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## Binding of Dimethyl Sulfoxide to Lysozyme in Crystals, Studied with Neutron Diffraction

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**ABSTRACT:** Crystals of hen egg white lysozyme soaked in 15% (v/v) dimethyl sulfoxide have been studied with single-crystal neutron diffraction to determine the effect of the solvent molecules on the protein configuration. A total of 9423 statistically significant Bragg reflections to a resolution of approximately 1.8 Å were used to locate 6 dimethyl sulfoxide molecules, and structure refinements including a model for the flat solvent lead to a final crystallographic agreement factor of 0.130. The mode of location of the dimethyl sulfoxide molecules was compared with that in previous studies employing ethanol. This showed that hydrophobic interactions can be an essential factor in fixing the probe molecules on the protein surface. There was, however, no sign of any significant change in the protein configuration; so although possibly at higher concentrations of dimethyl sulfoxide the protein will unfold, there was no indication of any precursor effect.

The interaction of proteins with solvent molecules is an essential part of many biological reactions, and the interplay between macromolecules and the solvent has therefore been a subject of many studies. These have been directed either toward the action of the protein on smaller molecules (substrates), such as those that occur in enzymatic reactions, or toward the influence of the solvent on the configuration of the macromolecule. A multitude of techniques has been used, ranging from thermodynamic studies supplying reaction rates or knowledge about protein stability to crystallographic studies giving the three-dimensional configuration of the protein.

Very detailed steric information about the interaction between the protein and very small molecules is, however, hard to come by. The normal approach is to use X-ray diffraction techniques, which will reveal a number of well-ordered water molecules attached to the protein. The scattering density of water is very similar to the scattering density of small molecules containing light atoms. As there is often some disorder due to the flexibility and high mobility of the components, and as even in the best of cases the resolution is limited, it can therefore be very difficult to distinguish between a series of hydrogen-bonded water molecules and a slightly larger solvent molecule.

One solution to this problem is to use selective deuteration combined with neutron diffraction analysis, and recently this was employed to study the interaction of ethanol and lysozyme (Lehmann et al., 1985). Crystals soaked in 25% deuterated ethanol (v/v) were used and gave the location of 13 ethanol molecules on the surface of the protein. In addition, a precise analysis of the protein structure was done, and this showed no changes in the molecular structure compared to the structure in the absence of ethanol. This led to a model for the denaturation of lysozyme by alcohol, where the change in the configuration is caused by the dehydration of the protein at high alcohol concentration.

The complete lack of change in the structure when ethanol is added was surprising, and it was therefore decided to repeat the experiment with a small molecule that might have a

stronger influence on its environment. Dimethyl sulfoxide (DMSO) was chosen. This molecule is both a good hydrogen-bond acceptor and has a side that should attach well to any accessible hydrophobic part of the protein. In addition, the dipole moment is 3.96 Debye, which is about the highest available for a small molecule. Altogether this molecule should therefore be well suited to perturb the protein. The effects can, however, in all cases be expected to be small, so an effort was made to include more data in this study and thus obtain an even higher precision than in the previous analysis.

### EXPERIMENTAL PROCEDURES

**Crystal Preparation and Data Collection.** A triclinic crystal of hen egg white lysozyme (Hodsdon et al., 1975; Mason et al., 1984) with a volume of 16.2 mm<sup>3</sup> was used for the measurement. It was grown from a 0.05 M acetate buffer (pH 4.7) containing 2% NaNO<sub>3</sub> and 0.5% protein. Trial experiments had shown that beyond 20% volume concentration of DMSO crystals dissolved, so for the measurement the crystal was soaked in 15% solution of 99% deuterated DMSO. This was added over a period of 5 days, and the measurements were started 2 months later. H<sub>2</sub>O solutions were used, as the neutron signal of this molecule is nearly zero, thus improving significantly the visibility of other small, deuterated molecules such as DMSO.

