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Structure Determination of Turkey Egg-White Lysozyme Using Laue Diffraction Data

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Abstract

The three-dimensional structure of turkey egg-white lysozyme (TEWL) has been solved and refined at 2.5 Å resolution using X-ray data collected by the Laue method. This is the first protein structure determination undertaken using Laue diffraction data. A re-examination of the existing structure of TEWL was necessary when attempts to refine an atomic model based on the C_α positions in the Protein Data Bank (entry 1LZ2) failed. The correct orientation and position of the turkey lysozyme molecules within the crystallographic unit cell were determined by molecular replacement using a refined model of the homologous hen egg-white lysozyme crystal structure. After modification of the model to reflect the differences in amino-acid sequence between the chicken and turkey enzymes, the structure was subjected to crystallographic refinement using the simulated-annealing refinement technique and conventional least-squares refinement. This yielded a final residual of $R = 20.7\%$. This crystal form is of potential interest for time-resolved crystallographic studies since the amino-acid residues involved in catalysis (Asp52 and Glu35) are accessible to solvent and not blocked by crystal contacts.

Introduction

The Laue method (Friedrich, Knipping & von Laue, 1912) gained new popularity in the 1970's (Tuomi, Naukkarinen & Rabe, 1974; Buras & Gerward, 1975; Steinberger, Bordas & Kalman, 1977) when synchrotron radiation became widely available for structural studies (Rosembaum, Holmes & Witz, 1971). This, along with developments in computational techniques to process Laue diffraction data (Wood, Thompson & Matthewman, 1983; Machin, 1985, 1987; Campbell, Habash, Moffat & Helliwell, 1986; Clifton *et al.*, 1985; Campbell *et al.*, 1987; Hajdu *et al.*, 1987, 1991; Rabinovich & Lourie, 1987; Cruickshank, Helliwell & Moffat, 1987, 1991; Helliwell *et al.*, 1989; Shrive, Hajdu, Clifton & Greenhough, 1990), led to an expansion in the use of the Laue technique. Bordas, Munro & Glazer (1976) showed that structure-factor amplitudes could be obtained from white-radiation fibre-diffraction studies of collagen and that biological materials could survive exposures to white synchrotron radiation. This lead to trials with protein crystals (Moffat, Szebenyi & Bilderback, 1984; Helliwell, 1984, 1985; Hajdu & Stuart, 1985; Hajdu *et al.*, 1986; Moffat, Bilderback, Schildkamp & Volz, 1986) which finally began to yield structural information from both protein and virus crystals (Hajdu *et al.*, 1987, 1989; Farber, Machin, Almo, Petsko & Hajdu, 1988; Campbell *et al.*, 1990; Sclichting *et al.*, 1990; Almo,

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Howell, Petsko & Hajdu, 1991). In these studies Laue data have been used in conjunction with difference-Fourier techniques where the initial structure of the protein of interest is available. The studies have demonstrated that Laue data can be used (i) to locate substrates (Hajdu *et al.*, 1987) and metal ions bound to proteins (Farber *et al.*, 1988) (ii) for crystallographic refinement (Almo *et al.*, 1991) and (iii) for time-resolved studies of enzyme reactions (Schlichting *et al.*, 1990).

A logical extension of this is *de novo* protein structure determination. Wood *et al.* (1983) determined and refined the structure of the small molecule berlinite (α-$AlPO_4$) with Laue data to an R factor of 0.19 for integrated intensities, and Harding and colleagues (Harding, Maginn, Campbell, Clifton & Machin, 1988; Clucas, Harding & Maginn, 1988) have also determined several small molecule structures using Laue diffraction data. However, in protein structure determination, problems arise in the location of heavy atoms bound to proteins by isomorphous difference Patterson syntheses (Hajdu *et al.*, 1991) because of the loss of low-resolution data due to the harmonic overlap problem (Cruickshank *et al.*, 1987) and the systematic distribution of data that occurs. Thus to date, the elucidation of a protein structure using the isomorphous replacement method with Laue data has not been successful.

The advantage and perhaps greatest potential of the Laue method of data collection lies in the speed with which a nearly complete data set can be measured. Using one of the currently available synchrotron sources, the intensity of the incident radiation permits data sets to be collected on the millisecond to second timescale and experiments (Szebenyi, Bilderback, LeGrand, Moffat, Schildkamp & Teny, 1988) have already demonstrated that exposure times of micro- to picoseconds are possible in the future. In favorable systems, this reduction in data-collection time may allow complex enzyme–substrate reactions to be investigated crystallographically (Hajdu, Acharya, Stuart, Barford & Johnson, 1988; Schlichting *et al.*, 1990). Turkey egg-white lysozyme (TEWL) is potentially such a system.

