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Effect of a sodium ion on the dehydration-induced phase transition of monoclinic lysozyme crystals

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A monoclinic lysozyme crystal grown in NaCl solution was transformed into a new monoclinic crystal form by controlled dehydration. This crystal-to-crystal phase transition was accompanied by 20–40% solvent loss and the transformed crystal diffracted to prominently high resolution. The structures of the native and transformed crystals were determined at 1.4 and 1.15 Å resolution, respectively. In the native crystal a sodium ion was bound to the loop region Ser60–Asn74; however, it was released in the transformed crystal and a water molecule occupied this position. In the transformed crystal a sodium ion was bound to the carboxyl group of Asp52, a catalytic residue. The same structural change was observed in the phase transition of a crystal soaked in a saturated NaCl solution. In contrast, a crystal soaked in 10% NaCl solution was transformed in a shorter time with a smaller loss of solvent and the structure of the sodium-binding site was conserved in the transformed crystal. The high concentration of NaCl is likely to stabilize the crystal structure against dehydration by forming salt linkages between protein molecules. This suggests that the sodium ion in the crystal regulates not only the structural change of the loop region Ser60–Asn74 but also the molecular rearrangement caused by dehydration.

1. Introduction

Protein crystals contain solvent in large proportions, typically 30–70% of the crystal volume (Matthews, 1968). Crystal packing is maintained by limited intermolecular contacts directly between protein molecules and is also mediated by solvent molecules. Since the intermolecular space is filled with solvent, loss of solvent severely damages the crystal packing. If protein crystals are dehydrated slowly, the crystal initially retains its original packing (Dobrianov *et al.*, 1999) but finally breaks up when the crystal can no longer retain its packing structure. However, in some protein crystals it has been observed that the crystal transforms into a new crystal without collapsing by changing its molecular arrangement. Such a crystal-to-crystal phase transition under controlled dehydration was first reported for lysozyme crystals (Salunke *et al.*, 1985) and the changes in protein structure and hydration have been intensively studied for a monoclinic lysozyme crystal (Madhusudan *et al.*, 1993; Nagendra *et al.*, 1998). The monoclinic lysozyme crystal containing two independent molecules transforms into a new monoclinic crystal with a unit-cell volume less than half that of the original crystal, since the new crystal contains one molecule in the asymmetric unit. On the other hand, the dehydration-induced phase transition of a triclinic crystal of lysozyme produced a new triclinic crystal with two molecules in the unit cell (Harata & Akiba, 2004).

X-ray analyses at atomic resolution of lysozyme crystal structures before and after the phase transition revealed a structural change in the loop region involving the sodium-binding site. The native triclinic crystal does not bind a sodium ion in the loop region Ser60–Asn74, but both molecules in the transformed crystal bind a sodium ion and a nitrate ion (Harata & Akiba, 2004). We subsequently observed the release of a sodium ion in the phase transition of a monoclinic lysozyme crystal (type I) grown in 2% sodium nitrate solution (Harata & Akiba, 2006). In the native crystal one of the two inde-

Table 1
Summary of data collection and structure determination.

Values in parentheses are for the highest resolution shell.

