Effect of stabilizing additives on the structure and hydration of proteins: a study involving monoclinic lysozyme

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In pursuance of a long-range programme on the hydration, mobility and action of proteins, the structural basis of the stabilizing effect of sugars and polyols is being investigated. With two crystallographically independent molecules with slightly different packing environments in the crystal, monoclinic lysozyme constitutes an ideal system for exploring the problem. The differences in the structure and hydration of the two molecules provide a framework for examining the changes caused by stabilizing additives. Monoclinic crystals were grown under native conditions and also in the presence of 10% sucrose, 15% trehalose, 10% trehalose, 10% sorbitol and 5% glycerol. The crystal structures were refined at resolutions ranging from 1.8 to 2.1 Å. The average B values, and hence the mobility of the structure, are lower in the presence of additives than in the native crystals. However, a comparison of the structures indicates that the effect of the additives on the structure and the hydration shell around the protein molecule is considerably less than that caused by differences in packing. It is also less than that caused by the replacement of $NaNO_3$ by NaCl as the precipitant in the crystallization experiments. This result is not in conformity with the commonly held belief that additives exert their stabilizing effect through the reorganization of the hydration shell, at least as far as the ordered water molecules are concerned.

1. Introduction

Different sugars and polyols are extensively used for stabilizing protein structures (Back et al., 1979; Timasheff, 1993; Wimmer et al., 1997). In view of its fundamental importance and possible applications, detailed thermodynamic and related studies have been carried out on the stabilizing effects of these compounds (Timasheff, 1993; Xie & Timasheff, 1997; Wimmer et al., 1997). These studies have indicated that higher stability is achieved by the preferential hydration of the protein molecule in the presence of the additives concerned. This implies a reorganization of the water molecules associated with the protein. However, detailed studies at near-atomic resolution aimed specifically at elucidating the postulated reorganization of the hydration shell are of recent origin. The first crystallographic attempt in this direction was recently made by us (Datta et al., 2001) as part of a long-range programme concerned with protein hydration and its consequences (Kodandapani et al., 1990; Madhusudan & Vijayan, 1991; Madhusudan et al., 1993; Nagendra et al., 1995, 1996, 1998; Sukumar et al., 1999; Biswal et al., 2000). In this attempt, the structures of tetragonal lysozyme grown in the presence of

Table 1
Data-collection statistics.

Values pertaining to the second set of experiments are given in bold in this and subsequent tables. Values in parentheses refer to the highest resolution shell.

	Native		10% sucrose		15% trehalose	10% trehalose	10% sorbitol	5% glycerol	
Space group	$P2_1$	**$P2_1$**	$P2_1$	**$P2_1$**	$P2_1$	$P2_1$	$P2_1$	$P2_1$	**$P2_1$**
a (Å)	27.89	**27.83**	28.01	**27.99**	28.07	27.99	27.78	27.86	**27.91**
b (Å)	62.76	**62.71**	62.91	**62.92**	62.94	62.76	62.71	62.71	**62.81**
c (Å)	60.27	**60.19**	60.48	**60.40**	60.41	60.21	60.29	60.02	**60.33**
β (°)	90.9	**90.5**	90.6	**90.6**	90.7	90.7	90.7	90.4	**90.6**
Z	4	**4**	4	**4**	4	4	4	4	**4**
Unit-cell volume (Å³)	105482	**105040**	106566	**106366**	106719	105760	105022	104858	**105754**
Solvent content (%)	32.1	**31.8**	32.7	**32.6**	32.8	32.2	31.8	31.7	**32.2**
Data resolution (Å)	2.0	**1.95**	2.0	**1.8**	2.1	2.0	2.0	1.9	**1.8**
Last shell (Å)	2.07–2.00	**2.02–1.95**	2.07–2.00	**1.86–1.80**	2.18–2.10	2.07–2.00	2.07–2.00	1.97–1.90	**1.86–1.80**
No. of measured reflections	39179	**58120**	47985	**135208**	45545	51393	61435	61157	**114902**
No. of unique reflections	13653 (1311)	**14462 (1432)**	14182 (1392)	**18248 (1789)**	12088 (1194)	13471 (1293)	13571 (1290)	15695 (1520)	**18861 (1862)**
No. of reflections with $I = 0$	302 (64)	**275 (82)**	102 (23)	**48 (19)**	317 (85)	91 (25)	147 (37)	119 (40)	**109 (35)**
Completeness (%)	96.7 (95.7)	**95.2 (94.9)**	99.3 (99.9)	**93.4 (90.9)**	97.6 (96.0)	95.1 (93.2)	96.4 (94.2)	95.8 (93.9)	**97.2 (95.1)**
R_{merge}(%)	5.2 (16.1)	**5.8 (17.9)**	4.6 (8.7)	**5.4 (12.4)**	8.0 (24.7)	9.1 (15.7)	7.5 (16.5)	4.6 (12.0)	**4.6 (13.9)**
Average $I/\sigma(I)$	11.8	**11.5**	11.2	**16.5**	7.3	10.4	8.5	10.7	**16.3**