While evaluating this system for time-resolved crystallographic studies, it became necessary to re-examine the original structure determination of TEWL (Bott & Sarma, 1976) available in the Protein Data Bank (entry 1LZ2). The refinement of an atomic model based on the existing C_α positions proved unsuccessful. The original structure had been determined using molecular replacement techniques prior to the development of restrained least-squares and simulated-annealing refinement methods.

In this paper we present the correct structure determination and refinement of TEWL and show that molecular replacement techniques for structure determination are possible with Laue diffraction data.

Materials and methods

Crystallization

Crystals of TEWL were grown using conditions similar to those described previously (pH 8.0 form) (Bott & Sarma, 1976). Single crystals of TEWL were grown by the sitting-drop method on a nine-well depression plate. 50 ml of protein solution (6 mg ml^{-1}) and 50 μl of a 9% NaCl (w/v) solution were equilibrated against a 4 ml reservoir of 18% NaCl (w/v) solution. The buffer was 100 mM ammonium acetate, pH 8.0. The hexagonal rod-shaped crystals (average size 0.8 × 0.4 × 0.4 mm) grew in about a week at room temperature. The crystals belong to the space group $P6_122$ or its enantiomorph $P6_522$ with unit-cell dimensions $a = b = 70.89$, $c = 84.6$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. For data collection the crystals were mounted in quartz capillary tubes with the long axis parallel to the tube. The capillaries were mounted in a flow-cell device (Wyckoff *et al.*, 1967) so that the crystals were at all times bathed in mother liquor.

Data collection

Initial Laue diffraction data were collected on the wiggler beam line (station 9.7) at the Synchrotron Radiation Source, Daresbury Laboratory, England. The storage ring was operated at 2.0 GeV, with a current of between 150–200 mA. The wiggler magnet was operated at 5.0 T, and produced an effective spectrum of 0.2–2.1 Å. Data were collected on a stationary crystal, exposed to the unfocused white beam, on a film pack of six pieces of CEA Reflex 25 film. With careful optical alignment prior to data collection, it is possible in principle to collect an almost complete set of data (95%) in a single 2 s exposure for this high-symmetry (hexagonal) system (Clifton, Elder & Hajdu, 1991). The incident beam was collimated to 200 μm and the crystal-to-film distance was 110 mm. Films were scanned on a rotating-drum microdensitometer with a 50 μm raster size and optical-density range of 0–2. The data were processed using software developed by P. A. Machin and the Computing Systems and Applications Group at Daresbury (Campbell *et al.*, 1987; Helliwell *et al.*, 1989). Briefly, this involves obtaining refined orientation parameters from the predicted pattern and then integrating the intensities of the reflections. The intensities from the different films in the film pack are then scaled using the equation $I_A = aI_B\exp(-b\lambda^3)$ and corrections for obliquity and Lorentz–polarization applied. Finally, the reflections are normalized, based on the intensities of symmetry-

equivalent reflections observed at different wavelengths (Campbell *et al.*, 1986).

A data set was produced with 2697 unique reflections [$F > 2\sigma(F)$] with an internal agreement factor (R_{merge}) of 7.4% on intensity. The reduced data represent 67% of the theoretical number of reflections between 5.0 and 2.5 Å resolution. Although about 95% of the data are recorded in a single exposure, 28% of the data are lost due to one of the following reasons: (*a*) the spots on the film being either harmonic (multiple reflections falling at the same point on the film) or spatial overlaps, (*b*) the reflection being generated by a wavelength that falls outside the range of wavelengths capable of being processed (due to the absorption edges of silver and bromine found in the film) or (*c*) due to the σ cutoff applied to the data [$2\sigma(F)$].

In order to obtain a more complete data set for refinement purposes, additional data were collected on a second crystal at the X31 station at EMBL, DESY, Hamburg (Wilson, 1989). The ring was operated at 5.5 GeV with a current of 30–45 mA. The crystal was exposed to a focused beam with an effective spectrum of 0.65–1.9 Å. All other data collection and reduction strategies were identical to those above. The final (combined) data set contained 2953 unique reflections with an R_{merge} of 8.4% on intensity. The distribution of data as a function of resolution is presented in Fig. 1.

