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Structures of orthorhombic lysozyme grown at basic pH and its low-humidity variant

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The structures of orthorhombic lysozyme grown at basic pH and its low-humidity variant have been solved and refined at 1.9 and 2.0 Å resolution, respectively. A comparison of the native structure with those of crystals grown at acidic pH does not show any systematic pH-dependent difference in the molecular geometry. The conformations, mutual orientation and interactions of the catalytic residues Glu35 and Asp52 also remain unchanged. However, comparison between the native and low-humidity forms in the orthorhombic form show that the changes in molecular geometry which accompany the water-mediated transformation to the low-humidity form are more pronounced in the C-terminal residues than in the other regions of the molecule. During the transformation from the native to the low-humidity form, the locations of only about half the water molecules in the hydration shell remain unchanged, but the hydration shell as a whole moves along with the protein molecule.

1. Introduction

We have been pursuing a programme involving water-mediated transformations in which protein crystals undergo reversible transformations, with abrupt changes in water content, when the environmental humidity is systematically varied, in order to explore the hydration and mobility of protein molecules and their relation to action, using hen egg-white lysozyme and bovine pancreatic ribonuclease A as model systems (Kodandapani *et al.*, 1990; Madhusudan & Vijayan, 1991; Madhusudan *et al.*, 1993; Radha Kishan *et al.*,1995; Nagendra *et al.*, 1995, 1996). Such studies on lysozyme have so far made use of the well known tetragonal and monoclinic forms (Kodandapani *et al.*, 1990; Madhusudan *et al.*, 1993; Nagendra *et al.*, 1996) grown at acidic pH (4.6). Here, we report similar studies on a form grown at basic pH, through the high-resolution X-ray analysis of orthorhombic lysozyme (Artymiuk *et al.*, 1982) and its low-humidity variant. This work also serves to explore any pH-related structural changes in lysozyme.

2. Experimental

HEW lysozyme was obtained from Sigma Chemical Company (three times crystallized, dialysed and lyophilized, containing approximated 95% protein, the rest being primarily sodium acetate and sodium chloride). Orthorhombic crystals of the enzyme were grown using the method described in the literature (Artymiuk *et al.*, 1982). Needle-like crystals grew in about three months from a 3%(*w*/*v*) protein, 2%(*w*/*v*) NaCl solution in deionized double-distilled water adjusted to pH 9.3 using NaOH. The low-humidity (88% relative humidity) form was obtained by replacing the mother liquor inside the capillary by a saturated aqueous solution of potassium chromate (Rockland, 1960; Weast & Astle, 1980/81). The transformation from native to low humidity was completed in about 24 h. A MAR Research imaging plate was used to collect intensity data from the crystals at 293 K. The crystal-to-detector distance was kept at 90 mm in the case of the native crystals and at 105 mm in the case of the low-humidity crystals. The data were processed using the *XDS* program package (Kabsch, 1988). Crystal data and data-

Table 1
Crystal data and data-collection statistics.

	Native	88% relative humidity form
Space group	$P2_12_12_1$	$P2_12_12_1$
a (Å)	30.49	30.50
b (Å)	59.58	55.39
c (Å)	68.66	68.85
Z	4	4
Unit-cell volume (Å^3)	124727	116315
Solvent content (%)	43.0	38.3
Data resolution (Å)	1.9	2.0
Number of observations	72266	35416
Number of unique reflections	10287	7683
Completeness of data (%)	99.3	92.2
Merging *R* for all reflections (%)	9.9	5.7
Data multiplicity (%)	7.1	4.7
Average $I/\sigma(I)$	26.7	26.9

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Table 2
Refinement parameters.

	Native	88% relative humidity form
Resolution limit (Å)	10.0–1.9	10.0–2.0
Number of reflections with $F > 2\sigma(F)$	8033	5319
Final R factor	0.190	0.199
R_{free}	0.267	0.291
R.m.s. deviation from ideal		
Bond lengths (Å)	0.014	0.016
Bond angles (°)	1.8	1.8
Dihedral angles (°)	25.1	25.4
Improper angles (°)	1.62	1.64
Number of protein atoms	1001	1001
Number of water molecules	154	136
Average B (Å^2)		
Protein atoms	20.3	23.2
Solvent atoms	46.9	44.9

Table 3
The r.m.s. deviations (Å) in Cα positions among the native structures.