	Native crystal			Transformed crystal		
	Type IIA	Type IIB	Type IIC	Type IIA	Type IIB	Type IIC
Crystal data						
Space group	$P2_1$					
Unit-cell parameters						
a (Å)	27.26	27.25	27.62	25.95	26.57	26.30
b (Å)	63.56	63.51	63.15	58.02	60.60	59.09
c (Å)	59.16	59.22	58.84	30.58	31.18	31.31
β (°)	92.84	92.79	91.24	110.40	111.36	110.79
V_M (Å^3 Da^{-1})	1.76	1.77	1.77	1.49	1.61	1.53
Data collection						
Temperature (K)	290	290	290	90	90	90
Resolution (Å)	1.40 (1.45–1.40)	1.40 (1.45–1.40)	1.40 (1.45–1.40)	1.15 (1.19–1.15)	1.15 (1.19–1.15)	1.15 (1.19–1.15)
Unique data	39166 (3767)	39224 (3766)	35851 (2720)	29957 (3026)	32729 (3281)	29647 (2299)
Completeness (%)	98.5 (94.9)	98.8 (95.7)	90.3 (68.4)	99.9 (99.9)	99.6 (99.9)	93.2 (72.6)
Redundancy	4.14 (2.72)	4.17 (2.74)	4.97 (2.25)	4.75 (2.87)	4.73 (3.09)	4.23 (2.71)
R_{merge}	0.104 (0.200)	0.101 (0.218)	0.119 (0.323)	0.061 (0.112)	0.104 (0.090)	0.088 (0.211)
Structure determination						
Resolution range (Å)	10–1.40 (1.45–1.40)	10–1.40 (1.45–1.40)	10–1.40 (1.47–1.40)	25–1.15 (1.19–1.15)	25–1.15 (1.19–1.15)	25–1.15 (1.20–1.15)
No. of reflections	39022 (3770)	39073 (3743)	35696 (3539)	29887 (2855)	32603 (3186)	29547 (2857)
No. of parameters	8815	8847	8938	4700	4964	4817
R (all data)	0.177 (0.242)	0.175 (0.231)	0.188 (0.329)	0.168 (0.205)	0.156 (0.171)	0.178 (0.271)
R_{free} (5% of data)	0.207	0.209	0.224	0.196	0.176	0.203
Coordinate error (Å)	0.044	0.044	0.049	0.031	0.031	0.037
Average error for U_{iso} (Å^2)	0.029	0.028	0.033	0.019	0.014	0.023

pendent molecules partially binds a sodium ion, while in the transformed crystal a water molecule replaces the sodium ion. In another monoclinic crystal (type II) grown in 10% NaCl solution at 313 K, two independent molecules fully bind the sodium ion. We recently showed the possibility of this crystal undergoing a phase transition by controlled dehydration (Harata & Akiba, 2006). Here, we report the structure of the transformed type II monoclinic crystal. In addition to the native crystal (type IIA), crystals soaked in 10% NaCl solution (type IIB) and saturated NaCl solution (type IIC) were used to clarify the effect of a sodium ion on the phase transition.

2. Materials and methods

The type II monoclinic crystal was prepared according to a previously reported method (Harata, 1994) with slight modification. 200 mg lyophilized protein (Seikagaku Kogyo Co.) was dissolved in 10 ml aqueous solution containing 10% NaCl and 5% 1-propanol. The solution (pH 7.6) was kept at 313 K for 1 h, passed through a 0.2 µm filter and divided into 1 ml portions in small culture dishes, which were allowed to stand at 313 K. Crystals were grown over two weeks while the solution slowly evaporated to ∼50% in volume. The solution was then cooled to room temperature, at which the crystal was stored for use.

X-ray experiments were carried out on a Bruker Smart6000 diffractometer equipped with a MacScience M06X rotating-anode generator (50 kV, 90 mA) and MONTEL Optics. Intensity data from three crystals, a native crystal (type IIA), a crystal soaked in 10% NaCl solution (type IIB) and a crystal soaked in a saturated NaCl solution (type IIC), were measured at 290 K using a crystal sealed in a glass capillary with a drop of mother solvent. For measurement of the phase transition using X-ray diffraction, a crystal was immersed in paraffin oil and visible solvent was carefully removed. The crystal was removed from the oil using a cryo-loop (Hampton Research Co.) and placed on the diffractometer in a nitrogen-gas stream at 273 K. The orientation of the crystal was roughly adjusted so that the a axis was almost parallel to the incident X-ray beam. To monitor the progress of the crystal transformation, the change in the X-ray diffraction was recorded at an arbitrary interval with an exposure time of 20 s per 1° rotation. When the phase transition was completed, the temperature was lowered to 90 K to prevent further release of solvent from the crystal and the intensity data were collected for the structure determination.

The structures of the native and transformed crystals were determined by molecular replacement using the atomic coordinates of the corresponding type I crystals: PDB codes 2d4i for the native crystal and 2d4j for the transformed crystal (Harata & Akiba, 2006). The structures of the type IIA, type IIB and type IIC crystals were refined at 1.4 Å resolution using *SHELX*-97 (Scheldrick, 1997), while the structures of the transformed crystals were refined at 1.15 Å resolution. Data-collection and structure-refinement statistics are given in Table 1.