Structure determination and refinement

The molecular replacement method (Crowther, 1972) was used to determine the structure of TEWL using data between 5–3 Å resolution and the previously determined structure of the homologous hen egg-white lysozyme (HEWL) molecule as the 'trial' structure (PDB entry number 1LYZ). The orientation of the known model structure (in this case HEWL) in the unit cell of the unknown molecule (TEWL) was determined using the *MERLOT* program package (Fitzgerald, 1988). Structure factors were calculated for the HEWL model with all side chains included in an ideal triclinic unit cell with dimensions $a = b = c = 120$ Å and $\alpha = \beta = \gamma = 90°$. Rotational searches of 5° were carried out by a systematic reorienting of the Patterson function of the HEWL model and a search for the maximum overlap with the Patterson function computed from the TEWL Laue data. The rotation function was scaled from 1–100, a value of 100 being the highest peak found in the map. Nine peaks were found above a value of 65. The most prominent peak was found at $\alpha = 20$, $\beta = 70$, $\gamma = 250°$, with a root-mean-square deviation above background of 5.10 and a scaled value of 100. The next highest peak had an r.m.s. deviation above background of 4.09 and a value of 80. A fine scan around the maximum yielded the best solution of $\alpha = 21.79$, $\beta = 72.0$, $\gamma = 250.0°$.

The position of the correctly orientated molecule within the TEWL cell was determined by calculating translation functions using vector sets between the 12 crystallographically related molecules in the hexagonal unit cell. It was also necessary to determine the space group. The space groups $P6_122$ and its enantiomorph $P6_522$ were distinguished by the vector set generated from molecules 1 and 5 at equivalent positions (x, y, z) and (y, $y-x$, $z+\frac{5}{6}$) which gives interatomic vectors at ($U = x - y$, $V = x$, $W = -\frac{5}{6}$) for $P6_122$ or (x, y, z) and (x, $y-x$, $z+\frac{1}{6}$) which gives interatomic vectors ($U = x - y$, $V = x$, $W = -\frac{1}{6}$) for $P6_522$. Examination of the Harker section for $w = \frac{1}{6}$ (*i.e.* $-\frac{5}{6}$, Fig. 2*a*) shows the presence of a prominent peak at $U = 0.228$, $V = 0.412$, 5.89σ above background, all other peaks shown are less than 3.5σ above the background. Examination of the Harker section $w = \frac{5}{6}$ (Fig. 2*b*) shows a relatively flat map, the highest peak being only 3.53σ above background. This indicates that $P6_122$ ($w = \frac{1}{6}$ map, Fig. 2*a*) is the correct enantiomorph.

Vector searches between molecules at equivalent positions (i) (x, y, z) and ($-x$, $-y$, $z+\frac{1}{2}$) examined at the Harker section $w = \frac{1}{2}$, (ii) (x, y, z) and (x, $x-y$, $\frac{1}{6}-z$) examined at the Harker section $w = -\frac{1}{6}$ and (iii) (x, y, z) and ($y-x$, y, $\frac{1}{2}-z$) examined at the Harker section $w = \frac{1}{2}$, enabled a unique solution to the translation function to be found, $x = 0.4125$, $y = 0.18$, $z = 0.368$. The correctly positioned model in the TEWL unit cell gives an R factor of 39.2% for all Laue data between 5–2.5 Å resolution.

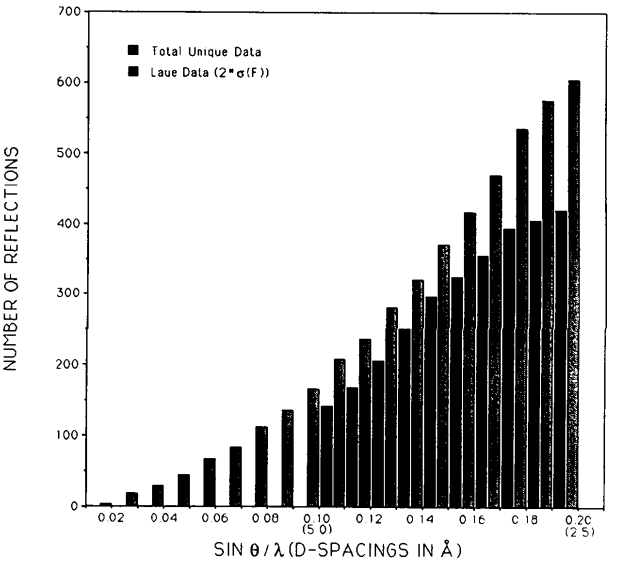

Fig. 1. Histogram showing the distribution of data as a function of resolution, where λ is taken as that of Cu $K\alpha$ radiation (λ = 1.541 Å).

