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The Structures of the Monoclinic and Orthorhombic Forms of Hen Egg-White Lysozyme at 6 Å Resolution

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Abstract

The structures of a monoclinic and an orthorhombic form of hen egg-white lysozyme (HEWL) have been determined at 6 Å resolution by the method of isomorphous replacement. At this resolution the conformations of the molecules are indistinguishable from that of the tetragonal form of HEWL. The two molecules in the asymmetric unit of the monoclinic form are related by a translation of approximately ($a/2 + c/2$). The tight packing of the molecules in the unit cell prevents substrate-binding studies being carried out on this crystal form. In the orthorhombic crystals the sugar-binding sites *A* and *B* are blocked but the lower part of the active-site cleft appears to be accessible. Thus, neither of these crystal forms is ideally suited to the binding of true substrate at sub-zero temperatures.

Crystallographic studies of the structure of the tetragonal crystal form of hen egg-white lysozyme (Blake, Mair, North, Phillips & Sarma, 1967) and its complexes with mono-, tri- and tetrasaccharides (Blake, Johnson, Mair, North, Phillips & Sarma, 1967; Ford, Johnson, Machin, Phillips & Tjian, 1974) have made a significant contribution to the understanding of enzyme activity (Phillips, 1966; Imoto, Johnson, North, Phillips & Rupley, 1972; Blake *et al.*, 1978). It now seems probable that further understanding can best be achieved by the examination of true substrate complexes of the enzyme stabilized at sub-zero temperatures (Douzou, 1977; Fink & Ahmed, 1976; Alber, Tsernogolou & Petsko, 1976). For success with this technique, the active site of the crystalline enzyme must be fully accessible to allow the diffused substrate to interact to form the enzyme–substrate complex. This condition is not met in the tetragonal form, where the lower half of the active site is occluded by a neighbouring molecule (Blake *et al.*, 1967). The active site of the triclinic form of the enzyme appears to be even less accessible (Kurachi, Sieker & Jensen, 1976). To determine whether any of the known crystal forms of hen egg-white lysozyme are suitable for the low-temperature experiments, we have carried out low-resolution X-ray analyses of a monoclinic and an orthorhombic crystal form.

Materials and methods

Crystallization

Monoclinic crystals of hen egg-white lysozyme (HEWL) were grown by a slight modification of the methods reported by Crick (1953) and Steinrauf (1959). Lysozyme (Sigma Chemical Co.) was slowly dissolved in water previously adjusted to pH 4·5 by HNO_3 to a concentration of 0·5% (*w*/*v*). After complete dissolution of the protein the solution was brought to 3% $NaNO_3$ and allowed to stand in glass vials. The crystals grew in the form of large rectangular plates. To obtain the orthorhombic crystals, HEWL was made up to a 3% (*w*/*v*) solution in water previously adjusted to pH 9·6 by NaOH, and then brought to 2% in NaCl. Large rod-shaped crystals

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grew in three months, by which time the pH had dropped to 7·5.

Methods

Heavy-metal derivatives of both crystal forms were prepared by soaking the crystals in solutions of heavy-metal complexes made up in the appropriate mother liquor of crystallization. One derivative, $K_3UO_2F_5$ (15 m*M*), was used for the monoclinic crystals and two, $Hg(OOC.CH_3)_2$ (20 m*M*) and $K_2Pt(CN)_4$ (5 m*M*), were used for the orthorhombic crystals.

Three-dimensional X-ray data for the native crystals and the potential isomorphous derivatives were collected on a computer-controlled Hilger & Watts Y230, four-circle diffractometer using an Elliott Fine-Focus Cu-anode X-ray tube, run at 40 kV, 30 mA. The diffracted intensities were measured with a proportional counter and output in the ordinate analysis mode (Watson, Shotton, Cox & Muirhead, 1970). Data sets for the orthorhombic crystals and derivatives contained about 800 reflections; those for the monoclinic crystals and derivatives about 1500 reflections. Each set contained measurements of Bijvoet pairs so that use could be made of anomalous contributions of the heavy metals in phasing. An empirical absorption curve (North, Phillips & Mathews, 1968) was measured for each crystal to correct the data for absorption. A number of standard reflections were measured at intervals to check for radiation damage, but as expected with low-resolution data no correction was needed.

The X-ray data sets were corrected for background, Lorentz and polarization effects and for absorption by standard programs using the Oxford University ICL 1906A computer. After correction the derivative data sets were scaled to the appropriate native set by making the sums of the intensities over all reflections equal. Patterson functions and Fourier maps were calculated by fast-Fourier transform programs.