3. Results and discussion

3.1. Crystal transformation

In the type II monoclinic lysozyme crystal with two independent molecules in the asymmetric unit, dehydration causes a crystal-to-crystal phase transition that produces a new monoclinic crystal with one molecule in the asymmetric unit. The original crystal, which shows pseudo-*B*-centred symmetry, is transformed into a crystal with a unit-cell volume less than half that of the native crystal. In the native crystal, one molecule is located at a position shifted by 15.54, 0.68 and −29.05 Å along the a, b and c axes, respectively, from the other. The two independent molecules related by pseudosymmetry become identical in the transformed crystal. In the phase-transition experiment, crystal solvent was slowly released through the paraffin coat in the nitrogen-gas stream and the phase transition started when the solvent amount became critical to maintaining the packing structure. Dehydration caused many fine cracks in the crystal. However, the transformed crystal still behaved as a single crystal on X-ray irradiation, although the peak width of the diffraction spots was considerably increased. The type IIB crystal was transformed in about 2 h, which was faster than the type IIA and type IIC crystals,

each of which took more than 5 h. As indicated by the V_M values in Table 1, the type IIA and type IIC crystals lost more solvent (V_M values of 1.49 and 1.53 Å^3 Da^{-1}, respectively) than the type IIB crystal (V_M value of 1.61 Å^3 Da^{-1}) during completion of the phase transition. The relatively high concentrations of NaCl in the type IIA and type IIC crystals may strengthen the intermolecular contacts