The HEWL model was rotated and translated into the TEWL unit cell. The seven amino-acid differences (F3Y, H15L, Q41H, R73K, V99A, D101G, Q121H) in the two structures were 'mutated' using the molecular-graphics program *FRODO* (Jones, 1978) and the preliminary TEWL model was then subjected to crystallographic refinement using the simulated-annealing technique with *XPLOR* (Brunger, Kuriyan & Karplus, 1987). Initial refinement used the data collected at the Daresbury Laboratory. The refinement protocol consisted of rigid-body minimization, C_α atom restrained minimization, a high-temperature simulation and a cooling-stage simulation followed by further conjugate gradient minimization (see Fig. 3). This reduced the R factor from 39.2 to 19.2% with an overall isotropic temperature factor for data between 5–2.5 Å resolution. Full details of the procedure and subsequent steps in the refinement using the more-complete merged data from the two crystals are given in the legend of Fig. 3. Further refinement was undertaken with manual rebuilding between each cycle of *XPLOR* refinement. Finally (step 16) individual isotropic temperature factors were refined using conventional least-squares refinement (Konnert, 1976; Konnert & Hendrickson, 1980). After 15 cycles of refinement an R factor of 20.7% was obtained. The increase in the residual from 19.2 (step 5) to 20.7% (step 16) on completion of the refinement arises from (i) the increased number of reflections used in the later stages of the refinement, (ii) the possible slight variation in the amplitudes following the merging of the two data sets and (iii) the restraints applied to the model in the *PROLSQ* program. The restraints placed on bond lengths and angles, torsion angles, planarity *etc.* are much tighter than those normally applied in *XPLOR* and a slight increase in the R factor normally results. No water molecules have been included in the structure because of the resolution range (5–2.5 Å) and completeness (73%) of the data available. Fig. 3 shows the decrease in R factor for the refinement process and the details of the individual steps. Fig. 4 is a $2F_o - F_c$ omit map (Dodson, 1981) for residues 52 (Asp) and 53 (Tyr) showing the general quality of the electron density map for the molecule.