Structure determinations

(*a*) *The monoclinic form*

The monoclinic crystals exist in two discrete forms that differ principally in the value of the β angle and in the detailed distribution of intensities. We report here the solution of the type II crystal form. The cell parameters of the two forms are: type I: space group $P2_1$, $a = 27{\cdot}62$, $b = 63{\cdot}56$, $c = 60{\cdot}51$ Å, $\beta = 90{\cdot}06°$, type II: space group $P2_1$, $a = 28{\cdot}12$, $b = 63{\cdot}61$, $c = 60{\cdot}52$ Å, $\beta = 91{\cdot}05°$. These parameters are in satisfactory agreement with those previously reported by Crick (1953), King (1959) and Steinrauf (1959). Two molecules are present in the asymmetric unit, giving V_M values (Matthews, 1968) of 1·83 Å^3 dalton^{-1} for type I and 1·84 Å^3 dalton^{-1} for type II. These values are considerably lower than that for the tetragonal form of hen egg-white lysozyme (2·04 Å^3 dalton^{-1}) and suggest a very low liquid content. There is a significant tendency for the low-order reflections to be weak when $h + l$ is odd, and this tendency is more marked in the type I crystals. As suggested by Crick (1953) and King (1959) this intensity distribution indicates that the two molecules in the asymmetric unit are related by a translation of approximately $a/2 + c/2$. In confirmation of this the native and heavy-atom Patterson maps all showed a large peak at $U = \frac{1}{2}$, $V = 0$, $W = \frac{1}{2}$, and the final electron density maps showed the two lysozyme molecules related by a translation of $\frac{1}{2},0,\frac{1}{2}$.

The presence of the large peak at $U = \frac{1}{2}$, $V = 0$, $W = \frac{1}{2}$ in the heavy-atom Patterson function of the uranyl derivative (shown in Fig. 1) indicated that the major sites of substitution were pseudo-related in the same way as the protein.

Direct methods of phase determination were applied to the analysis of that derivative, using *MULTAN* (Germain, Main & Woolfson, 1971) as described by Wilson (1978). The isomorphous differences were normalized, and the 172 terms with E values greater than unity were selected as input. Using the 2000 triple-product relationships with the highest reliability estimates and with two general reflections of unknown phase as the starting set, eight phase sets were generated. The E map calculated for the phase set with the highest figure-of-merit contained as the highest features five of the six sites revealed by the Fourier methods outlined above and the sixth site was represented by a peak just below the error level in the E map (see Table 2). The six uranyl sites were subjected

Table 1. *Heavy-atom parameters for the* $K_3UO_2F_5$ *derivative of monoclinic hen egg-white lysozyme*

Mean figure-of-merit (the cosine of the standard error in the phase angle) = 0·755.

Site	Relative occupancy	x	y	z
1*	0·543	0·247	0·368	0·917
2	0·301	0·768	0·395	0·412
3	0·332	0·197	0·166	0·657
4	0·243	0·644	0·197	0·113
5	0·579	0·186	0·195	0·239
6	0·359	0·554	0·209	0·708

* $\Delta F = 0{\cdot}262$, $E = 45$, $E' = 15$, $R_{F_{HLE}} = 0{\cdot}296$, where ΔF is the mean isomorphous difference, $(1/n)\sum_0^n ||F_{PH}| - |F_P||$, x,y,z are fractional cell coordinates, $R_{F_{HLE}} = \sum||F_{HLE}| - |F_{H(\text{calc})}||/\sum|F_{HLE}|$ (Dodson & Vijayan, 1971), E is the r.m.s. lack of closure between $|F_{PH}|$ and $|F_P + F_H|$ for centric reflections, and E' is an estimate of the corresponding r.m.s. error in the anomalous-scattering measurements.

