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The effect of stabilizing additives on the structure and hydration of proteins: a study involving tetragonal lysozyme

S. Datta, B. K. Biswal and M. Vijayan*

Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India

Correspondence e-mail: mv@mbu.iisc.ernet.in

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In order to elucidate the effect of stabilizing additives on the structure of proteins and the associated ordered water molecules in the hydration shell, the crystal structures of tetragonal lysozyme grown in the presence of sucrose, sorbitol and trehalose have been refined. Also refined are the structures of orthorhombic and monoclinic lysozyme grown under the conditions in which tetragonal lysozyme is normally grown. A comparison of the two sets of structures with the structure of native tetragonal lysozyme shows that the effect of the additives on the structure of the protein molecule is less than that of the normal minor changes associated with differences in molecular packing. Surprisingly, the same is true of the effect on the hydration shell, represented by the ordered water molecules attached to the protein. Thus, it appears that the cause of the stabilizing effect of the additives needs to be sought outside the immediate neighbourhood of the protein molecule. Sorbitol and trehalose do not coherently interact with the protein. One sucrose molecule binds at the active-site cleft of the enzyme

1. Introduction

Sugars and polyols are often used to enhance the stability of proteins (Back *et al.*, 1979; Timasheff, 1993; Wimmer *et al.*, 1997). In addition to its fundamental importance, the stabilization process has received considerable attention in recent years on account of its practical utility in terms of preservation, particularly in relation to the food industry (Rajeshwara & Prakash, 1996). A number of studies on the mechanism underlying enhanced protein stability in the presence of these additives have been reported in recent years (Timasheff, 1993; Lin & Timasheff, 1996; Priev *et al.*, 1996; Xie & Timasheff, 1997; Sola-Penna & Meyer-Fernandes, 1998). These investigations, mainly based on physicochemical and thermodynamic techniques, indicated that the stabilizing effect of these compounds is achieved by modulation of the solvent structure around the protein molecule. It is generally believed that the stabilization of the protein conformation is achieved not by the specific binding of the additives, but by their preferential exclusion from the protein surface and the consequent preferential hydration of the protein.

To date, no systematic crystallographic study appears to have been carried out to explore at near-atomic resolution the effect of these additives on protein structure and hydration. Such a study would also reveal the direct interactions, if any, of the stabilizing compounds with the protein and the water around it. Hen egg-white lysozyme is a good model system for such a study, as its structure in many crystal forms is well characterized and also as a number of investigations have

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell.

	Sucrose 30%	Sorbitol 20%	Trehalose 30%	Native tetragonal	Ortho-rhombic	Monoclinic
Space group	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P2_12_12_1$	$P2_1$
Unit-cell parameters						
a (Å)	79.00	79.34	79.17	79.20	30.56	27.42
b (Å)	79.00	79.34	79.17	79.20	58.99	62.80
c (Å)	38.15	37.89	38.12	37.94	68.26	60.94
β (°)						92.7
Z	8	8	8	8	4	4
Unit-cell volume (Å^3)	238094	238511	238931	237983	123054	104819
Solvent content (%)	38.6	39.8	39.9	39.6	41.6	31.5
Data resolution (Å)	1.90	1.85	1.90	1.90	1.80	1.80
Last shell (Å)	1.97–1.90	1.92–1.85	1.97–1.90	1.97–1.90	1.86–1.80	1.86–1.80
No. of measured observations	79880	120501	25368	46026	41051	68752
No. of unique reflections	9892 (973)	10776 (1043)	9275 (917)	9803 (954)	11864 (1089)	19151 (1889)
No. of reflections with $I = 0$	139 (39)	177 (44)	253 (68)	175 (45)	449 (125)	290 (88)
Completeness of data (%)	98.9 (99.9)	99.5 (100)	92.3 (93)	98.0 (98.2)	98.8 (94.4)	99.5 (99.2)
Merging R for all reflections (%)	6.2 (21.3)	8.6 (32.8)	6.4 (22.7)	7.7 (19.7)	8.1 (39.1)	7.7 (21.6)
Average $I/\sigma(I)$	14.1	10.6	11.3	10.1	8.1	9.3