sucrose, trehalose and sorbitol were refined. These structures, including the arrangements of water molecules in them, were then compared with those in monoclinic and orthorhombic lysozyme prepared in conditions under which tetragonal crystals normally grow. This comparison clearly showed that the effect of the stabilizing additives on the protein structure and, more importantly, on the organization of ordered water molecules attached to the protein, are substantially less than that of normal differences caused by variations in molecular packing.

In view of the importance of the phenomenon and the voluminous literature on solution studies on it, we felt it important to carry out further crystallographic studies. Even in high-resolution crystal structures, the positions of individual water molecules are associated with a comparatively high degree of uncertainty. Therefore, results obtained on the basis of positions of individual water molecules should be treated with extreme caution. Statistical inferences based on comparative studies, as in the work involving tetragonal lysozyme mentioned earlier, are more reliable. Monoclinic lysozyme is an ideal system for carrying out such studies. The monoclinic crystals contain two crystallographically independent molecules that have slightly different packing environments. The differences in the structure and the hydration shells of these two molecules would represent the effects normally associated with differences in packing environments. They could provide a framework for assessing the differences caused by the presence of stabilizing additives in the medium. Furthermore, monoclinic lysozyme crystals grown from solutions of different compositions are also available (Nagendra *et al.*, 1996; Datta *et al.*, 2001). Comparison of their structures with those of crystals grown in the presence of stabilizing additives could provide information on the relative effects of normal additives and stabilizing additives on the structure and hydration of lysozyme molecules. Therefore, a detailed X-ray study of monoclinic lysozyme crystals grown in the presence of sucrose, trehalose, sorbitol and glycerol was carried out.

2. Experimental

Hen egg-white lysozyme purchased from Sigma Chemical Company was used for crystallization. Sorbitol, trehalose and glycerol were obtained from LOBO Chemie Pvt. Ltd, Mumbai, India and sucrose from Qualigens Fine Chemical, Mumbai, India. Crystallization experiments were carried out in 0.1 *M* sodium acetate buffer pH 4.6. Separate stock solutions of 100, 200, 300, 400 and 500 mg ml^{-1} sucrose, trehalose, sorbitol and glycerol were prepared in the buffer containing 7.5%(*w/v*) NaNO$_3$. A 2% solution of lysozyme in the same buffer was also prepared. In each crystallization experiment, equal volumes of the protein solution and one or the other of the stock solutions were gently mixed and left undisturbed at 293 K. Crystals did not grow at high concentrations of the additives. Especially in the case of glycerol, crystals were only obtained in experiments involving 5% of the additive. Good crystals could be grown at 10% concentrations of the other additives. Only in the case of trehalose could crystals be grown at a higher additive concentration (15%). For purposes of comparison, native crystals were also prepared under identical conditions.

Intensity data were collected at 293 K from the native crystals and crystals grown in the presence of 5% glycerol, 10% sucrose, 10% trehalose, 10% sorbitol and 15% trehalose on a MAR imaging plate mounted on an RU-200 Rigaku rotating-anode X-ray generator. The crystal-to-detector distance was maintained at 100 mm in all experiments. Care was taken to ensure that the data-collection conditions were identical in all cases. The data sets were processed in an identical manner using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The data-collection statistics are given in Table 1.