The recording was done at room temperature at the neutron diffractometer D19 (Thomas et al., 1983) of the Institut Laue-Langevin with a wavelength of 1.547 Å. The sample holder is a Eulerian cradle, and the neutron detection system is a two-dimensional position-sensitive detector covering 4° in the horizontal and ±32° in the vertical direction. The measurement was done by moving the detector in steps of 2° to a maximum angle of 46°, and for each detector position the crystal was rotated to yield a section out of slightly more than a hemisphere of reciprocal space. Because the step in the detector position was only half the width of the detector aperture, many of the reflections were measured twice, and this gave a continual check of internal agreement and detector stability.

The unit cell was determined from 166 reflections recorded for detector angles between 8 and 42°. The space group is

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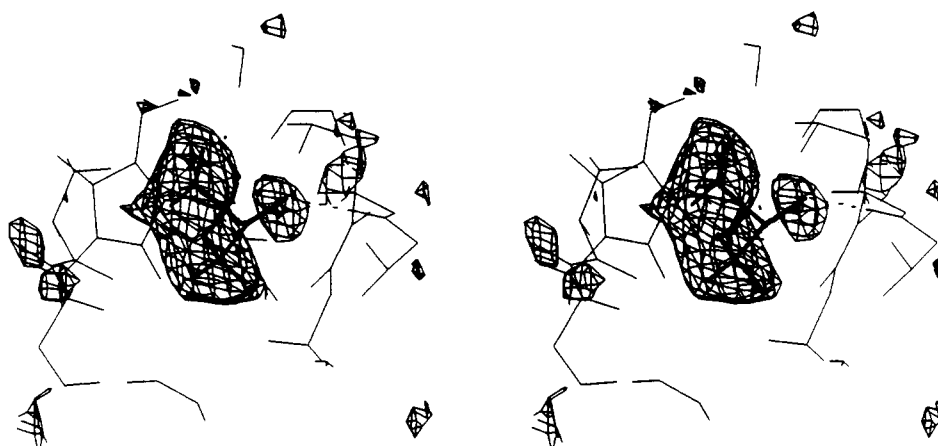


FIGURE 1: Stereo diagrams of the neutron scattering density for DMSO 1. One positive contour is given with a level, which is a fourth of the highest density found in maps, where part of the chain had been removed. The level thus corresponds to about half of the density found in an average side chain. The sulfur atom is a weak scatterer of neutrons, and consequently its scattering density is below the level chosen for the plot.

$P1$ , and the cell constants are  $a = 27.280 \pm 0.007 \text{ \AA}$ ,  $b = 32.045 \pm 0.008 \text{ \AA}$ ,  $c = 34.269 \pm 0.007 \text{ \AA}$ ,  $\alpha = 88.16 \pm 0.01^\circ$ ,  $\beta = 108.79 \pm 0.01^\circ$ , and  $\gamma = 111.60 \pm 0.01^\circ$ .

For the data reduction, strong reflections were used to form a library of peak shapes. These were then employed to obtain an optimal estimate of the intensity of the weak reflections by using an algorithm that tries to minimize the relative error  $\sigma(I)/I$  (Wilkinson et al., 1988), where  $I$  is the integrated Bragg intensity and  $\sigma(I)$  is the standard deviation based on counting statistics. In all, 16 009 reflections were obtained, which after averaging gave 10 233 independent values. This corresponds to all data to a  $d$  spacing of  $1.9 \text{ \AA}$ . In addition, due to the shape of the detector, a considerable number of higher resolution data were included, corresponding to 50% of the reflections to  $1.7 \text{ \AA}$ , and 850 beyond this limit. Before the reflection intensities were averaged, they had been corrected for absorption by use of a Gaussian integration and a calculated  $\mu$  of  $3.26 \text{ cm}^{-1}$ . The transmission factors were in the range from 0.46 to 0.59.

Because of the low energy of the neutrons, no radiation damage was observed, so there was no change in the intensity of the standard reflections, which were recorded at half-daily intervals over the measurement period of 12 days. Second-order contamination of the data coming from scattering of neutrons with half the wavelength was found to be less than 2%.

**DMSO Location and Structure Refinement.** Throughout the analysis the Hendrickson-Konnert program modified for neutron diffraction (Wlodawer & Hendrickson, 1982) was used. The starting parameters for the lysozyme molecule were the coordinates from the neutron diffraction study of the deuterated crystal (Mason et al., 1984), and in the first calculations only the protein and four well-defined nitrate groups were included in the calculation. When the refinement had converged, the DMSO molecules were found by using difference maps.