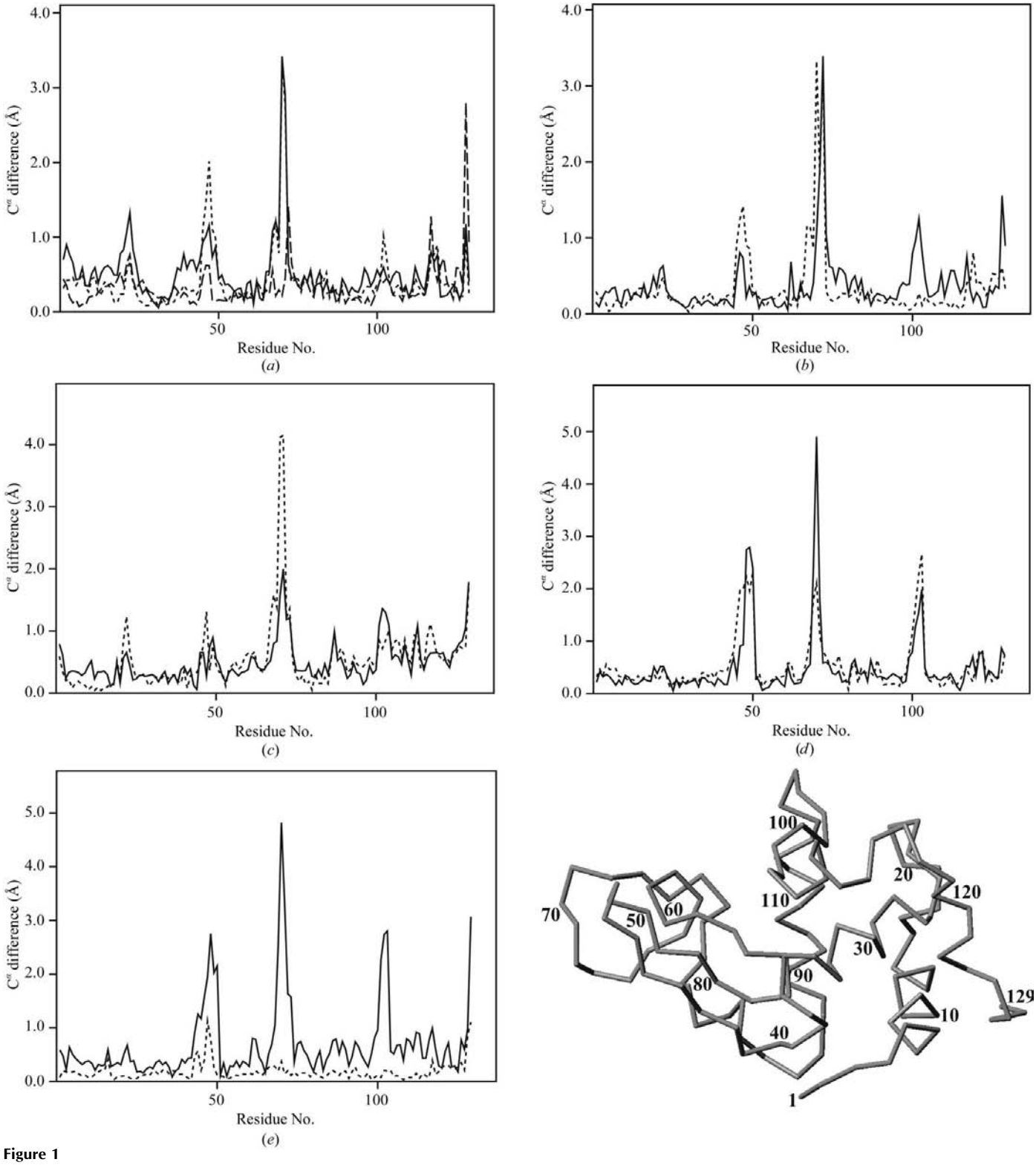

Figure 1
Plot of C$^\alpha$ differences calculated after the superposition of lysozyme structures. (*a*) Difference between the two molecules in the asymmetric unit of the type IIA crystal (solid line), type IIB crystal (short dashes) and type IIC crystal (long dashes). (*b*) Difference between the IIA and IIC crystals: molecule 1 (solid line) and molecule 2 (dashed line). (*c*) Difference between the type IIA crystal and the transformed type IIA crystal: molecule 1 (solid line) and molecule 2 (dashed line). (*d*) Difference between the type IIC crystal and transformed type IIC crystal: molecule 1 (solid line) and molecule 2 (dashed line). (*e*) Comparison of the transformed type IIA crystal with the transformed type IIB crystal (solid line) and transformed type IIC crystal (dashed line).

mediated by solute ions and these crystals may need to lose a greater proportion of their solvent than the type IIB crystal in order to trigger the phase transition by destabilizing the crystal packing. In the transformed crystals, the diffraction power was increased prominently, especially in the type IIB crystal. The native crystals diffracted to 1.4 Å resolution, while the transformed crystals diffracted to a resolution higher than 1.1 Å. The molecular structure and arrangement of the transformed crystal were better ordered because of the two independent molecules in the native crystal becoming identical. The loss of bulk solvent increases the intermolecular contacts and the close packing suppresses the fluctuations of side-chain groups and local structures. Protein molecules may also be better ordered as rigid-body motion is reduced, as suggested by a thermal motion analysis (Harata & Akiba, 2006). The decrease in bulk solvent may increase the ion concentration in the crystal and the formation of intermolecular salt linkages mediated by ions stabilizes the crystal packing. The reduction in the unit-cell volume is also responsible for the increase in average diffraction intensity. These findings explain why controlled dehydration of protein crystals sometimes improves diffraction quality (Kiefersauer *et al.*, 2000; Heras & Martin, 2005). In the intermediate stage of the phase transition, the arrangement of the microcrystals is disturbed and less ordered because two types of microcrystals coexist in one crystal (Harata & Akiba, 2004). The order of the microcrystals may not be completely recovered in the transformed crystal. As a result, the peak width of the diffraction spots is unfavourably increased because of the less ordered arrangement of microcrystals. Therefore, further improvement in crystal quality is possible if we could find a condition that transforms the crystal without disturbing the ordered arrangement of the microcrystals.