The atomic coordinates of native monoclinic lysozyme (Nagendra *et al.*, 1996; PDB code 1uco) obtained using 2% NaNO$_3$ as the precipitant were used as the starting model for refinement. All the structures were refined in an identical

Table 2
Refinement parameters.

	Native		10% sucrose		15% trehalose	10% trehalose	10% sorbitol	5% glycerol	
Resolution limit used in refinement (Å)	30.0–2.0	**30.0–1.95**	30.0–2.0	**30.0–1.8**	30.0–2.1	30.0–2.0	30.0–2.0	30.0–1.9	**30.0–1.8**
No. of reflections with $F > 0$	13344	**14177**	14066	**18200**	11759	13379	13423	15576	**18746**
Final R factor (%)	17.8	**18.5**	17.3	**19.7**	17.1	18.4	18.2	20.5	**19.8**
R_{free} (%)	23.3	**23.0**	21.2	**22.7**	21.9	21.3	23.2	24.3	**22.6**
No. of protein atoms	2002	**2002**	2002	**2002**	2002	2002	2002	2002	**2002**
No. of nitrate ions	5	**5**	6	**6**	8	6	5	7	**7**
No. of water molecules	279	**284**	297	**349**	245	290	268	309	**372**
R.m.s. deviation from ideal									
Bond length (Å)	0.005	**0.005**	0.005	**0.005**	0.005	0.005	0.005	0.005	**0.005**
Bond angles (°)	1.3	**1.4**	1.3	**1.4**	1.3	1.3	1.3	1.3	**1.3**
Dihedral angles (°)	23.2	**23.6**	23.1	**23.4**	23.2	23.2	23.4	23.3	**23.3**
Improper angles (°)	0.7	**0.8**	0.7	**0.7**	0.7	0.7	0.7	0.7	**0.7**

manner using *CNS* (Brünger *et al.*, 1998). In each case, only protein atoms in the starting model were used in the refinement. The whole molecule was treated as a rigid body in the initial cycles of refinement. This was followed by refinement using simulated annealing, calculation of the electron-density map and rebuilding of the molecule using *O* (Jones *et al.*, 1991). Subsequent refinement of the atomic parameters led to R factors in the range 22–24%. In the subsequent stages of refinement, peaks greater than 3σ in $F_o - F_c$ maps and 1σ in $2F_o - F_c$ maps were identified as water O atoms. In the later cycles, the cutoff values were reduced to 2.5 and 0.8σ, respectively. Omit maps (Vijayan, 1980; Bhat & Cohen, 1984), in addition to $F_o - F_c$ and $2F_o - F_c$ maps, were also used in the later stages of refinement. Bulk-solvent correction and anisotropic scaling were used throughout. The stereochemical quality of the structures was validated using *PROCHECK* (Laskowski *et al.*, 1993). 5–8 nitrate ions were located in each structure. The geometric parameters of the nitrate ion were obtained from the HICCUP database (Kleywegt & Jones, 1998). In each case several trial calculations were carried out to ensure that a nitrate ion, and not a set of water molecules, best explains the relevant density. A summary of the refinement parameters is given in Table 2. In the final refined models, 87.6–88.5% of the residues lie in the most favoured regions of the Ramachandran plot (Ramachandran *et al.*, 1963). Only 1–3 residues are found in the generously allowed or disallowed regions.

In order to ascertain the repeatability of the results, a second set of experiments was carried out using sucrose and glycerol as representatives of the additives used in the original set of experiments. In addition to growing crystals in separate solutions containing 10% sucrose and 5% glycerol, native crystals were also grown simultaneously under identical conditions. X-ray data from the three crystals were collected in the same way as in the original measurements, except that an Osmic mirror was now used for collimation. The three structures were refined in the same way as the six structures in the original set of experiments. Data-collection statistics and refinement parameters pertaining to the second set of experiments are also given in Tables 1 and 2.