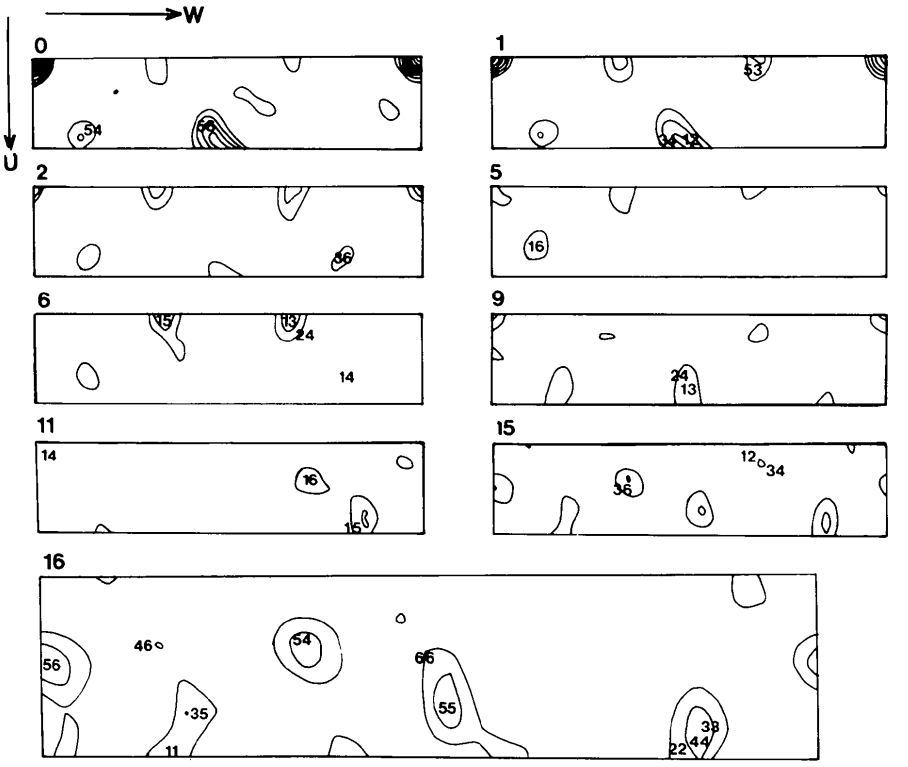

Fig. 1. The Patterson function for the UO_2F_5 derivative of monoclinic lysozyme showing the vector positions for the six sites. The sections are sampled in intervals of $V/32$ with U running from 0–½ and W from 0–1.

to F_{HLE} refinement (Dodson & Vijayan, 1971), and the final parameters are listed in Table 1.

(*b*) *The orthorhombic form*

The orthorhombic crystals have the following cell parameters: space group $P2_12_12_1$, $a = 59{\cdot}4$, $b = 68{\cdot}7$, $c = 30{\cdot}8$ Å, $V = 1{\cdot}257 \times 10^5$ Å^3. They contain one lysozyme molecule as the asymmetric unit, giving a V_M of 2·12 Å^3 dalton^{-1}. This form has very similar cell dimensions to those reported by Jollès & Berthou (1972) from crystallization at room temperature, pH 6–10, but is quite distinct from the high-pH form

Table 2. *The five highest features in the E map calculated for the uranyl derivative of monoclinic lysozyme*

Also included is a sixth peak for reasons described in the text. The 'background' level of the synthesis is ±13 units.

Site	Height (arbitrary units)	*x*	*y*	*z*
1	57	0·192	0·195	0·234
2	26	0·576	0·196	0·782
3	25	0·214	0·366	0·906
4	19	0·181	0·159	0·562
5	15	0·624	0·189	0·112
6	11	0·678	0·375	0·430

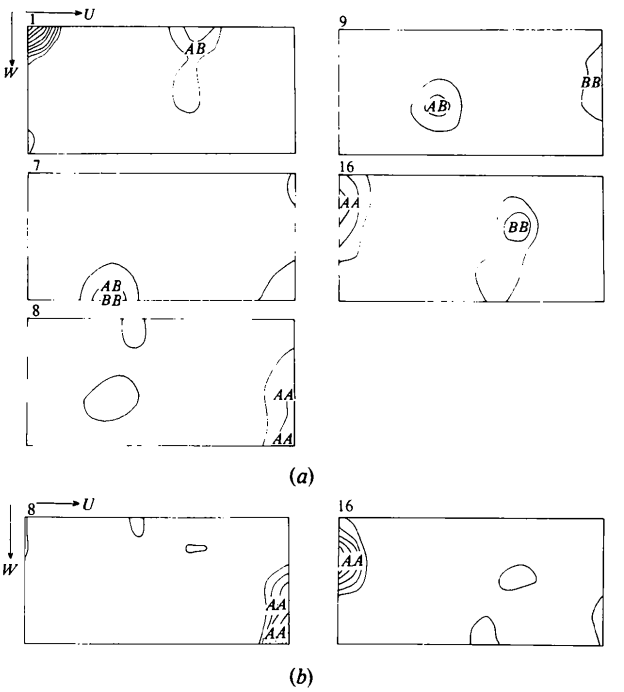

Fig. 2. The Patterson function for (*a*) the $Hg(Ac)_2$ derivative and (*b*) the $Pt(CN)_4$ derivative for orthorhombic lysozyme. The maps are in sections of V, with V running from 0–$\frac{16}{32}$; U and W run from 0–½. Only the sections showing peaks (with the exception of the origin) are shown.