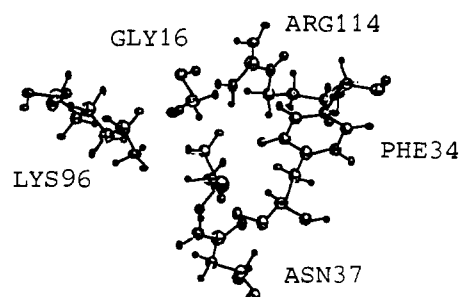
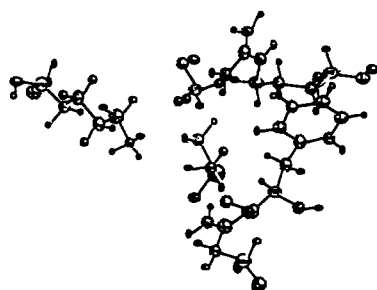
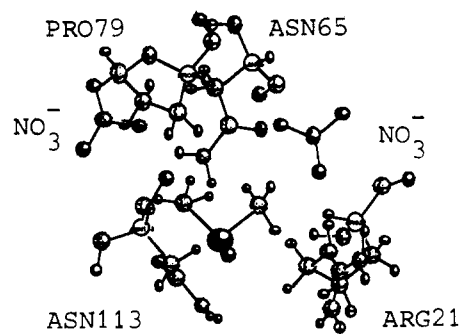
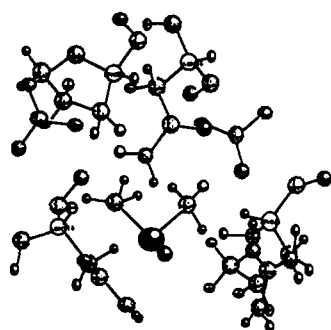
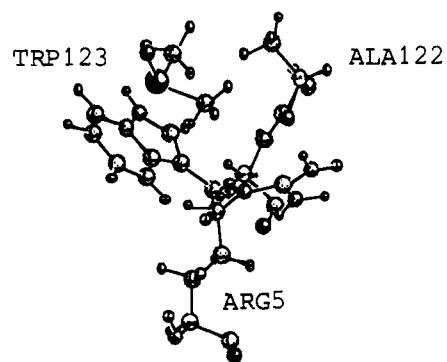
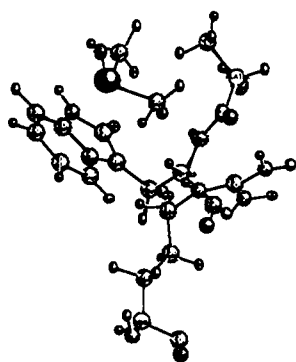
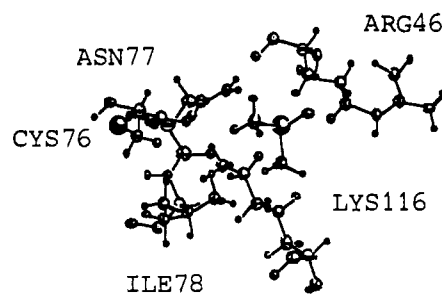
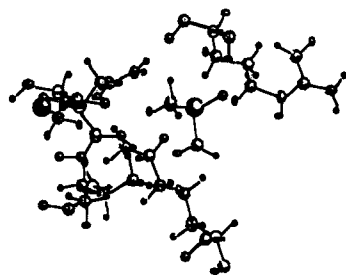
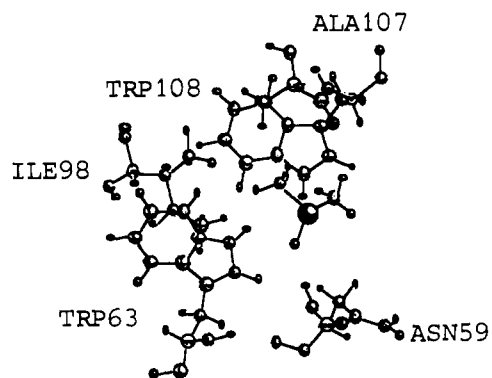
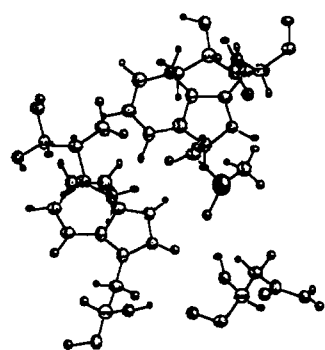
In this study only the methyl groups of the DMSO were deuterated and should be easily identifiable. The scattering lengths of the different atomic species involved are  $b_D = 0.667$ ,  $b_C = 0.665$ ,  $b_N = 0.921$ ,  $b_O = 0.581$ ,  $b_S = 0.285$ , and  $b_H = -0.374$ , all in units of  $10^{-12} \text{ cm}$  (Koester & Rauch, 1981). The peak heights of all atoms in a map of the scattering density are thus comparable, except those for H, which are negative. The consequence is that in a nondeuterated environment the protein is easily visible, as the scattering density of the protein