3. Results and discussion

The structures refined in the present study are those of native monoclinic lysozyme and the monoclinic crystals grown in the presence of 10% (0.29 *M*) sucrose, 15% (0.44 *M*) trehalose, 10% (0.29 *M*) trehalose, 10% (0.55 *M*) sorbitol and 5% (0.54 *M*) glycerol from a 1% (0.7 m*M*) solution of the protein. Each crystal contains two crystallographically independent molecules, designated as molecule *A* and molecule *B*. The slight differences between the molecules are presumably caused by the differences in their packing environments (Nagendra *et al.*, 1996). These differences provided a useful framework for examining the changes, if any, caused in the structures of molecule *A* and molecule *B* on account of the presence of stabilizing additives in the medium. In the present work, crystals were grown with 3.75% (0.44 *M*) NaNO$_3$ as the precipitant. Crystals grown in the presence of 2% (0.24 *M*) NaNO$_3$ were used in our earlier study (Nagendra *et al.*, 1996). The structure of monoclinic lysozyme prepared with 5% (0.86 *M*) NaCl as the precipitant is also available (Datta *et al.*, 2001). These studies provide information on changes brought about by variations in normal additives and it would be instructive to compare them with those brought about by the presence of stabilizing additives.

4. Structure of the protein molecule

The structures considered here involve 16 lysozyme molecules distributed over eight crystals. Of these, two belong to the native crystals grown using 3.75% NaNO$_3$ as the precipitant and have been chosen as the reference for comparison. The r.m.s. deviations in C$^\alpha$ positions on superposition of the remaining 14 molecules with respect to the two in the native structure are given in Fig. 1. The r.m.s. deviation between the two molecules in the native crystals is 0.45 Å. It is clearly seen that the r.m.s. deviations of C$^\alpha$ positions of molecule *A* in all other structures with respect to molecule *A* in the native structure are substantially lower than the r.m.s. deviations between molecules *A* and *B*. The same is true with respect to molecule *B*. However, the r.m.s. deviations of molecule *B* in

different structures with respect to molecule A and those of molecule A in different structures with respect to molecule B are close to the deviation between A and B in the same structure. Thus, the effect of additives, including the stabilizing additives, appears to be much less than that of differences in crystal packing. The largest effect of the composition of the crystallization medium is exhibited by the crystals grown with NaCl instead of NaNO$_3$ as the precipitant. R.m.s. deviations involving side-chain atoms also lead to results similar to those obtained in calculations using C$^{\alpha}$ positions.

The r.m.s. deviations involving structures obtained from the second representative data sets (Fig. 2a) follow the same trend. The r.m.s. deviations in C$^{\alpha}$ positions between the same molecule in corresponding structures (for example, the native structure derived from the first and second data sets) vary between 0.07 and 0.19 Å, while those between different molecules (A versus B) are in the 0.45–0.51 Å range. As can be seen from Fig. 2(a), similar values are obtained when the molecules in the native structure derived from the second data set are compared with those in the structures of the crystals grown in the presence of sucrose and glycerol derived from the second sets.

5. Effect on hydration

Water molecules were located in exactly the same way in all the structures under study in order to facilitate meaningful comparison. The numbers of molecules identified, which also depends on other factors such as resolution and quality of the data, have comparable values in the structures. The number varies between 269 and 297 in the structures refined at 2 Å resolution, whereas it is 245 and 309 in the structures refined at