Table 3. *Heavy-atom parameters for the* $K_2Pt(CN)_4$ *and* $Hg(OOC.CH_3)_2$ *derivatives of orthorhombic hen egg-white lysozyme*

Mean figure-of-merit = 0·779.

Derivative	Site	Relative occupancy	x	y	z	ΔF	E	E'	$R_{centric}$
$K_2Pt(CN)_4$	*A*	0·404	0·010	0·131	0·168	0·137	35	12	0·378
$Hg(OOC.CH_3)_2$	*A*	0·386	−0·004	0·123	0·177	0·231	50	20	0·479
	B	0·414	0·323	0·095	0·130				

ΔF = mean isomorphous difference = $(1/n)\sum_0^n \| |F_{PH}| - |F_P| \|$, x,y,z are fractional cell coordinates,
$R_{centric} = \sum_{\text{centric terms only}} (|F_{PH} - F_P| - |F_{H(calc)}|)/\sum |F_{PH} - F_P|$.

described by Palmer, Ballantine & Ferold (1948) or the high-temperature one (Jollès & Berthou, 1972).

The difference Patterson map of the $K_2Pt(CN)_4$ derivative was readily solved in terms of a single site of substitution, and that of $Hg(OOC.CH_3)_2$ in terms of two sites. Sections of the difference Patterson maps are shown in Fig. 2. Refinement of the heavy-metal sites was carried out in three dimensions using centrosymmetric refinement. Difference Fourier maps at the completion of the refinement, and those using joint phases, revealed no further sites nor any significant features at the position of the refined sites. The final heavy-metal parameters and refinement indices are listed in Table 3.*

Results and conclusions

An electron density map of the monoclinic form was calculated using phases obtained from the isomorphous and anomalous contributions from the uranyl derivative alone (figure-of-merit 0·75). The map of the orthorhombic form used combined phases from the mercury and platinicyanide derivatives (figure-of-merit 0·78).

Interpretation of the maps was carried out by making use of the characteristic division of the tertiary structure of the hen egg-white lysozyme molecule into two distinct parts (Blake, Mair *et al.*, 1967). Residues 1–40 and 85–129 combine to form a subdomain of largely α-helical structure, while the subdomain formed by residues 41–84 is almost entirely extended-chain structure. In the low-resolution maps of tetragonal hen egg-white lysozyme (Blake, Fenn, North, Phillips & Poljack, 1962) and human lysozyme (Blake & Swan, 1971) these two subdomains appear as a cluster of rod-shaped segments of density, and a complex sheet-like region of density, respectively, with the active site marked by a pronounced surface depression lying between them. In the electron density map of the monoclinic and orthorhombic crystal forms, molecules with these characteristics were clearly present. The asymmetric unit of the monoclinic form contained two such molecules which were related by a translation of approximately $a/2 + c/2$.

The electron density corresponding to a single lysozyme molecule was isolated and it was possible to identify particular rods of density with the major α-helices in the lysozyme structure. The coordinates of the N and C termini of the density corresponding to helices 5–15, 24–34 and 88–96 were read off the map and used to calculate the rotation matrix required to transform the atomic coordinates of the hen egg-white lysozyme molecule from the tetragonal cell into the monoclinic and orthorhombic cells. The matrices were subsequently improved by plotting the α-carbon positions on the monoclinic and orthorhombic maps and then moving certain 'guide' α-carbons more centrally into the electron density. The final fits of the connected α-carbon positions determined from the tetragonal crystal form of hen egg-white lysozyme to the Fourier maps of the monoclinic and orthorhombic crystal forms are shown in Figs. 3 and 4 respectively.