been carried out on the effect of additives and cosolvents on its structure and hydration (Back *et al.*, 1979; Gekko, 1982; Wimmer *et al.*, 1997). Indeed, we ourselves have been studying the high-resolution crystal structures of different forms of lysozyme and their low-humidity variants in order to delineate the role of water molecules in the plasticity and action of the protein (Kodandapani *et al.*, 1990; Madhusudhan & Vijayan, 1991; Madhusudan *et al.*, 1993; Nagendra *et al.*, 1995, 1996, 1998; Sukumar *et al.*, 1999; Biswal *et al.*, 2000). Here, we report the crystal structures of tetragonal lysozyme grown in the presence of two sugars, namely sucrose and trehalose, and one polyol, sorbitol. The purpose of this study is to understand the stabilizing effect of these additives at the molecular level. We also report the structures of monoclinic and orthorhombic lysozyme grown under the conditions used for obtaining the tetragonal form. This permits us to compare the changes in the structure and hydration of the protein arising from the presence of additives with those that normally occur on account of variations in the packing environment.

2. Experimental

2.1. Crystallization

Hen egg-white lysozyme was purchased from Sigma Chemical Company and was used directly for crystallization without further purification. Sorbitol and trehalose were obtained from LOBA Chemie Pvt. Ltd, Mumbai, India. Sucrose was obtained from Qualigens Fine Chemicals, Mumbai, India. All crystallization experiments were carried out using 0.04 *M* acetate buffer pH 4.6. Separate stock solutions containing 50, 100, 200, 300, 400 and 500 mg ml^{-1} of sucrose, sorbitol and trehalose in the buffer were prepared. In a typical experiment, 80 mg of lysozyme powder was dissolved in an appropriate stock solution. The same volume of the same solution containing 100 mg NaCl per millilitre was added dropwise into the protein solution and left undisturbed. Tetragonal lysozyme crystals grew in 2–3 weeks. No crystals grew when the concentration of the additive was 50% and crystals rarely grew at 40% concentration. Crystals were obtained in experiments involving 30% or less concentration of the additive. For comparison, native tetragonal crystals were also grown from solutions containing no additives. In the crystallization experiment involving 5% sorbitol, needle-like crystals were observed in addition to tetragonal crystals. These crystals turned out to be orthorhombic. They were then used as seeds in crystallization setups without any additive in the acetate buffer. The crystals that resulted from these experiments turned out to be monoclinic.

2.2. Data collection

Preliminary checks on crystals grown in different experiments were carried out using a MAR imaging plate mounted on an RU-200 Rigaku rotating-anode X-ray generator with a copper target. In general, crystal quality tended to decrease with increasing concentration of the additive. Also, the proportion of good quality crystals was lower at high additive concentrations. The resolution of data fell to lower than 2 Å at concentrations of sorbitol higher than 20%. In the case of the other two additives, a couple of good-quality crystals could be observed even at 30% concentration. The orthorhombic crystals grown at a low concentration of sorbitol and native monoclinic crystals obtained using the orthorhombic crystals as seeds diffracted well beyond 2 Å resolution.

Intensity data from native tetragonal crystals and those grown in the presence of 30% sucrose, 20% sorbitol, 30% trehalose and from the orthorhombic and the monoclinic crystals were collected on the same MAR imaging plate at 293 K. The crystal-to-plate distance was kept at 100 mm in all cases. Each frame, involving a rotation of 1°, was recorded for 6 min. Every effort was made to ensure that data were collected successively during the same period from all the crystals under identical conditions. Data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) in an identical manner. The data-collection statistics are given in Table 1.

2.3. Structure refinement

The atomic coordinates of the enzyme molecule in the high-resolution structure of tetragonal lysozyme crystals grown in the APCF apparatus (Vaney *et al.*, 1996) were used as the

Table 2
Refinement parameters.

