arg 5, as could the sulfate of SDS, though the latter might also be bound to the side chain of Arg 125.

A third bromoethanol molecule (No. 3*R*) is located near the active site of lysozyme between Asp 52 and Gln 57. In fact this bromoethanol molecule is observed only in the difference map of the 0.43 M bromoethanol treated crystal and not in the 0.0 M. No SDS was found in the vicinity of this site, but on the other hand, one SDS molecule was found to bind at the interface between two adjacent proteins, at a position where no bromoethanol could be detected [4].

A further point of similarity between the difference maps obtained from lysozyme crystals renatured after treatment with bromoethanol or with SDS concerns the so called packing regions [4]. These are regions in the crystal structure where surface loops of polypeptide chain from neighbouring molecules lie close to one anoth-

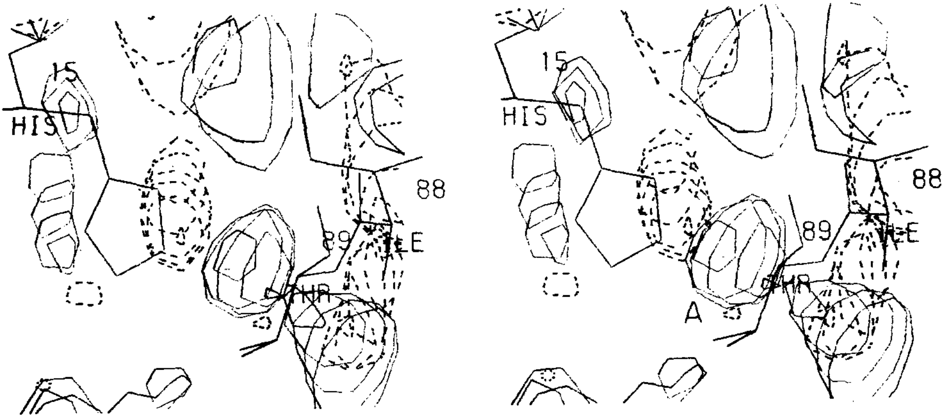

Fig. 3. Stereo superposition of 10 sections of the electron-density difference map between 0.0 M bromoethanol renatured molecule (point *D* in Fig. 1) and the native crosslinked one in the region of the internally trapped bromoethanol molecule interaction site (peak *A*)

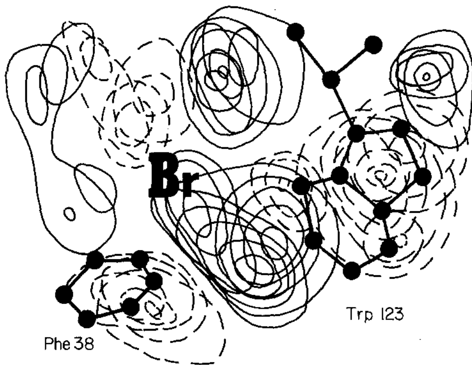

Fig. 4. Superposition of 11 sections of the electron-density difference map between partially renatured lysozyme crystal (point *C* in Fig. 1) and the cross-linked one. The superimposed model is of the native molecule, and the map shows the hydrophobic pocket of Phe 38 and Trp 123

er. The maps for both denaturants show some of the strongest indications of conformational changes in these regions.

The electron density difference maps derived from crystals renatured after SDS or bromoethanol denaturation have many features. Some of them can be clearly ascribed to conformational changes in the protein, and others have been assigned to denaturant molecules. However, there are still some peaks which we have not yet been able to interpret with confidence. We plan to refine the coordinates of the renatured protein, and to prepare a difference map between the refined renatured molecule and native cross-linked one, with a view to obtaining a more complete description of this protein-denaturant complex.

Discussion

Inspection of Table 1 shows that there is a clear difference between the crystals at point *A* and *B* as compared to those at *C* and *D* cases. Whereas the first two, studied

before complete denaturation, show very high similarity to the crosslinked crystals, the last two, which lie on the refolding pathway, are less similar to the native state. This is reflected in the observed lower X-ray resolution, higher *R* factors, higher temperatures factors and the standard deviations of the difference electron density maps.

The most striking result of this study is the high degree of similarity found in crystals renatured after treatment with bromoethanol and with SDS, with regard to both the conformational changes in the protein and the locations of the attached denaturant molecules. These similarities appear to us to have structural implications.

First, our results are consistent with what may be quite a general mechanism for binding of hydrophobic-hydrophilic denaturants to proteins. In this mechanism the initial attachment occurs between the hydrophilic portion of the detergent (sulfate in SDS, OH group in bromoethanol) and a polar group on the surface of the protein. This interaction need not cause any appreciable changes in protein conformation as can be seen from complexes *A* and *B*. At higher detergent concentrations further interactions between the hydrophobic portion of the detergent and hydrophobic regions of the protein occur. It is these interactions, involving the interior of the protein, that lead to significant unfolding.

In this connection it is of interest to note that bromoethanol site 3*R* (Table 4) in the renatured protein is close to site 2*D* (Table 2) found before denaturation. Similarly, site 2*R* (Table 4) which is common to bromoethanol and SDS after renaturation is near site 1*D* (Table 2). In both cases, the bromoethanol molecules are found on the surface of the protein before denaturation, and are more burried in the renatured molecule, while maintaining their hydrophilic contacts with the surface.

Though different detergent molecules may bind through the same mechanism, they need not occupy identical locations, because of differences in size and shape. Thus SDS may be too long and too hydrophobic to fit confortably into the active site at 2*D* (Table 2) or the associated location 3*R*, (Table 4) whereas bromoethanol may be too small to be wedged between two molecules in the crystal as is SDS at the site 4*R* (Table 4).

Another inference one might draw from our results is that the similar conformational changes in the protein observed with the two denaturants arise from some flexibility inherent in the lysozyme structure. In particular the trapping of a denaturant molecule in the hydrophobic core of the protein (site 1*R*, Table 4) implies a major conformational change brought about by separation of the two wings of the protein. This was discussed in detail in our previous paper, dealing with SDS denaturation studies. The similar results with bromoethanol strongly support our earlier suggestion that the hinging mechanism, between independent whole lobes, is an important feature of the refolding pathway.

Finally, the marked conformational changes observed in the maps at packing regions suggest a possible explanation for the sharpness of changes in the crystal volume and X-ray resolution observed with bromoethanol and SDS. Separation of the two wings of the protein would cause a large change in molecular size and consequently produce close contacts between neighbouring molecules in the crystal. These close contacts are evidently accommodated by distortions in the fairly flexible loops of polypeptide chain at the packing regions. However, for the lysozyme mole-

cule to unfold it would appear that a certain minimum concentration of denaturant is required to force these distortions to occur in a cooperative transition throughout the crystal.

We are grateful to Dr. Gerson Cohen for providing us with his data processing programs, to Drs. David Haas, Paul Sigler, Thomas Creighton and Micael James for helpful discussions, and to Mr. Samuel Getteno for his invaluable technical assistance.

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Received December 24, 1976/Accepted April 12, 1977