Deviations for all atoms are given in parentheses.

	Tetragonal	Triclinic	Monoclinic	
			Molecule *A*	Molecule *B*
Orthorhombic	0.38 (1.00)	0.52 (1.18)	0.36 (1.26)	0.53 (1.19)
Tetragonal		0.65 (1.31)	0.32 (0.99)	0.52 (1.26)
Triclinic			0.64 (1.37)	0.71 (1.55)
Monoclinic molecule *A*				0.46 (1.28)

collection statistics for both the forms are given in Table 1.

3. Structure solution and refinement

The structure of the native crystals was solved by the molecular-replacement method (Rossmann & Argos, 1975) using the program *AMoRe* (Navaza, 1994) with the molecule in the native tetragonal crystals (the coordinates, kindly made available by Professor D. C Phillips, were used without any modification) as the search model. The refined coordinates of the native form was used as the starting model for the low-humidity form. Both the structures were refined in a similar manner using *X-PLOR* 3.1 (Brünger, 1992). *FRODO* (Jones, 1978) was used for model building. Simulated annealing was used once during the course of refinement in each case. $2F_o - F_c$, $F_o - F_c$ and omit-type maps (Vijayan, 1980; Bhat & Cohen, 1984) were extensively used to check and correct the current model and to locate water molecules. R_{free} (Brünger, 1992) was closely monitored in the course of refinement. The refinement parameters are given in Table 2.

4. Results and discussion

4.1. Effect of pH

Orthorhombic lysozyme is the only well refined crystal form of lysozyme grown at alkaline pH. The other three well known forms, tetragonal (Vaney *et al.*, 1996), triclinic (Walsh *et al.*, 1998) and monoclinic (Nagendra *et al.*, 1996) are obtained at around pH 4.6. Therefore, a major reason for analysing the orthorhombic form has been to delineate the effect of change in pH on the structure, if any. A superposition of the Cα traces of the four native structures is shown in Fig. 1, while the r.m.s. deviations in atomic positions among them are listed in Table 3. There are no overall perceptible differences among the structures, except in loops and regions of the molecule which are known to be flexible (Madhusudan & Vijayan, 1991). The r.m.s. deviations also do not indicate any major effect of pH. In many instances, the molecules in the low-pH forms differ more among themselves than they do from those in the orthorhombic form.

The enzymatic activity of lysozyme is highest around pH 5. The activity falls sharply on either side of the optimal pH (Stryer, 1981). In the active enzyme, Glu35 is believed to be protonated and Asp52 ionized. Presumably, this situation prevails in the tetragonal, monoclinic and triclinic crystals grown around pH 4.6. In orthorhombic lysozyme, however, the carboxyl groups of both the residues are expected to be ionized. This change in the ionization state does not appear to lead to any significant systematic structural perturbation. As can be seen from the superposition of the active-site residues shown in Fig. 2, Glu35 and Asp52 have remarkably the same conformation and mutual disposition in the acidic forms and the basic orthorhombic form. The hydrogen bonds they are involved in are also the same. Many other residues in the active site exhibit considerable conformational variability, but it cannot be correlated with difference in pH in any sensible manner.