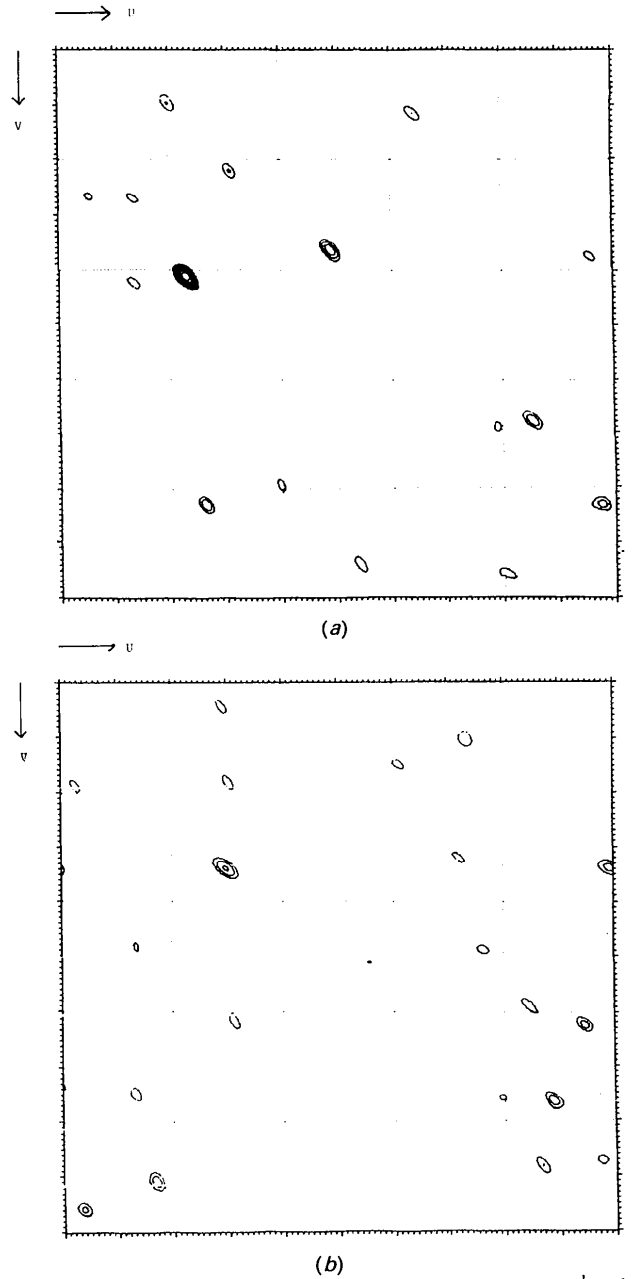

Fig. 2. Harker sections of the translation function for (*a*) $w = \frac{1}{6}$ and (*b*) $w = \frac{5}{6}$ for the vector set generated between molecules 1 and 5. The sections are contoured at 0.5σ starting at 2.5σ above background.

Discussion

TEWL structure at pH 8.0 *determined using Laue data*

The TEWL structure contains five helices (residues 5–15, 25–35, 80–84, 89–98, 108–113) and a region of β-pleated sheet (residues 42–46, 50–53, 58–60), and is, as expected from the > 90% homology between them, very similar to the refined HEWL model used in the structure solution (Fig. 5). The average r.m.s. deviation in all the TEWL backbone atoms from the HEWL model, after refinement, was 0.65 Å (determined using the least-squares fitting program in *SYBYL*, a graphics program from Tripos Associates Inc., a subsidiary of Evans and Sutherland). This figure mainly results from a series of small changes as can be seen in Fig. 5. In the comparison of main-chain atoms approximately 10% of the atoms

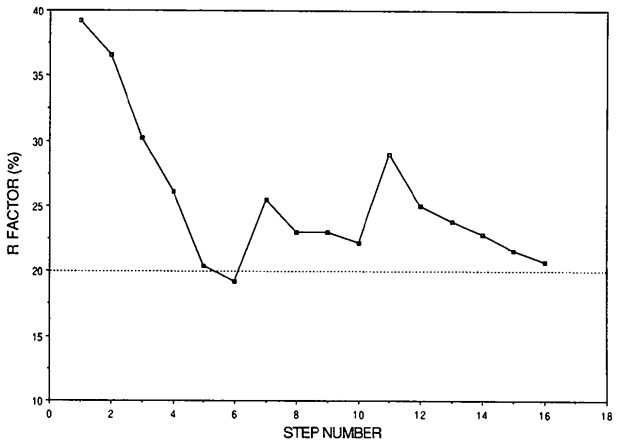

Fig. 3. Refinement of turkey egg-white lysozyme. Graph of *R*-factor reduction *versus* refinement progress. The *R* factor is the crystallographic agreement factor, defined as $\Sigma|F_{obs} - F_{calc}|/\Sigma F_{obs}$ where the observed quantities are measured reflection amplitudes and the calculated quantities are computed from the atomic coordinates of the current structure model. The sums are over all observed reflections. Refinement was carried out with data between 5–2.5 Å resolution. Steps 1–5 used the initial data collected at the Daresbury laboratory (2697 reflections) while steps 6–16 used the merged data from two crystals (2953 reflections), see *Data collection* for details. Refinement protocol: step 1, 40 cycles of rigid-body *R*-factor minimization. Step 2, 100 conjugate gradient minimization steps with soft repulsive potential, followed by 40 conjugate gradient minimization steps with CHARMM nonbonded potential, C_α restraints at 83.68 kJ mol^{-1} Å^{-2}, B = 12.0 Å^2, ΔF = 0.05 Å (where ΔF is the maximum movement of atoms allowed before updating the structure-factor calculation). Step 3, 2 ps simulation, T = 2000 K, time step = 1 fs, velocity scaling every 25 fs, ΔF = 0.2 Å. Step 4, 0.5 ps simulation, T = 300 K, time step = 1 fs, velocity scaling every 25 fs, ΔF = 0.2 Å. Step 5, 160 conjugate gradient minimization steps, ΔF = 0.01 Å. Step 6, manual re-build using $2F_o - F_c$ and $F_o - F_c$ Fourier syntheses. Step 7, 80 conjugate gradient minimization steps with CHARMM non-bonded potential, C_α restraints at 83.68 kJ mol^{-1} Å^{-2}, ΔF = 0.05 Å. Step 8, temperature bath used, temperature raised to 4000 K and cooled to 300 K, no heat stage simulation, time step = 0.5 fs, number of steps = 50. Step 9, 80 conjugate gradient minimization steps, ΔF = 0.01 Å. Step 10, manual re-build. Step 11, 40 cycles conjugate gradient minimization. Step 12, 40 conjugate gradient minimization steps with soft repulsive potential, followed by 80 conjugate gradient minimization steps with CHARMM nonbonded potential, C_α restraints at 83.68 kJ mol^{-1} Å^{-2}, ΔF = 0.05 Å. Step 13, 1 ps simulation, T = 2000 K, time step = 1 fs, velocity scaling every 25 fs, ΔF = 0.2 Å. Step 14, 0.25 ps simulation, T = 300 K, time step = 1 fs, velocity scaling every 25 fs, ΔF = 0.2 Å. Step 15, 80 conjugate gradient minimization steps, ΔF = 0.01 Å. Step 16, 15 cycles of the restrained least-squares Konnert–Hendrickson refinement were performed. Isotropic temperature factors were refined. The final *R* factor was 20.7%. The refinement was terminated at this stage after examination of $2F_o - F_c$ maps. The model was found to reflect the resolution (2.5 Å) and completeness (73%) of the data. The following is a summary of the restraints applied and observed.