averages  $0.019 \times 10^{-12} \text{ cm}^3/\text{\AA}^3$ , while the scattering density of water is  $-0.006 \times 10^{-12} \text{ cm}^3/\text{\AA}^3$ . Moreover, the deuterated DMSO molecule has a scattering density of  $0.053 \times 10^{-12} \text{ cm}^3/\text{\AA}^3$ , and as it is located in water, again it should be easily observable. The DMSO should be seen as an extended region of positive scattering density compared to any cluster of ordered water molecules, which gives a pattern of positive and negative peaks in the difference maps. If the waters are disordered, the signal disappears because of spatial averaging of positive and negative regions, while for DMSO molecules, the main result of disorder is a smearing where it becomes difficult to identify the exact orientations of the S-O and the  $\text{CD}_3$  groups.

A total of six molecules of DMSO were found, and Figure 1 shows the density for DMSO 1. The orientation and position were determined by using the MRC version of the program FRODO (Jones, 1978) and were at this stage based entirely on the density distribution. Later on, DMSO 4, which according to the density could be oriented in at least two directions, was fixed so that its orientation made chemical sense. The geometry of the DMSO molecule was obtained from a single crystal study by Thomas et al. (1966).

Further refinements were then carried out, including 9423 reflections for which the intensity was more than 3 times the standard deviation. Atomic coordinates and the thermal motion parameters were adjusted for all atoms in the lysozyme molecule, the nitrate ions, and the DMSO molecules, and in addition the site occupation factors for the DMSOs were allowed to vary. To use also the low-order data, a description of the disordered, flat solvent was included in the structure factor equation (Lehmann et al., 1985). The solvent scattering was calculated to be equal in magnitude and opposite in sign to the scattering from a pseudomolecule with the same envelope as the protein, but with a constant scattering density similar to that of the solvent. The pseudomolecule was obtained by placing hard spheres at the atomic positions. To obtain a smooth surface, it was convoluted with a sphere representing a water molecule. Although this only gives an approximative description of the solvent, it can very easily be added to the least-squares refinement program (Lehmann, 1985). The scattering density was set to  $0.003 \times 10^{-12} \text{ cm}^3/\text{\AA}^3$ , corresponding to 15% DMSO.

Structure refinements were then continued until convergence. At this point the agreement factor on the structure amplitudes was 0.130 for the 9423 reflections, with 227 of the