3.2. Structures of native and soaked crystals

The backbone structures of the two independent molecules in the monoclinic crystals are not identical (Saraswathi *et al.*, 2002), as also indicated by the root-mean-square difference of equivalent C^{α} atoms, which is 0.66 Å in the native type II crystal. A relatively large structural difference is observed for residues Asp66–Ser72, as shown in Fig. 1(*a*). The largest positional difference of C^{α} atoms is 3.4 Å for Pro70. Structural differences larger than 1.0 Å are also observed in the loop regions Asn19–Ser24 and Arg45–Gly49. Soaking in 10% NaCl solution caused no obvious structural changes. The root-mean-square differences of equivalent C^{α} atoms are 0.068 and 0.039 Å for the two independent molecules. In contrast, transfer of the crystal into saturated NaCl solution caused significant structural changes (Fig. 1*b*). In the superposition on the native structure, the root-mean-square differences are 0.538 and 0.574 Å for the two molecules. Relatively large structural changes are observed in the loop regions Arg45–Gly49, Gly67–Ser72 and Ser100–Gly104, as well as in the C-terminal region, each of which shows a positional difference of C^{α}

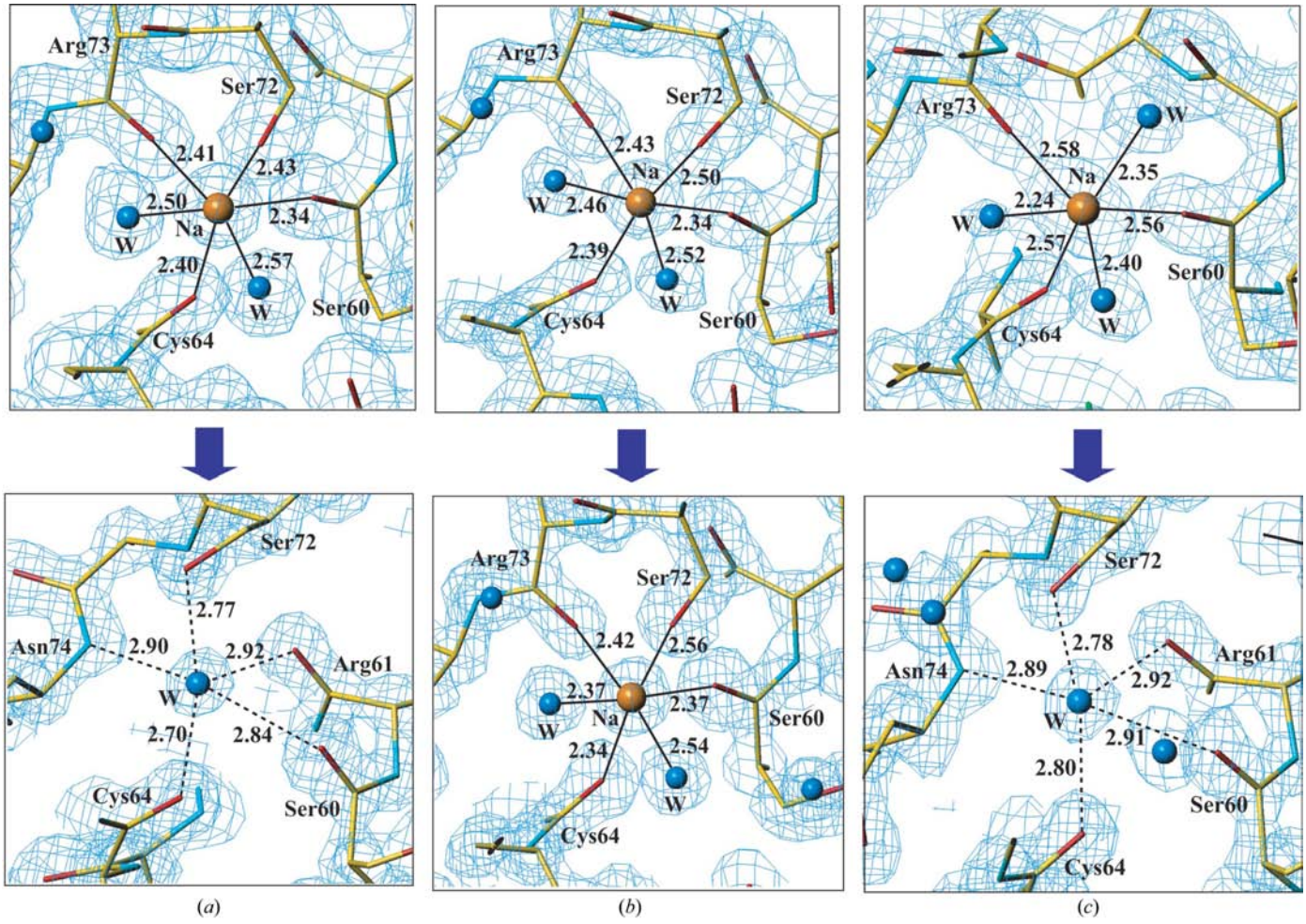

Figure 2
Structures of the sodium-binding site before (above) and after (below) the transition in the type IIA (*a*), type IIB (*b*) and type IIC (*c*) crystals. Water O atoms are denoted W. Dashed lines indicate possible hydrogen bonds. Contours are drawn at the 1.5σ level.

atoms more than 1.0 Å. The crystal was obtained by the slow evaporation of a solution containing 10% NaCl. The final NaCl concentration is presumed to be ∼20%. Soaking in saturated NaCl solution further increased the concentration of NaCl in the crystal. This may have affected the electrostatic interaction of ionized side-chain groups and induced structural change.

In the native crystal, a sodium ion is bound to the loop region Ser60–Leu75 in both independent molecules, as shown in Fig. 2(*a*). In both the type IIA and IIB crystals, six O atoms of three main-chain peptide groups (Ser60, Cys64 and Arg73), two water molecules and the hydroxyl group of Ser72 are coordinated to the sodium ion to form a distorted octahedral structure. In contrast, the hydroxyl group of Ser72 in the type IIC crystal points away from the sodium ion; a water molecule instead occupies the position and coordinates to the sodium ion. The average distance between the sodium ion and coordinated O atoms is 2.44 Å in the type IIA and IIB crystals and 2.45 Å in the