4.2. Changes in protein structure during transformation

In addition to the perturbations in the three-dimensional structure, the water-

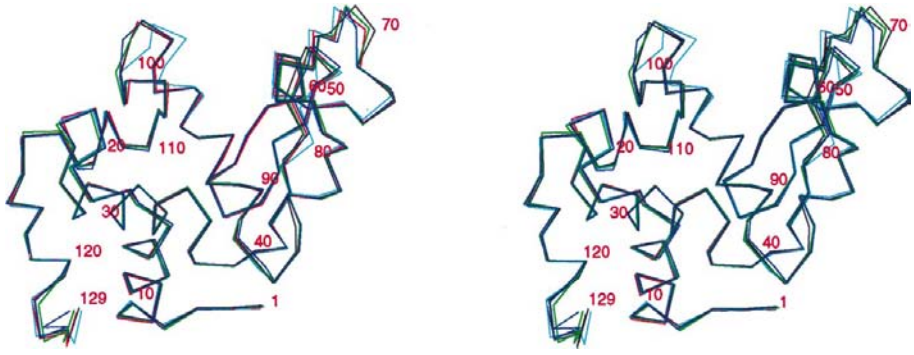

Figure 1
Stereoview of the superposition of Cα positions in the native orthorhombic (red), tetragonal (black), triclinic (cyan) and monoclinic (green for molecule *A* and blue for molecule *B*) forms.

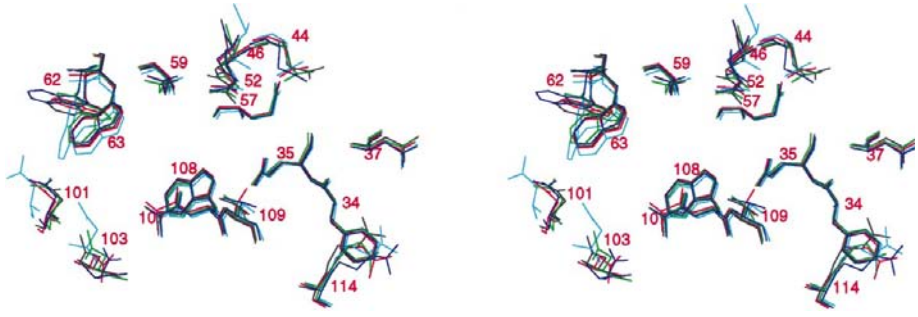

Figure 2
Stereoview of the superposition of active-site residues in the native forms. The colour code is same as that used in Fig. 1.

Table 4
Water-mediated transformation in different crystal forms of lysozyme.

	Orthorhombic	Tetragonal	Monoclinic	
			Molecule *A*	Molecule *B*
Resolution (Å)				
Native	1.9	1.3	2.0	2.0
Low humidity	2.0	2.1	1.75	1.75
Solvent content (%)				
Native	43	39	32	32
Low humidity	38	37	22	22
Number of water molecules in the hydration shell				
Native	128	147	125	131
Low humidity	114	121	173	173
Number of equivalent water molecules in native and low-humidity forms	56	77	54	54
R.m.s deviations in atomic positions between the native and low-humidity form (Å)				
Cα atoms	0.43	0.26	0.46	0.53
Main-chain atoms	0.41	0.35	0.55	0.57
Side-chain atoms	1.21	1.36	1.45	1.67
All atoms	0.88	0.98	0.99	1.23

mediated transformation from native to the low-humidity form is accompanied by the movement of the protein molecule as a whole. The rotational matrix and the translation describing this movement is given below.

$$\begin{pmatrix} 0.9997 & 0.0142 & -0.0193 & 0.8604 \\ -0.0129 & 0.9978 & 0.0657 & -1.0232 \\ 0.0202 & -0.0654 & 0.9977 & 0.6371 \end{pmatrix}$$

The matrix represents a rotation of 4° about an axis with direction cosines 0.938, 0.346 and 0.284. The overall translation is around 1.5 Å. Orthorhombic lysozyme is the third crystal form of the enzyme for which the structure of the native and low-humidity forms have been determined, the other two being tetragonal lysozyme (Kodandapani *et al.*, 1990) and monoclinic lysozyme (Madhusudan *et al.*, 1993; Nagendra *et al.*, 1996). Therefore, the changes in the orthorhombic form during water-mediated transformation are best discussed in relation to those in the other two. As indicated in Table 4, the two crystallographically independent molecules in native monoclinic lysozyme become equivalent in the low-humidity form. Furthermore, the reduction in the solvent content (32 to 22%) is very substantial. Consequently, the r.m.s. deviations in atomic positions between the native and the low-humidity structures are the highest in this form. The solvent contents and the changes in them during the transformations have comparable values in tetragonal and orthorhombic lysozyme. However, the deviations in main-chain atoms are lower in the tetragonal form, probably indicating the greater stability of the molecule in this form than in the orthorhombic form.