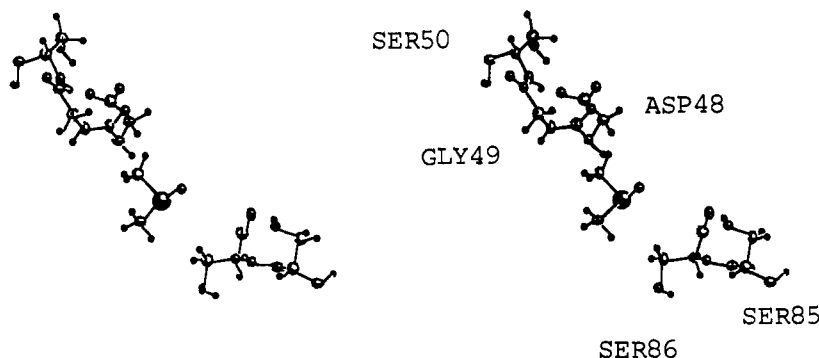


FIGURE 2: Stereo plots of the six DMSO molecules and their environment. Panels correspond to DMSO 1-6, respectively. The residues shown are those given in Table II with a few added for clarity. Small dots are hydrogen atoms, shaded spheres are nitrogen and oxygen atoms, while large spheres are sulfur atoms.

Table I: Thermal Parameter,  $B$ , and Site-Occupation Factors for the DMSO Molecules<sup>a</sup>

no.	$B$	$Q$	no.	$B$	$Q$
1	23.0	0.88	4	35.4	0.44
2	32.4	0.80	5	31.7	0.43
3	50.1	0.70	6	40.4	0.39

<sup>a</sup>The Debye-Waller factor describing the thermal motion is expressed as  $\exp(-B \cdot \sin^2(\theta/\lambda)^2)$ , with  $B$  in units of  $\text{\AA}^2$ .

6858 bond distances deviating more than  $2\sigma$  from the ideal, where  $\sigma$  for the distances range from 0.020 to 0.045  $\text{\AA}$ .

## RESULTS AND DISCUSSION

**Molecular Interactions.** Table I summarizes the site-occupation factors,  $Q$ , and thermal parameters,  $B$ , derived from the least-squares refinement. In all cases the site-occupation factors are high, so there are certainly further molecules with lower values of  $Q$ . However, additional inspection of the difference maps indicated that only these molecules were significantly above the noise level in the maps, so no attempts were made to include more molecules in the calculations. It is of course quite likely that some water molecule configurations are partly replaced by DMSO. In this case the form of the deuterated molecule will be difficult to identify, being now a sum of positive peaks from  $\text{CD}_3$  and negative peaks from the H in  $\text{H}_2\text{O}$ .

The inspection of the maps also clearly showed a few patterns of hydrogen-bonded water molecules, but these were not analyzed in detail, as their absence from the refinement is not expected to affect the results for the DMSO molecules. In any case, only the best-located water molecules will be found in a map based on nondeuterated water, and these few molecules will be discussed and compared with the similar positions found in lysozyme soaked in ethanol (Lehmann et al., 1985) and in ethylene glycol (Lehmann, unpublished results) when the analysis of the latter is completed.

There is no clear correlation between  $Q$  and  $B$ . In general, the  $B$  values are considerably higher than the mean value of 12.5  $\text{\AA}^2$  for the protein, but this is not unexpected. It can easily be due to both disorder in the DMSO molecule and the relatively high rotational freedom of the methyl groups.

The specific interactions between the DMSO molecules and the protein are summarized in Table II, and Figure 2 gives stereo plots of the environment of the six DMSO molecules. These show all the types of bonding expected for DMSO.

In only three cases is there clear evidence of hydrogen bonding with the O atom of DMSO accepting a hydrogen bond. Two of these, DMSO 1 and DMSO 2, are relatively short with an  $\text{O}\cdots\text{H}$  bond of 1.8  $\text{\AA}$ . Obviously this leads to

Table II: Contacts and Distances ( $\text{\AA}$ ) in Parentheses between the DMSO Molecules and the Lysozyme Molecule<sup>a</sup>