2.1 and 1.9 Å resolution, respectively. Comparable numbers of ordered water molecules were located in the other two monoclinic lysozyme crystals used in the present analysis. As in the earlier investigations from this laboratory, a water molecule within a distance of 3.6 Å from a protein N or O atom was considered to belong to the hydration shell of the
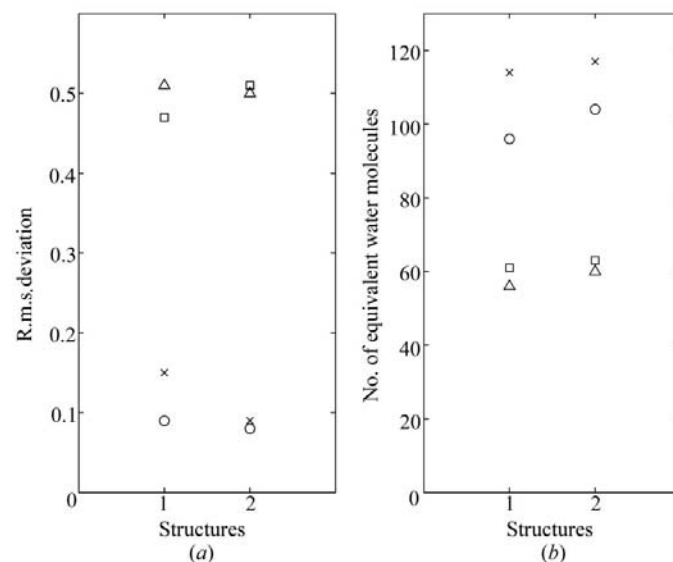

Figure 2
R.m.s. deviations in C$^{\alpha}$ positions (a) and number of equivalent water molecules in the hydration shell (b) derived from the second set of experiments. The symbols in (a) have the same meaning as in Fig. 1. 1 and 2 here refer to crystals grown in the presence of 10% sucrose and 5% glycerol, respectively. Equivalent water molecules between molecules A and A, A and B, B and A, and B and B are represented by circles, squares, triangles and crosses, respectively in (b).
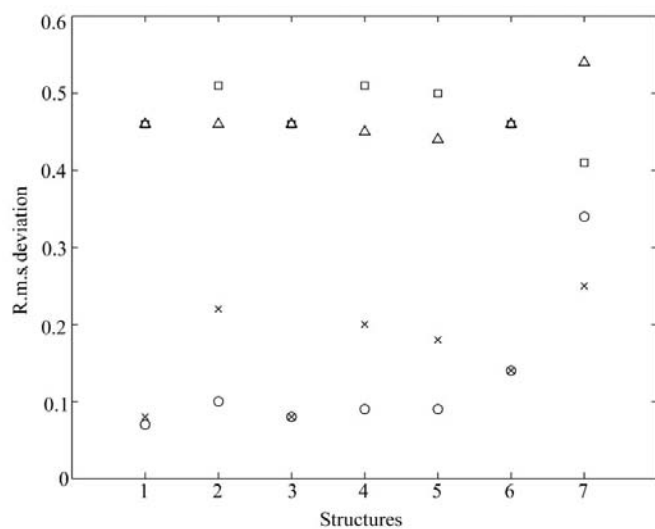

Figure 1
R.m.s. deviations in C$^{\alpha}$ positions from the native structure of structures of crystals grown in the presence of 10% sucrose (1), 15% trehalose (2), 10% trehalose (3), 10% sorbitol (4), 5% glycerol (5), a lower concentration of NaNO$_3$ (6) and NaCl instead of NaNO$_3$ as the precipitant (7). Deviations between molecules A and A, A and B, B and A, and B and B are represented by circles, squares, triangles and crosses, respectively.
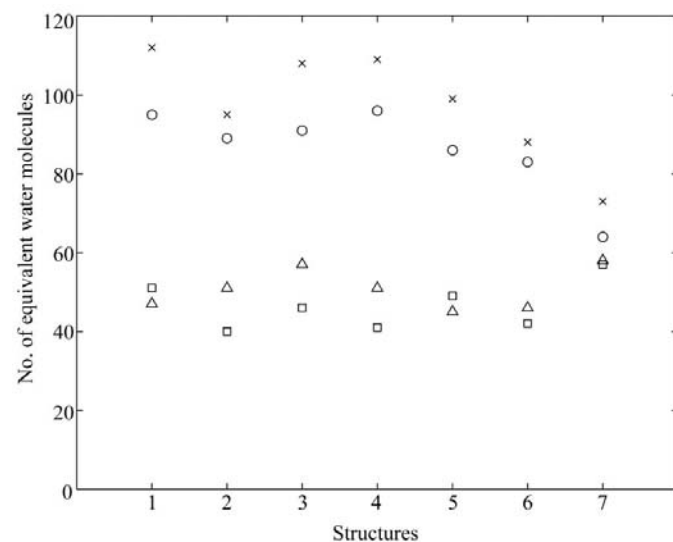