	Sucrose 30%	Sorbitol 20%	Trehalose 30%	Native tetragonal	Ortho-rhombic	Monoclinic
Resolution limit used in refinement (Å)	30.0–1.9	30.0–1.9	30.0–1.9	30.0–1.9	30.0–1.9	30.0–1.9
No. of reflections with $F > 0$	9730	9792	9009	9613	9881	16134
Final R factor (%)	19.5	19.0	19.4	18.9	18.8	17.6
R_{free} (%)	23.3	21.3	22.5	23.4	22.3	20.7
R.m.s. deviation from ideal						
Bond length (Å)	0.005	0.004	0.005	0.005	0.005	0.005
Bond angles (°)	1.3	1.3	1.3	1.4	1.4	1.4
Dihedral angles (°)	22.2	22.8	22.6	22.9	23.5	23.3
Improper angles (°)	0.7	0.6	0.7	0.8	0.8	0.8
No. of protein atoms	1001	1001	1001	1001	1001	2002
No. of water molecules	145	151	150	142	134	290

starting model for the refinement of the four tetragonal structures under study. Those for orthorhombic lysozyme grown at pH 9.5 (Sukumar *et al.*, 1999) and for monoclinic lysozyme obtained using $NaNO_3$ as the precipitant (Nagendra *et al.*, 1996) were the starting models employed for refining the structures of the orthorhombic and monoclinic crystals, respectively. Although the resolution of data from the six crystals varied from 1.8 to 1.9 Å, data in the 30–1.9 Å resolution shell were used in the refinement of all six structures for the sake of uniformity. The structures were refined in an identical manner using *CNS* (Brunger *et al.*, 1998). In each case, non-protein atoms were removed from the starting model. Initially, the structure was refined treating the whole molecule as a rigid body. This was followed by molecular-dynamics refinement using the simulated-annealing technique. Electron-density maps were calculated at this stage to correct and rebuild the model, wherever necessary, using *FRODO* (Jones, 1978). This was followed by the refinement of atomic parameters. Identification of water molecules began at the stage at which the R value converged to 0.23. This was performed in two stages. In the first stage, water molecules were selected based on peaks of at least 3σ in $F_o - F_c$ and 1σ in $2F_o - F_c$ electron-density maps. Cycles of positional and B-factor refinement and correction of the model using Fourier maps were repeated. In the second stage, the selection was based on peaks of at least 2.5σ in $F_o - F_c$ and 0.8σ in $2F_o - F_c$ maps. This was continued until no significant density was left in the electron-density maps. The possibility that a few of the sites corresponded to positions of ions cannot be ruled out. Dauter *et al.* (1999) have identified eight chloride ions and one sodium ion in their 1.53 Å resolution structure of tetragonal lysozyme. In the present investigation, water molecules have been identified close to their positions in each tetragonal structure. Electron density, interpreted as that of a water molecule, appeared close to four, three and four 'chloride' locations around molecules *A*, *B* and the molecule in the orthorhombic form, respectively. None of these water O atoms showed abnormally low temperature factors, nor was there any residual electron density at their locations. Hence, they were treated as water molecules. Towards the end of the refinement, the model was checked against omit maps (Vijayan, 1980; Bhat & Cohen, 1984) to confirm the location of the water molecules and to minimize the effect of model bias. The stereochemical quality of the structures were validated using the program *PROCHECK* (Laskowski *et al.*, 1993). In the case of the sucrose–lysozyme complex, one sucrose molecule was located in the electron-density map. An $F_o - F_c$ difference Fourier map permitted initial unambiguous positioning of the sucrose molecule. Although the electron density corresponding to the fructose moiety was not clear in this map, subsequent refinement improved the quality of the map. The geometric parameters of the sucrose molecule were obtained from the HIC-Up database (Kleywegt & Jones, 1998). Refinement parameters for all structures are given in Table 2.

2.4. Accessibility and superpositions

Solvent-accessible surface areas were estimated using the program *MSP* (Connolly, 1993). A probe radius of 1.2 Å was used throughout the calculations. The program *ALIGN* (Cohen, 1997) was used for superposition of different structures.

3. Results and discussion

A comparison of the three tetragonal structures grown in the presence of high concentrations of sucrose, sorbitol and trehalose with the native tetragonal structure should bring out the effect of additives on the structure and hydration of the protein molecule. On the other hand, a comparison among the native tetragonal, orthorhombic and monoclinic crystals should serve to highlight the effect of packing. The monoclinic crystals contain two crystallographically independent molecules and thus a total of four molecules with different packing environments are available for comparison in the present study.