In addition to the overall changes, it is also interesting to examine variations in changes in different segments of the molecule. As the magnitudes of changes during the transformations are different in the three forms, the deviations at individual Cα positions were normalized against their r.m.s value in each structure. These normalized values are shown in Fig. 3. The changes in the loops, particularly in the main loop 74–79, are comparatively large in all structures. It is noteworthy in the case of orthorhombic lysozyme that the changes are pronounced in about 25 C-terminal residues. Unlike in the other forms, the changes in this segment are significantly larger than in the other contiguous segments of the polypeptide chain.

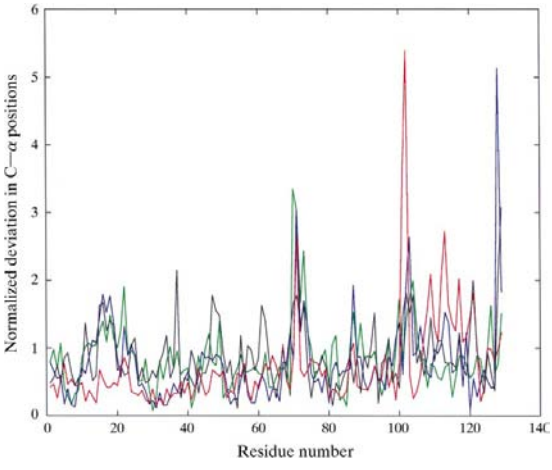

Figure 3
Normalized deviations in Cα positions between the native and low-humidity forms. The colour code is the same as in Figs. 1 and 2.

5. Changes in the hydration shell

As in previous studies (Kodandapani *et al.*, 1990; Madhusudan & Vijayan, 1991; Madhusudan *et al.*, 1993; Radha Kishan *et al.*, 1995), a water molecule is assumed to belong to the hydration shell of the protein if it is at a distance of 3.6 Å or less from a protein N or O atom. The number of such water molecules in the hydration shells of different crystal forms of lysozyme are listed in Table 4. In addition to the intrinsic hydration properties of the enzyme, this number obviously also depends upon other factors such as the diffraction quality of the particular crystal form.

A water molecule in one structure and that in another are considered equivalent if (i) they interact with at least one common protein atom and (ii) the distance between two water molecules is less than 1.8 Å, when the two protein molecules along with their hydration shells are superposed (Kodandapani *et al.*, 1990). Understandably, the equivalence in water molecules in the hydration shell between the native and the low-humidity forms is the lowest in monoclinic lysozyme (Table 4). The solvent content and its change during water-mediated transformations have similar values in tetragonal and orthorhombic lysozyme. However, 77 water molecules remain equivalent in the native and low-humidity forms of tetragonal lysozyme, while the corresponding number is lower (56) in orthorhombic lysozyme. This probably points to the greater stability of the hydration shell in tetragonal lysozyme than in orthorhombic lysozyme. The set of 56 water molecules in the low-humidity form was superposed as a whole onto the corresponding set in the native structure, which yielded the following rotation matrix and translation.

$$\begin{pmatrix} 0.9998 & 0.0134 & -0.0141 & 0.7057 \\ -0.0124 & 0.9974 & 0.0710 & -1.1283 \\ 0.0150 & -0.0708 & 0.9974 & 0.8058 \end{pmatrix}$$

This matrix represents a rotation of 4.2° about an axis with direction cosines 0.962, 0.192 and 0.192 and a translation of 1.6 Å. These values are very close to those obtained on the superposition of the two protein molecules. Thus, as noted earlier (Kodandapani *et al.*, 1990), despite differences within the hydration shell, the hydration shell as a whole appears to move along with the protein molecule during water-mediated transformation.

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