Figure 3
Number of equivalent water molecules in the hydration shell when the native structure is compared with the structures of crystals grown in the presence of 10% sucrose (1), 15% trehalose (2), 10% trehalose (3), 10% sorbitol (4), 5% glycerol (5), a lower concentration of NaNO$_3$ (6) and NaCl instead of NaNO$_3$ as the precipitant (7). The symbols have the same meaning as in Fig. 2(b).

Table 3
Average B values (Å^2).

	Main chain		Side chain	
	A	B	A	B
Native	22.0	20.4	24.0	22.0
	24.4	**22.5**	**25.6**	**23.5**
10% sucrose	19.6	18.7	22.3	20.7
	22.9	**21.7**	**24.4**	**23.1**
15% trehalose	19.2	17.5	21.4	19.5
10% trehalose	20.0	18.9	22.5	21.1
10% sorbitol	19.3	17.8	20.8	19.0
5% glycerol	16.7	15.0	18.1	16.3
	22.0	**20.4**	**22.6**	**20.9**

protein. It has been suggested that stabilizing additives exert their influence by modifying the hydration of proteins. Therefore, what is looked for is a change in the hydration shell. A water molecule in the hydration shell of a protein molecule and one in the shell of another protein molecule are considered equivalent if they have a common interaction with a protein atom and if the distance between the two water molecules is less than 1.8 Å when the two protein molecules are superposed along with their hydration shells. The numbers of equivalent water molecules between shells associated with the protein molecules in the native structure on the one hand and those associated with molecules in the other structures are given in Fig. 3. The number of equivalent water molecules works out to be 45 when the hydration shells of molecules A and B in the native structure are compared. This provides a measure of similarity of hydration shells that survives the difference in crystal packing. Interestingly, the number of equivalent water molecules between the hydration shell of molecule A in the native structure and those of molecule A in the crystals grown in the presence of stabilizing additives ranges between 81 and 96. The corresponding numbers in the case of molecule B are 88 and 112, respectively. On the other hand, the number of equivalent water molecules between the hydration shells of molecule B in the crystals containing the additives and those of molecule A in the native crystals ranges between 40 and 51. The corresponding numbers are 45 and 57 when the hydration shells of molecule A in the crystals containing the additives and that of molecule B in the native crystals are compared. Thus, the effect of the stabilizing additives on the hydration shell is considerably less than that resulting from changes in packing environment. It is similar to that caused by the change in the $NaNO_3$ concentration in the medium, but is less than that caused by the replacement of $NaNO_3$ by NaCl as the precipitant.

The resolution of the second data sets is higher than that of the original sets, presumably on account of the use of the Osmic mirror in the former. Consequently, the numbers of water molecules identified are higher in the structures derived from the second data sets (Table 2). However, as can be seen from Fig. 2(b), the numbers of equivalent water molecules between pairs of molecules in the three structures follow the same trend as those in the structures derived from the original data sets.

As in the case of the earlier study involving tetragonal lysozyme (Datta *et al.*, 2001), features such as the hydration of the binding site, burial of water molecules by the protein, functional groups which are invariably hydrated *etc.* (Biswal *et al.*, 2000) were carefully examined. No systematic difference in these features involving water molecules is readily discernible between native monoclinic lysozyme and the monoclinic crystals grown in the presence of stabilizing additives.