3.1. Molecular structure

R.m.s. deviations in C^α positions, main-chain atoms and side-chain atoms as obtained on pairwise superposition of the molecules in the four tetragonal structures and those in the native tetragonal, orthorhombic and monoclinic crystals are given in Table 3. The values clearly show that the effect of additives on the overall molecular structure is small; in fact, the r.m.s. deviations in atomic positions caused by additives are considerably lower than those caused by differences in the crystal packing. The variations in the deviations in C^α positions in the two sets of molecules along the polypeptide chain are illustrated in Fig. 1. The variations follow the same pattern, although the magnitudes of deviation caused by changes in

packing are higher than those caused by additives. In both cases, loops known to be flexible (Biswal *et al.*, 2000) show larger deviations. The observed pattern of deviations therefore represent the known differential flexibility of different regions of the enzyme molecule and not any specific effect of additives.

It has been shown earlier that 45 of the 103 non-Gly, non-Ala, non-Pro residues in lysozyme have totally conserved side-chain conformations in 20 crystallographically independent enzyme molecules (Biswal *et al.*, 2000). The same conformations are retained in the seven independent molecules in the present study. Of 309 relevant residues in the three structures grown in the presence of additives, only 21 have conformations significantly different (Biswal *et al.*, 2000) from that in the native tetragonal crystals, compared with 28 in the three molecules in the orthorhombic and monoclinic crystals, indicating again the higher effect of differences in packing than of additives. In both cases, a majority of the residues with changed conformation are arginine and lysine which have long and flexible side chains.

Internal hydrogen bonds provide another indication of structural conservation. The hydrogen bonds described as invariant by Biswal *et al.* (2000) exist in all seven molecules. The refined structure of the native tetragonal crystal in the present study contains 228 main-chain–main-chain, 70 main-chain–side-chain and 15 side-chain–side-chain hydrogen bonds. Of these, those retained in the structures containing sucrose, sorbitol and trehalose are 225, 57 and 8; 222, 62 and 12; and 222, 62 and 14, respectively. The corresponding numbers in the orthorhombic crystals and molecules *A* and *B* of the monoclinic crystals are 219, 49 and 9; 222, 51 and 10; and 223, 47 and 10, respectively. These numbers indicate that main-chain–main-chain hydrogen bonds are largely unaffected by the additives as well as changes in the crystal packing. The effect of additives on hydrogen bonds involving side chains is, however, lower than that caused by packing. Some hydrogen bonds are disrupted by the binding of a sucrose molecule to the enzyme.

Table 3
R.m.s. deviations (Å) in C^{α}, main-chain and side-chain atoms.

(*a*) Tetragonal forms without and with additives.

	Lysozyme–sucrose	Lysozyme–sorbitol	Lysozyme–trehalose
Native tetragonal	0.11, 0.12, 0.89	0.10, 0.10, 0.80	0.09, 0.11, 0.92
Lysozyme–sucrose	—	0.13, 0.13, 0.74	0.12, 0.13, 0.86
Lysozyme–sorbitol	—	—	0.10, 0.11, 0.58

(*b*) Native forms.

	Orthorhombic	Monoclinic *A*	Monoclinic *B*
Native tetragonal	0.41, 0.46, 1.38	0.31, 0.32, 1.35	0.44, 0.48, 1.52
Orthorhombic	—	0.43, 0.48, 1.34	0.45, 0.55, 1.41
Monoclinic *A*	—	—	0.45, 0.49, 1.52

3.2. Hydration

Thermodynamic studies on the effect of cosolvents and additives on proteins have stressed the reorganization of water molecules around protein molecules. The high-resolution X-ray results reported here enable one to explore the reorganization, if any, of ordered water molecules in the presence of additives in comparison to that normally caused by changes in crystal packing. As indicated in Table 2, the number of water molecules identified for each protein molecule has comparable values in different structures. Assuming the specific gravity of water in the crystals to be 1, a rough-and-ready estimate indicates that they represent about a half (monoclinic form) to a third (orthorhombic form) of the water molecules present in the crystals. A more important parameter is the number of molecules in the hydration shell of each protein molecule. As in the previous studies in this laboratory, the primary hydration shell of a protein molecule is considered to be made up of ordered water molecules at a distance of 3.6 Å or less from a protein O or N atom. The numbers of water molecules in the hydration shells are 114, 131 and 127 in the crystals grown in the presence of sucrose, sorbitol and trehalose, respectively. The corresponding numbers in the native tetragonal, the orthorhombic and molecules *A* and *B* of the monoclinic crystals are 127, 127, 138 and 143, respectively. All the tetragonal crystals, except that containing a bound sucrose molecule, have very similar numbers of water molecules in the hydration shell. Therefore, there is no evidence for preferential hydration in the presence of additives, at least as far as the ordered bound water molecules are concerned.