	Restraints applied	R.m.s. deviations from ideality
σ bond length (Å)	0.020	0.009
σ angle distance (Å)	0.040	0.038
σ planar distance (Å)	0.050	0.030

are greater than 1.0 Å apart. The largest variations occur in residue ranges 64–74 and 101–105 (see loops in Fig. 5*b*). Both these regions are involved in extensive intermolecular crystal packing, which is not found in the HEWL model. This could explain why there are such large variations between the two structures in these regions.

The main difference between the crystals of HEWL and TEWL is the packing of the enzymes in their respective crystalline environments. In the tetragonal form of HEWL, the protein is packed such that the lower half of the active site is blocked and only limited inhibitor-binding studies have been possible (Johnson & Phillips, 1965). However, examination of the crystal packing for TEWL shows that the long-active site is partially blocked by intermolecular contacts near Gly101 (binding site *A*) but that the region of the active site involved in hydrolysis (Glu35 and Asp52) is accessible to the bulk solvent *via* channels that run parallel to the *x* and *y* crystallographic axes. It should therefore be possible to initiate catalysis in crystals of TEWL by diffusing penta-*N*-acetyl glucosamine into preformed crystals of the enzyme in a flow cell (Howell, Warren, Amatayakul-Chantler, Petsko & Hajdu, 1991).

Structure determination at pH 7.0

In a concurrent but separate study, the structure of TEWL at pH 7.0 has also been determined (Parsons, 1988; Phillips, Somers, Bhat & Parsons, 1990; Parsons & Phillips, 1991). This structure was solved to 2.2 Å resolution by conventional multiple

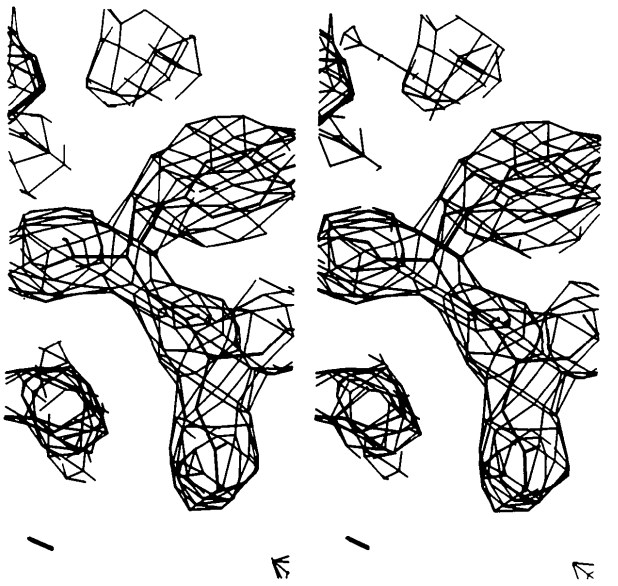

Fig. 4. Stereoview of a $2F_o - F_c$ omit map showing the quality of the electron density map for residues Asp52 and Tyr53. The electron density map is contoured at 1σ above background.