type IIC crystal. In the type I crystal grown at room temperature in a pD 4.5 solution with a relatively low salt concentration (2% sodium nitrate), one molecule binds water molecules in this loop region (Harata & Akiba, 2006). The other molecule partially binds a sodium ion and residues Gly71–Asn74 show alternate conformations for the sodium-bound form and the water-bound form. Partial binding of the sodium ion associated with a disordered structure of the loop region has also been observed in tetragonal lysozyme crystals (Sauter *et al.*, 2001). In contrast, full binding of the sodium ion has been reported for the tetragonal crystal grown in the presence of 1.25 *M* NaCl (Vaney *et al.*, 1996). It is plausible that binding of the sodium ion is in equilibrium in solution and that the equilibrium is largely shifted to the sodium-binding side in the solution from which the type II monoclinic crystal was grown.

3.3. Structure of the transformed crystal

The phase transition changed the molecular structure and arrangement so that the two independent molecules in the asymmetric unit of the native crystal become identical. The V_M value decreased from 1.76 Å^3 Da^{-1} in the native crystal (type IIA) to 1.49 Å^3 Da^{-1} in the transformed crystal. Therefore, the protein molecules are more densely packed in the transformed crystal than in the native crystal. As a result, the number of intermolecular direct contacts between protein molecules is increased because of the decrease in the bulk-solvent region. There are 19 intermolecular pairs of amino acids with polar contacts less than 3.3 Å for a molecule in the type IIA crystal and 23 for a molecule in the transformed crystal. Many intermolecular polar contacts less than 3.3 Å are conserved in the transformed crystal; eight of 19 contacting pairs of amino acids for one molecule and ten of 20 for the other molecule are observed in the transformed crystal. The phase transition affected the backbone structure of the protein molecule. The root-mean-square positional differences of C^α atoms before and after the crystal transformation are in the range 0.59–0.79 Å. In the type IIA and IIC crystals a prominent structural change was observed in the loop region Asp66–Asn74 involving the sodium-binding site (Fig. 1*c*). A similar structural change has been observed in the phase transition of the type I crystal (Nagendra *et al.*, 1996; Harata & Akiba, 2006). In the type IIB crystal, structural changes larger than 2.0 Å are observed in the loop regions Thr43–Thr51, Asp66–Asn74 and Val99–Asn113 (Fig. 1*d*).