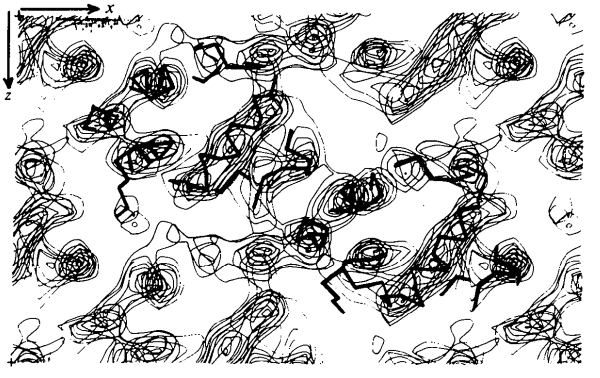

Fig. 3. Sections $\frac{5}{12}$ to $\frac{9}{12}$ of the monoclinic lysozyme map with connected α-carbon coordinates. Molecule 1 is on the left of the figure.

* Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1LZH, 2LZH, R1LZHSF and R2LZHSF), and are available in machine-readable form from the Protein Data Bank at Brookhaven or one of the affiliated centres at Cambridge, Melbourne or Osaka. The data have also been deposited with the British Library Lending Division as Supplementary Publication No. SUP 37002 (4 microfiche). Free copies may be obtained through The Executive Secretary, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England.

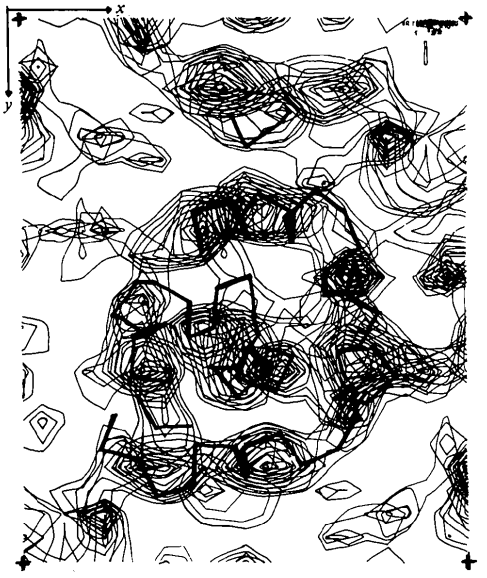

Fig. 4. Sections $\frac{1}{32}$ to $\frac{8}{32}$ of the orthorhombic lysozyme map.

The fit of the coordinates to the density is very close for the two molecules in the asymmetric unit of the monoclinic crystals and for the single molecule in the asymmetric unit of the orthorhombic crystals, and indicates that the lysozyme molecule has, in these crystals, an identical or almost identical tertiary structure to that in the tetragonal crystals (Blake, Mair *et al.*, 1967). Table 4 gives the relevant rotation matrices.

Table 4. *Rotation/translation matrices for converting tetragonal hen egg-white lysozyme coordinates (Imoto et al., 1972) into the monoclinic and orthorhombic unit cells*

Monoclinic molecule 1			
	0·84284	0·432758	−0·31330
R	−0·17852	−0·32186	−0·92980
	−0·50770	0·83960	−0·19316
T	−3·43693	35·81177	−20·37522
Monoclinic molecule 2			
	0·80357	0·32489	−0·49872
R	−0·42394	−0·27572	−0·86270
	−0·41779	0·90467	−0·08382
T	17·66917	33·20193	5·91291
Monoclinic molecule 2 → molecule 1			
	0·97571	−0·20760	0·06998
R	0·21560	0·96659	−0·13867
	−0·03885	0·15039	0·98786
T	−14·19590	0·72997	−30·52292
Orthorhombic			
	−0·62971	−0·70595	−0·32420
R	−0·08321	0·47623	−0·87538
	0·77236	−0·52425	−0·35863
T	−10·92757	50·73715	33·83519

The electron density map for the monoclinic form revealed that the active sites of both molecules appeared open along their entire lengths. In spite of this, however, attempts to diffuse in *N*-acetylglucosamine resulted in crystal shattering even at low concentrations. It would appear that the tight crystal packing is unable to accommodate the changes in the conformation of lysozyme that occur on binding substrate or inhibitor (Blake, Johnson *et al.*, 1967), and that this crystal form is therefore unsuitable for substrate-binding studies.