isomorphous replacement techniques with monochromatic data. It has been refined using the restrained least-squares refinement procedure of Konnert–Hendrickson (Konnert, 1976; Konnert & Hendrickson, 1980) to an R factor of 19.3% with 120 water molecules and individual isotropic B values. Coordinates were exchanged after the completion of each study, and the rotation and translation solutions found in the monochromatic study were found to agree with those presented in this paper. An analysis of the r.m.s. deviations in all backbone atoms between the monochromatic structure and the structure determined with Laue diffraction data shows that the two structure determinations agree to within 0.62 Å. The deviations between the two structures could be caused in part by the difference in resolution ranges 2.2 Å (pH 7.0 structure) *versus* 2.5 Å (pH 8.0 structure, this paper). The r.m.s. deviation of this TEWL structure from the HEWL structure is 0.71 Å. Again differences between the structures are generally small with the largest differences occurring in residue ranges 47–49 and 100–107. A more detailed comparison of the monochromatic turkey and hen lysozyme structures will be presented elsewhere (Parsons & Phillips, 1991).

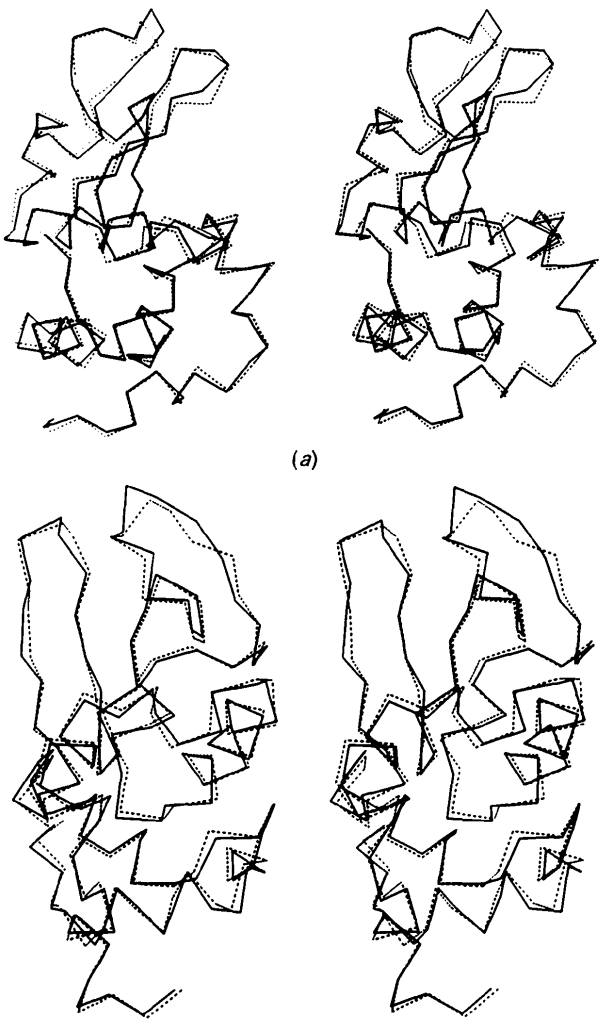

Fig. 5. Two stereoviews of the refined structure of TEWL showing the superposition of the C_α TEWL (solid lines) and HEWL (dashed lines) structures and the overall structure of the molecule. In (*a*), the lysozyme molecule is positioned looking down the first helix (residues 5–15) at the bottom left. The active-site cleft is to the right of the molecule, the binding sites *A–F* running from right to left across the picture. In (*b*), loop 64–74 is at the top right of the molecule and loop 101–105, the top left.

Comparison with previously determined structure (PDB entry: 1*LZ*2)

Bott & Sarma (1976) used the molecular replacement technique and the structure of the homologous hen egg-white lysozyme with either all side chains or side chains beyond C_β removed as their search molecule. Owing to the use of just the strongest terms for data from 10 to 6 Å resolution, several potential solutions were found for the orientational element of the molecular replacement problem. The solution that gave the largest peak was chosen since it appeared in the same region in all calculations performed, independent of the number of reflections used or the calculated transform that was used (all side-chain atoms or truncated at C_β). A solution to the translation function was found using packing considerations. The model finally chosen gave $R = 47\%$ at 6 Å with four intermolecular C_α—C_α contacts less than 5.0 Å.

Although the actual error in the orientation of the molecule with regard to the correct structure determination can be considered as just a re-orientation of 17° and a translational shift with regards to the origin of the unit cell (Phillips *et al.*, 1990; and this paper), the result means that the orientation of the active site with respect to the channel along the crystallographic z axis is different. The earlier model predicted that the active site would face directly onto the channel (Fig. 6*b*). In our model, the active site is rotated by about 180° away from the channel shown in Fig. 6(*a*), the consequence being that the active site is partially blocked at site *A* as described above.