In addition to the number of protein-bound water molecules, their organization is also of crucial importance in the present study. As in our previous studies, a water molecule in one form and that in another form may be considered as equivalent if they interact with at least one common protein atom and if the distance between them is less than 1.8 Å when the two protein molecules along with their hydration shells are superposed. The positions of water molecules associated with proteins are highly variable and only a few ordered water molecules remain invariant with respect to their locations across crystal forms (Biswal *et al.*, 2000). Therefore, one can, in general, discuss variations only in terms of average effects. In this context, the number of equivalent water molecules in pairs of crystallographically independent molecules, listed in Table 4, is a good parameter. The numbers of equivalent water molecules are clearly very high among the tetragonal crystals irrespective of the presence of the additives. They are substantially higher than those among molecules in different packing environments but with the same solution around the molecules.

The binding-site groove is a heavily hydrated region of the molecule (Madhusudan *et al.*, 1993). Water molecules are necessary not only as catalytic equipment (Rupley *et al.*, 1983; Rupley & Careri, 1991), but also to maintain the active-site geometry (Nagendra *et al.*, 1998). The number of water molecules in the binding groove of lysozyme varies between 20 and 28. 14 of them remain invariant among the tetragonal

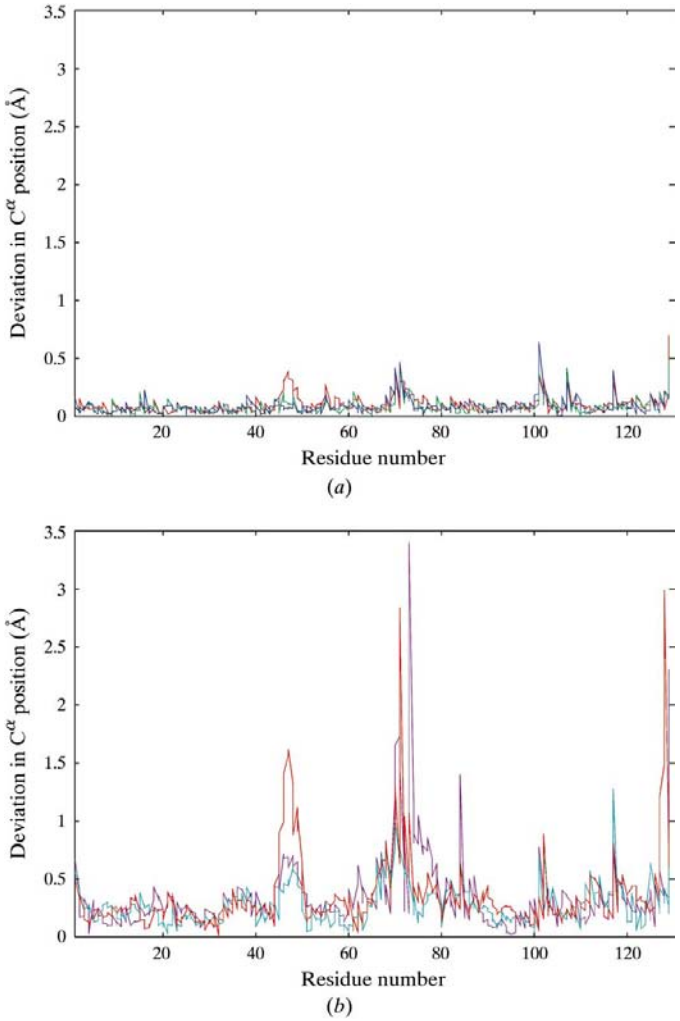

Figure 1
(*a*) Deviations of C^{α} positions in crystals grown in the presence of sucrose (red), sorbitol (green) and trehalose (blue) from those in the native tetragonal crystals. (*b*) Deviations of C^{α} positions in orthorhombic crystals (magenta) and molecule *A* (cyan) and molecule *B* (orange) of monoclinic crystals from those in native tetragonal crystals. These plots were prepared using *GNUPLOT* (Williams & Kelley, 1999)