no.	O-S	C1	C2
1	O near Asn59 H (1.8)	Ala107 O (3.6) Trp108 C <sup>β</sup> (3.6)	Trp63 N <sup>ε</sup> (4.0) Ile98 C <sup>γ</sup> (4.1) Ala107 C <sup>β</sup> (3.6) Trp108 C <sup>α</sup> (4.3)
2	O near Arg45 H (1.8)	Ile78 C <sup>γ</sup> (3.7) Lys116 C <sup>γ</sup> (4.0)	Cys76 O (3.2) Asn77 O (3.6) Ile78 C <sup>γ</sup> (4.0)
3	no contact	Trp123 C <sup>α</sup> (4.0)	Arg5 C <sup>δ</sup> (3.7)
4	S near Asn113 C <sup>γ</sup> (3.2)	Asn65 N <sup>δ</sup> (3.8) Pro79 C <sup>β</sup> (3.9) NO <sub>3</sub> N (3.1)	Arg21 N <sup>ε</sup> (3.2) NO <sub>3</sub> N (3.2)
5	O near main Phe34 (3.3)	Phe34 C <sup>α</sup> (3.7) Lys96 C <sup>ε</sup> (3.2) Arg114 C <sup>η</sup> (3.4)	Gly16 C <sup>α</sup> (3.8) Asn37 C <sup>γ</sup> (4.1)
6	O perhaps near Ser86 O (3.2)	no contact	Gly49 C <sup>α</sup> (4.5)

<sup>a</sup>Distances are given to O or to S and to the two C atoms of the methyl groups; except for the two well-defined hydrogen bonds, the nearest non-hydrogen atom is given.

a good fixation and high occupation factor for the molecules. The third hydrogen bond toward DMSO 6 is strongly bent and longer, and the molecule has a much lower site-occupation factor.

The second mode of binding is the attachment of the methyl groups to the hydrophobic parts of the protein surface or interior. Tryptophan associations occur for DMSO 1 and DMSO 3, but most commonly the methyl group is imbedded in a small cavity formed by hydrophobic residues or the  $\text{CH}_2$  stem of residues such as Lys, Arg, or Asn. A good example of this is DMSO 2, where one methyl group is enclosed in the bent Lys116, while Asn77 and Ile78 form contacts with the other methyl group.

When the S-O group is not involved in hydrogen bonding, it points toward the solvent region. In none of these cases is there any clear indication from the difference maps of well-defined water molecules nearby, but these would probably only be easily observable in a completely deuterated solution. We can safely assume, though, that hydrogen bonding must take place. The direction of the S-O vector, which is near the direction of the large dipole moment of DMSO, can then be determined simply by the fixation of the two methyl groups. However, local gradients can also play a role. This is evident for DMSO 4, which is placed in a cavity formed by three lysozyme molecules. Two sites on one side of the DMSO are filled by nitrate groups. The main parts of the other side of the cavity are Arg21 and Arg112, producing a region of positive charge. The S-O group is aligned in the direction between the two, thus probably following the electric gradient.

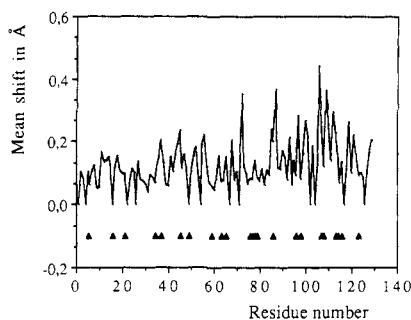


FIGURE 3: Mean deviation in angstroms between the atomic coordinates in the protein soaked in DMSO and in ethanol, respectively, as a function of residue number. The values are the average values for the side-chain atoms, excluding hydrogen. Triangles mark the location of the interactions with the DMSO molecules.

Four of the six DMSO molecules, namely, DMSO 2, DMSO 4, DMSO 5, and DMSO 6, are located near the contact regions between two or more lysozyme molecules, and it is therefore difficult to state how DMSO-protein interactions would be for the protein in solution. One would expect that the interface regions provide less free space for small molecules to move in, and it is therefore likely that this is what holds the DMSO molecules enough to make them visible by diffraction techniques. In solution DMSO will undoubtedly still fix itself on the surface, but certainly in most cases in a less organized manner. It is worth noting that the higher concentration of DMSO in these regions might help to explain the dissolution of the crystals at concentrations above 20%. As the sites get more densely occupied, the effects of the large dipole moment of DMSO could well reduce the intermolecular attraction enough to separate the protein molecules.

**Comparison with Ethanol Binding.** In the previous measurement with ethanol as soaking agent, 13 molecules were located with site-occupation factors between 1.0 and 0.2. The best bound of these was near the active site and connected with residues 58, 59, 63, 98, 107, and 108. In the present analysis the best-bound molecule, DMSO 1, is located in the same region with hydrogen bonding to the main-chain hydrogen and Asn59. This region has a rich blend of aliphatic, aromatic, and polar groups and is obviously well suited for binding of small molecules, which have both hydrophobic and hydrophilic parts.