6. *B* values and stability

The average B values of main-chain and side-chain atoms in the crystal structures presented here are listed in Table 3. Individual B values derived from protein structures determined even at reasonably high resolutions, as in the present case, are subject to substantial errors. However, when averaged over hundreds of atoms, these parameters provide a rough and ready estimate of the flexibility of the protein, especially when the same molecules in somewhat different environments are compared. Furthermore, those of the two crystallographically independent molecules in the structure provide an internal check on their stability. The B factors in molecule A are higher than those in molecule B, presumably on account of the difference in mobility resulting from the difference in the packing environment. That this is so in every one of the six crystal structures shows that errors in B values are not large enough to obscure genuine differences in mobilities. Interestingly, in every case the average B values in the crystals grown in the presence of stabilizing agents are lower than those in the native crystals. Thus, there appears to be a perceptible decrease in mobility of the protein in the presence of the stabilizing additives.

The average B values derived from the second set of refinements are also listed in Table 3. Displacement parameters are more susceptible to external effects than positional coordinates and it is not often meaningful to compare the B values obtained under one set of experimental conditions with those derived under a different set of conditions. The average B values obtained from the second set of experiments are somewhat higher than those derived from the original set. However, within the former, as in the case of the original set of values, the values are lower in the crystals grown in the presence of additives than in the native crystals, although the differences are not as high as in the original set. Also, in every case the overall B value calculated using Wilson's plot is lower in crystals grown in the presence of additives than in the native crystals, both in the original set and in the second set (data not given).

Based on detailed NMR studies, Wimmer *et al.* (1997) have suggested that the mobility of a set of alanyl and threonyl residues increases whereas that of another set decreases in the presence of sorbitol. The B values of the concerned residues do not corroborate this suggestion. These B values follow the same pattern as that followed by those of the whole molecule. The NMR studies also suggest that water is displaced from the enzyme surface close to Ile88 upon addition of sorbitol. The X-ray results, however, do not indicate any such displacement.

7. Bound nitrate ions

Unlike in the case of the earlier study involving tetragonal crystals (Datta *et al.*, 2001), there is no evidence of bound sugar, sorbitol or glycerol molecules in the structures presented here. It may be recalled that a bound sugar molecule was found at the binding site of the enzyme in the tetragonal crystals grown in the presence of sucrose. Even in tetragonal lysozyme, where good-quality crystals could be grown at higher concentrations of sugars and sorbitol, molecules of the other additives do not bind coherently enough to be detected through X-ray diffraction studies.

Unlike the stabilizing additives, nitrate ions bind extensively to the molecule in the structures reported here. The number of nitrate ions located in the six molecules varies between five and eight. In a recent study, Vaney *et al.* (2001) have identified seven sites for nitrate ions in monoclinic lysozyme, some often occupied by more than one nitrate ion. All the nitrate ions identified in the present study occupy one or the other of the seven identified sites. Interestingly, although the molarity of $NaNO_3$ in the crystallization solution is similar to those of the stabilizing additives, several nitrate ions bind to the protein molecule while none of the additive molecules do. This clearly points to preferential binding of the salt ions.

8. Conclusions

The crystal structures reported here suggest that the structure of the enzyme is unaffected by the presence of stabilizing additives in the surrounding solution. However, the average *B* values indicate decreased mobility in the presence of additives. More significantly, the additives appear to have hardly any effect on the distribution of ordered water molecules attached to the protein molecule. In fact, the effect of additives is much less than that caused by normal differences in packing. It is similar to that caused by a change in the concentration of $NaNO_3$ in the crystallization medium and less than that resulting from the replacement of $NaNO_3$ by NaCl as the precipitant. Admittedly, the molar concentrations of the additives (0.29–0.55 *M*) at which monoclinic crystals could be grown are comparatively low. However, their stabilizing effects manifest themselves even at these concentrations (Xie & Timasheff, 1997; Wimmer *et al.*, 1997). The lowering of the *B* values also provide evidence of their effect. Furthermore, studies involving tetragonal lysozyme, where much higher concentrations of the additives (0.88–1.1 *M*) were possible, also showed that their effect on the observed hydration shell of the protein is much lower than that resulting from differences in packing. Thus, stabilizing sugars and polyols do not appear to cause any appreciable rearrangement of ordered water molecules attached to the protein. The basis for their undoubted stabilizing effect should perhaps be sought beyond the first hydration shell of the protein.

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