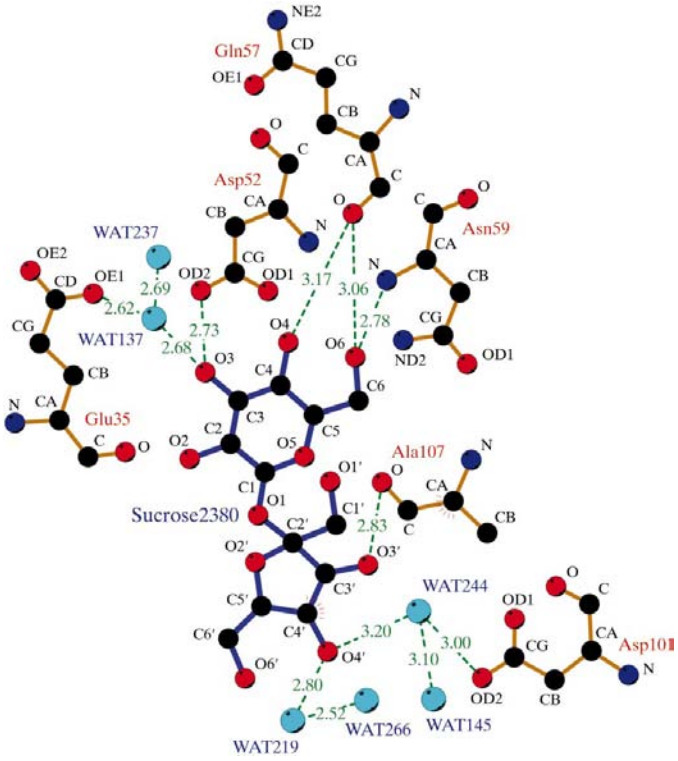

Figure 3
Schematic representation of the interactions of sucrose with the enzyme molecule. This figure was prepared using *LIGPLOT* (Wallace *et al.*, 1995).

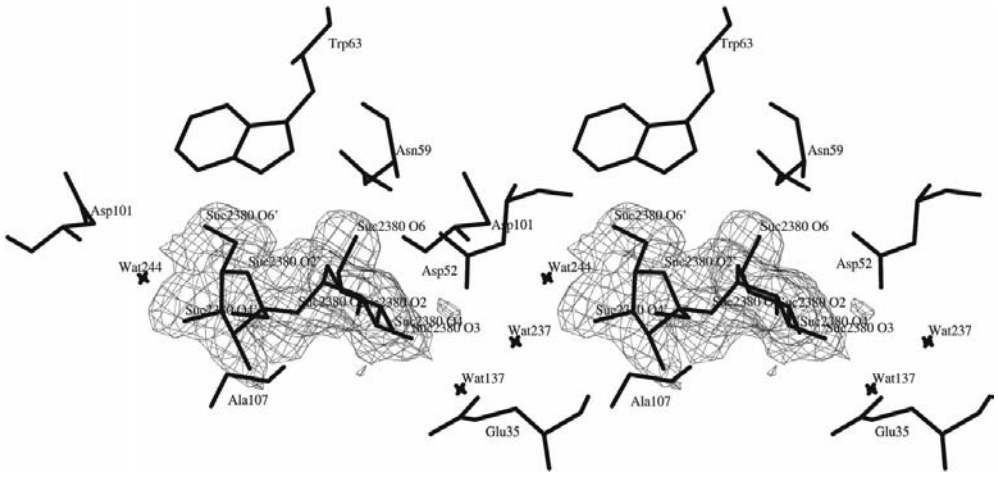

Figure 2
Stereoview of the $F_o - F_c$ omit map showing the density for the sucrose molecule in the lysozyme–sucrose complex. The residues in the neighbourhood are also shown. The map has been contoured at 2.2σ. This figure was prepared using *FRODO* (Jones, 1978).

crystals, while the corresponding number is seven among the tetragonal, the orthorhombic and the monoclinic crystals. This again indicates that additives do not cause any major reorganization in the binding site. There are four water molecules in the active site common to all the seven lysozyme molecules under study. One of them forms part of a water network connecting the catalytic residues Glu35 and Asp52. This water molecule is the same as that identified as invariant in the earlier study of 20 independent lysozyme molecules (Biswal *et al.*, 2000).