A similar pattern is found for DMSO 3, which overlaps completely with the second-best-bound ethanol molecule. This had a site-occupation factor of 0.6. For all the other DMSO molecules, except DMSO 4, one end of the DMSO is located similarly to an ethanol molecule, either with one end of the ethanol near a methyl group of DMSO or with the ethanol sitting across the methyl group. Obviously, an important part of the protein-solvent interaction derives from attachment of hydrophobic groups to the surface of the protein. Only DMSO 4 is not near any of the ethanol sites, and as discussed above this molecule is most likely fixed by electrostatic forces.

**Changes in the Molecular Configuration.** In the previous analysis where lysozyme was soaked in 25% ethanol (Lehmann et al., 1985), no change in the protein structure was found when compared with the native structure. The final difference map made at the end of the present refinement was therefore studied very carefully. As for the other studies of triclinic lysozyme (Mason et al., 1984; Lehmann et al., 1985), there was some minor disorder near Arg45, and the Trp62 is distributed over several sites. This effect was therefore accounted for in the refinement by the introduction of two partially occupied sites for Trp62. There is furthermore some indication of disorder near Val121. In the early part of the analysis it

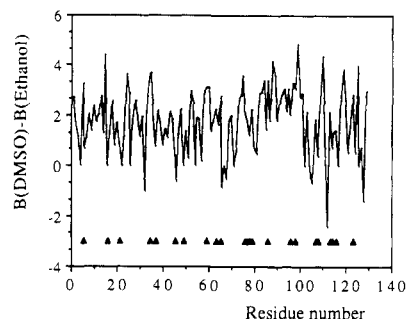


FIGURE 4: Difference between mean thermal motion  $B$  (defined in Table I) for the DMSO and ethanol data. The values are the average values for the side-chains atoms, excluding hydrogen. Triangles mark the location of the interactions with the DMSO molecules.

was assumed that there was a DMSO molecule near this location, but as the refinement moved the molecule away from the observed density, this DMSO was removed from the analysis.

For the parts of the protein in direct contact with the DMSO molecules there was no sign of changes. Only the side chain of Ser86 had an indication of disorder, with one position favoring a hydrogen bond with DMSO 6, but it is not clear whether the same pattern is found in the native structure (S. A. Mason, personal communication).

An overall indication of this lack of change in configuration is given in Figures 3 and 4, where the positions and thermal motions of the protein soaked in DMSO and ethanol, respectively, are compared for the non-hydrogen atoms of the side chains. For the positions the mean shift for all atoms was 0.17 Å, which at the present resolution must be considered to be negligible. The temperature parameters were slightly higher for the DMSO data, but this might partly be explained by the slightly higher measurement temperature, which was ambient in the present case but 8 °C for the ethanol data, and partly attributed to experimental errors, which will tend to be absorbed in the  $B$  values. The mean  $B$  values were 12.5 and 10.8 Å<sup>2</sup> for DMSO and ethanol data, respectively, hardly enough to invoke any major change. Moreover, there does not seem to be any correlation between the local fluctuations and the location of the solvent molecules.

## CONCLUSION

The configuration of proteins is known to be dependent on the content of solvent molecules in the aqueous solution, and normally important changes can be provoked as these are varied. For the concentration of DMSO used in this study we did not expect any major change from the native structure but were mainly searching for precursor effects.

A detailed study of the structure of the protein in 15% DMSO and comparisons with earlier structure determinations of this protein using neutron diffraction showed no changes, but did give some indication that an important part of the solvent binding on the protein surface is via hydrophobic interactions.

The absence of changes agrees with earlier observations made with ethanol as a probe and indicates that denaturation of lysozyme will occur abruptly in a dehydration type of process and not by infiltration of the solvent into the protein interior prior to a major structural change.