The backbone structures of the transformed crystals are almost the same in the type IIA and IIC crystals (Fig. 1*e*), as indicated by the root-mean-square difference of equivalent C^α atoms, which was 0.25 Å. In contrast, structural changes are observed between the type IIB crystal and the other two crystals. The root-mean-square C^α differences are 0.97 Å for the transformed type IIA crystal and 1.01 Å for the transformed type IIC crystal. Large structural differences are observed in the flexible loop regions Thr43–Thr51, Asp66–Asn74 and Val99–Asn113, as well as in the C-terminal region, each of which shows a C^α atom difference larger than 2.0 Å. The backbone structure in the transformed type IIA or IIC crystal is basically the same as that of the transformed type I crystal, with the loop region Ser60–Asn74 having the structure of the water-bound form. The root-mean-square deviations for equivalent C^α atoms between the transformed type I structure and the structures of the transformed type IIA and IIC crystals are 0.32 and 0.40 Å, respectively. The type IIB crystal transformed without the loss of the sodium ion that is bound to the loop region and the structure of the sodium-binding site is conserved in the transformed crystal.

In the transformed type IIB crystal, a sodium ion is bound in the loop region Ser60–Leu75 in the same manner as observed in the crystal before transformation (Fig. 2*b*). Six O atoms from the three peptide carbonyl groups (Ser60, Cys64 and Arg73), the hydroxyl group of Ser72 and two water molecules are coordinated with an average distance of 2.45 Å from the sodium ion. In contrast, in the transformed type IIA and IIC crystals the sodium ion is lost and a water molecule occupies the position that is capable of forming

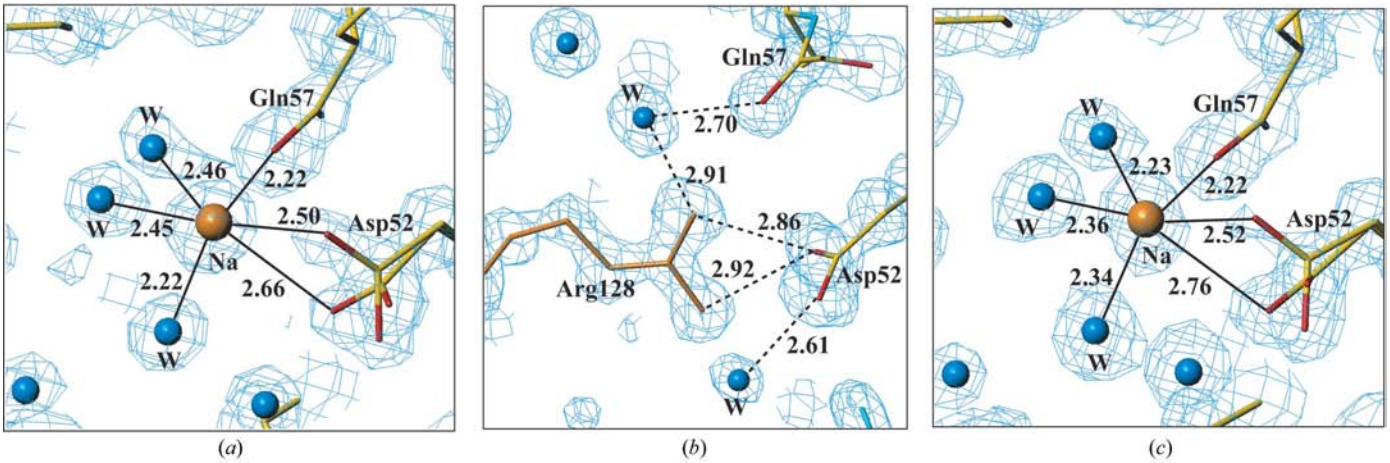

Figure 3
Structure of the active centre in the transformed crystal: type IIA (*a*), type IIB (*b*) and type IIC (*c*). The side chains of Asp52 in the transformed type IIA and IIC crystals are disordered and each of them shows two alternate conformers. In the type IIB crystal (*b*) the side chain of Arg128 of an adjacent molecule is shown in orange.