The number of buried water molecules is a measure of the cavities within the protein molecule. The seven lysozyme molecules have four buried water molecules located at identical positions. They include the one identified as buried in all lysozyme structures (Biswal *et al.*, 2000). All of the seven water molecules identified as invariant are also present in the seven structures. Six of the seven N and O atoms hydrated in all the 20 molecules used in the earlier study are hydrated in all of the structures under consideration. Thus, there does not appear to

Table 4
Number of equivalent water molecules among different structures.

	Lysozyme–sucrose	Lysozyme–sorbitol	Lysozyme–trehalose
Native tetragonal	78	91	93
Lysozyme–sucrose	—	76	80
Lysozyme–sorbitol	—	—	88

	Orthorhombic	Monoclinic *A*	Monoclinic *B*
Native tetragonal	55	55	58
Orthorhombic	—	61	59
Monoclinic	—	—	65

be any systematic difference in structural features involving water molecules between native crystals and those grown in the presence of additives.

3.3. Interaction of sucrose with lysozyme

One sucrose molecule is bound to the active site of the enzyme molecule in the crystal grown in the presence of the sugar (Fig. 2). Sucrose, Glcα1-2Fru, is a disaccharide made up of glucose and fructose. The interactions of the sugar molecule with the protein are illustrated in Fig. 3. The glucose moiety occupies subsites *C* and *D*, while fructose interacts exclusively with subsite *C*. The crystal structures of several carbohydrate complexes of lysozyme are available. The α (Blake *et al.*, 1967) and the β (Beddell *et al.*, 1970) forms of GlcNAc bind at the *C* subsite. The disaccharide GlcNAcβ1-4Glc, like sucrose, occupies the *C* and *D* subsites (Beddell *et al.*, 1970). However di-NAG occupies subsites *B* and *C* (Kurachi *et al.*, 1976). The trisaccharides tri-NAG (Cheetham *et al.*, 1992) and NAM-NAG-NAM (Kelly *et al.*, 1979) occupy subsites *ABC* and *BCD*, respectively. There have been studies on the binding of small molecules to lysozyme, notably those involving ethanol (Lehmann *et al.*, 1985) and DMSO (Lehmann & Stanfield, 1989). In both cases, the binding appeared to be non-specific. Several lysozyme structures solved using data collected at very low temperatures are available (PDB codes 1a2y, 1at6, 1bhz, 1lz8, 1lz9, 3lyt, 3lzt and 5lyt). They involved the use of cryoprotectants, but in no case were the cryoprotectant molecules located in the lattice.

A superposition of the sucrose bound molecule on the molecule in the native tetragonal structure and search in locations within 2.7 Å from sugar O atoms and 2.8 Å from sugar C atoms indicated that the sucrose molecule replaces eight bound water molecules. That is the reason for the smaller number of water molecules in the hydration shell in the crystals grown in the presence of sucrose than in those in other crystals. It also turns out that two of the displaced water molecules have positions close to those of glucose O1 and O6. Like the sugar O atoms, these water molecules interact with Ala107 O and Asn59 N. Such mimicry of sugar hydroxyl groups by water molecules has been widely observed (Ravishankar *et al.*, 1999; Elgavish & Shannan, 1998; Delbaere *et al.*, 1990, 1993).

4. Conclusions

The results presented here conclusively demonstrate that the structure of the lysozyme molecule remains unaffected by the presence of the sugars sucrose and trehalose and the polyol sorbitol in the surrounding medium. The additives do not even affect the level of hydration as indicated by the numbers of ordered water molecules associated to the protein. Still more surprisingly, they do not cause any significant reorganization of water molecules in the hydration shell. In fact, the changes in the hydration shell caused by the additives are smaller than those normally caused by differences in packing arrangement. Among the three additives, only one interacts with the protein in a coherent manner. A sucrose molecule binds at the binding groove of the enzyme molecule.

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