## ACKNOWLEDGMENTS

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## Is $\gamma$ -Chymotrypsin a Tetrapeptide Acyl-Enzyme Adduct of $\alpha$ -Chymotrypsin?<sup>†</sup>

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**ABSTRACT:** Refinement of the structure of  $\gamma$ -chymotrypsin based on X-ray crystallographic data to 1.6-Å resolution has confirmed the overall conformation of the molecule as reported previously [Cohen, G. H., Silverton, E. W., & Davies, D. R. (1981) *J. Mol. Biol.* 148, 449-479]. In addition, the new refinement suggests that  $\gamma$ -chymotrypsin, which is operationally defined by its crystalline habit, may not be the free enzyme but rather a complex, possibly an acyl-enzyme adduct, with the tetrapeptide Pro-Gly-Ala-Tyr (or a close homologue). The crystallographic refinement provides a detailed geometrical description of the enzyme-substrate-solvent interactions that occur in the presumptive adduct.

$\gamma$ -Chymotrypsin ( $\gamma$ -CHT) is a variant of  $\alpha$ -chymotrypsin ( $\alpha$ -CHT) originally identified by its different crystalline habit (Kunitz, 1938). Conversion of  $\alpha$ -CHT to  $\gamma$ -CHT occurs at a pH ( $\sim$ 7.0-8.0) at which the enzyme is active and was initially thought to be irreversible, suggesting that  $\gamma$ -CHT might be an autolytically degraded form of  $\alpha$ -CHT. It was subsequently shown, however, that  $\alpha$ -CHT can be obtained from  $\gamma$ -CHT, indicating that the two forms of the enzyme are covalently identical (Corey et al., 1965). Crystallographic studies have also shown that the structures of  $\alpha$ - and  $\gamma$ -CHT are very similar (Matthews et al., 1967; Cohen et al., 1981).

The prior crystallographic refinement of  $\gamma$ -CHT by Davies and co-workers (Cohen et al., 1981) was based on 15 240 intensities to 1.9-Å resolution. Here we have extended the resolution to 1.6 Å and included 23 255 reflections. The new refinement confirms the overall conformation of the molecule as reported previously (Cohen et al., 1981). In addition, the refinement suggests that the form of crystalline  $\gamma$ -chymotrypsin used in the present study is not the free enzyme but is a complex, possibly an acyl-enzyme adduct, with the tetrapeptide Pro-Gly-Ala-Tyr. The crystallographic refinement provides a detailed geometric description of the enzyme-substrate-solvent interactions that occur in this complex.

The present study was prompted by the observation that crystals of  $\gamma$ -chymotrypsin are exceptionally resistant to changes in pH, the pregrown crystals being stable from pH 2 to pH 11 [B.W.M., reported in Cohen et al. (1981)]. This provided an unusually favorable opportunity to study the

behavior of a crystalline protein and its bound solvent as amino acid side chains were titrated. The present paper derives from the high-resolution refinement of the structure at pH 7.0. Comparisons of this structure with the protein at pH 2.0 and pH 10.5 will be reported elsewhere.

## EXPERIMENTAL PROCEDURES

$\gamma$ -Chymotrypsin was crystallized essentially as described (Cohen et al., 1981). The enzyme (Worthington CDG 6204-5) was dissolved to 25 mg/mL in water. To 0.5-mL aliquots were slowly added equal volumes of saturated ammonium sulfate, adjusted to pH 7.0, and the mixture was left at room temperature. (In the standard procedure, unbuffered ammonium sulfate, pH 5.5, is used.) Seeding with microcrystals is often helpful to promote nucleation, but was not used in the present instance.

X-ray data were measured to 1.6-Å resolution by oscillation photography (Schmid et al., 1981) (Table I).

Refinement was commenced with the previous model (Cohen et al., 1981) for the protein but with all water molecules deleted. Cycles of automatic refinement with the TNT system (Tronrud et al., 1981) were alternated with inspection of difference maps on an interactive graphics system in the usual manner. Solvent molecules were added conservatively. They were required to have density of at least  $5\sigma$  in a difference map and to be located within H-bonding distance of appropriate donors or acceptors.

## RESULTS

As the refinement progressed it became obvious that there was significant unexplained electron density in the vicinity of

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