hydrogen bonds to the peptide amino group of Asn74, the hydroxyl group of Ser72 and three carbonyl O atoms of Ser60, Arg61 and Cys64 (Figs. 2*a* and 2*c*). The peptide group linking Arg73 and Asn74 is rotated by about 180°, allowing the amino group to form a hydrogen bond to the water molecule. In the transformed triclinic crystal, a nitrate ion is bound to the sodium ion either directly or mediated by water (Harata & Akiba, 2004). However, no chloride ion is located in the loop region of the transformed type II crystals. Therefore, anions are not likely to be responsible for the structural change in the sodium-binding site. The structure of the loop region is not the same in the native and transformed crystals, even though the sodium ion is bound with the same coordination structure in both crystals. In the transformed crystal Pro70 and Gly71 are shifted by the rotation of the $N-C^{\alpha}$ bond of Val69 and the $C-C^{\alpha}$ bond of Ser72. The latter rotation enables the coordination of the hydroxyl group of Ser72 to the sodium ion. A large movement of several residues should be necessary for the 180° rotation of the peptide group and may only be possible when the crystal packing is dynamically changing. This suggests that the intermediate state of the phase transition causes high mobility not only in crystal packing but also in molecular structure and enables structural change of the loop region.

A sodium ion is found at the active site in the transformed type IIA and IIC crystals. As shown in Fig. 3, the sodium ion is bound to the carboxyl group of Asp52 and the main-chain carbonyl group of Gln57, and three water molecules are coordinated with average distances of 2.42 Å for the transformed type IIA crystal and 2.41 Å for the transformed type IIC. The side chain of Asp52 is statistically disordered. The carboxyl O atom of one conformer occupies a position in the distorted octahedron, while the O atom of the other conformer is located at another position. Therefore, the six oxygen positions that form the octahedron are not fully occupied. In contrast, no sodium ion is found at the active site in the transformed type IIB crystal (Fig. 3*b*). The carboxyl group of Asp52 forms a salt linkage to the guanidyl group of Arg128 of an adjacent molecule. The same salt linkage is also observed in the native crystal. Therefore, the type IIB crystal is transformed not only without changing the structure of the sodium-binding site but also while retaining the intermolecular contact between Asp52 and symmetry-related Arg128. In the transformed type IIA and IIC crystals the guanidyl group of Arg128 is moved away from the carboxyl group of Asp52, allowing the binding of the sodium ion. The guanidyl group in the transformed type IIA crystal still contacts the carboxyl group of Asp52 at a distance of 3.05 Å, whereas in the transformed type IIC crystal the guanidyl group points to the side chain of Asn46, which is too distant to form a hydrogen bond.

Many anion-binding sites have been identified in the high-resolution structures of lysozyme crystals (Steinrauf, 1998; Vaney *et al.*, 2001). However, only a few binding sites have been found for the sodium ion because of the difficulty of distinguishing it from a water molecule in the electron-density map. The sodium ion was identified by the octahedral coordination of six O atoms with an average distance of about 2.4 Å (Vaney *et al.*, 1996; Dauter *et al.*, 1999; Sauter *et al.*, 2001; Harata & Akiba, 2004). Except for the sodium ion bound to the loop region Ser60–Asn74, sodium ions that form intermolecular salt linkages have only been observed in the hexagonal crystal (Brinkmann *et al.*, 2006). The sodium ion bound to Asp52 was first observed in the present structures of the transformed type IIA and IIC crystals. These crystals are presumed to have a much higher ion content than the IIB crystal and may be more stabilized by intermolecular interactions mediated by ions. The high concentration of NaCl seems to be required for the binding of the sodium ion to Asp52 by ejecting the guanidyl group. Therefore, the concentration of NaCl is primarily responsible for the binding or release of the sodium ion in the transformed crystal.

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