A difference map was calculated between the two monoclinic native types with coefficients $(F_I - F_{II}) \exp i\alpha_{II}$, where F_I and F_{II} are the structure factors of the two native forms and α_{II} is the single isomorphous-replacement phase for type II. As can be seen from this map, which is shown in Fig. 5, the great majority of the difference features are associated with molecule 1, there being negligible changes in molecule 2. The map can be interpreted in terms of a rotation of about 10° in molecule 1 in a direction which increases the pseudosymmetry in the type I crystals. This analysis is supported by the increase in the height of the $(\frac{1}{2},0,\frac{1}{2})$ peak from 52% of the origin peak in the type II native Patterson to 65% of the origin peak in type I. The relationship between the two molecules of the asymmetric unit in type II is shown in Fig. 6. There would be considerable interest in the high-resolution refinement of these two forms of monoclinic lysozyme to observe the effect on thermal parameters of slightly different crystal packing in order to assess the relative contributions of static disorder and molecular motions. The variations in solvent structure between these two closely related crystal forms would also give much useful information on the factors governing the interaction of water with the enzyme.

Since the submission of this manuscript, Hogle, Rao, Mallikarjunan, Beddell, McMullan & Sundaralingam (1981) have published the structure determination of the monoclinic crystal form at 4 Å resolution. A

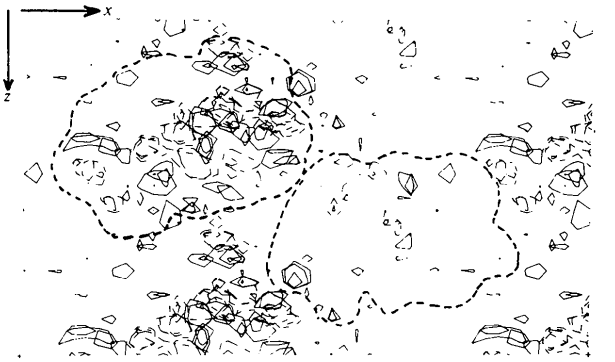

Fig. 5. Sections 0 to 14 of the type I–type II difference map with the molecular boundaries for the two molecules marked. The majority of the difference features are associated with molecule 1.

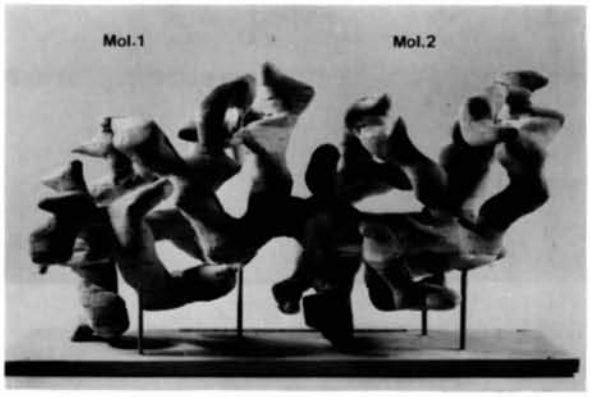

Fig. 6. A balsa-wood model of the asymmetric unit of type II monoclinic lysozyme. The division of the asymmetric unit into two molecules approximately related by a translation can be seen.

detailed comparison of the coordinates obtained in the present work and those derived by Hogle *et al.* (1981) is inappropriate at this stage because both coordinate sets are those of tetragonal lysozyme (Blake, Mair *et al.*, 1967) rotated into low-resolution maps. However, when account is taken of the different origins chosen in the two independent structure determinations it is clear that the two rotation matrices agree quite well.

Like us, Hogle *et al.* (1981) report two related crystal forms, but a comparison of cell dimensions shows that theirs are distinct from ours (for example β angles of 90·5 and 90·8° compared with 90·06 and 91·95° respectively). This may indicate that it is possible to obtain a wide variety of slightly different crystal forms corresponding to small changes in the orientation of molecule 1 to molecule 2 within the asymmetric unit.

Inspection of the electron density map for the orthorhombic form showed that access to the active site was obstructed in the region of sugar-binding sites *A*, *B* and perhaps *C*. On the other hand, sites *D*, *E* and *F* are open to the solvent channels in the crystal. This is potentially interesting since in the tetragonal form of hen egg-white lysozyme sites *E* and *F* are blocked (Blake, Mair *et al.*, 1967), and so substrate-binding studies will yield information complementary and additional to that which is already available. Equilibration of the crystals with a saturated solution of *N*-acetylglucosamine produced surface cracks in the crystals, but they diffracted normally. A 15° precession photograph of the *hk*0 zone showed small discrete intensity changes relative to that of the native enzyme which may indicate saccharide binding.

Data have been collected on this orthorhombic form of hen egg-white lysozyme to 1·9 Å and it is intended to refine the structure. The starting model will be the coordinates of the tetragonal form of hen egg-white lysozyme rotated into the 6 Å isomorphous map as described above. It is also intended to collect high-resolution data on the crystals soaked in saccharide.

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