Concluding remarks

We have shown that Laue diffraction data are capable of being used for protein structure determinations using the molecular replacement technique. It was initially surprising that the rotation function yielded a solution since the Laue data lacked the low-resolution reflections due to harmonic overlaps

and had a very non-uniform distribution of the data in reciprocal space. Our previous work (Hajdu *et al.*, 1991) on heavy-atom Patterson functions with Laue data had been unsuccessful. However, since the trial structure is nearly exact, the correlation of a near perfect Patterson calculated from the model structure with that of the very noisy Laue Patterson map did yield sufficient information for the solution to be found and for the structure to be determined.

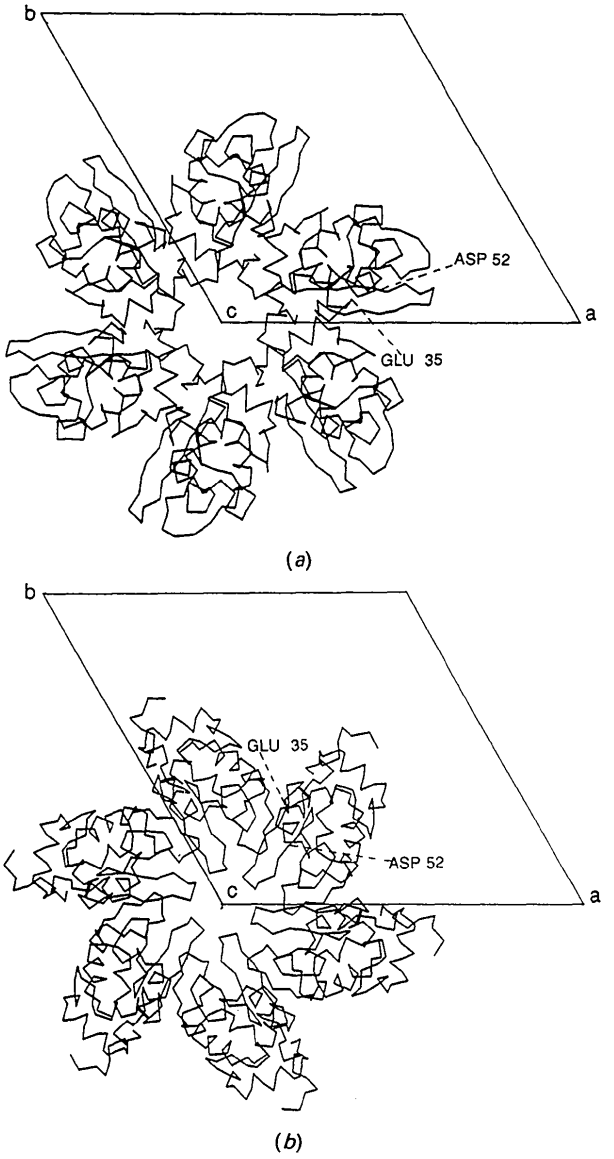

Fig. 6. Diagrams of the crystal packing of TEWL looking down the *z* axis for the correct (*a*) and (*b*) incorrect structures showing the location of the active-site residues (Asp52 and Glu35). A channel exists which is parallel to the *c* axis (indicated by the letter *c* in the diagram). The channel is approximately 20 Å in diameter in (*a*). Only six of the 12 molecules present in the unit cell are displayed.

The present success of the Laue technique in the solution of a crystal structure of a protein when the structure of a homologous molecule was known suggests that the method will prove valuable for studying proteins and their mutants at high resolution, and for determining structures of protein or virus crystals that decay rapidly in a monochromatic beam or for which a limited number of crystals exist. Although one should not necesarily advocate routine use of a method that provides only partial data for the structure determination of macromolecules since this may lead to errors in the structure, data collected by the Laue method have subsequently been used successfully in the structure determination by molecular replacement of glycosomal glyceraldehyde phosphate dehydrogenase from *T. brucei* where limited amounts of protein and only a few crystals were available (Vellieux, Hajdu, Groendijk & Hol, 1991).

The main advantage of the Laue method, however, is the fast data-collection times, which it is hoped can be exploited to examine enzyme–substrate complexes and other transient species. TEWL is a candidate for such time-resolved studies.*

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* Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 3LZ2, R3LZ2SF), and are available in machine-readable form from the Protein Data Bank at Brookhaven. The data have also been deposited with the British Library Document Supply Centre as Supplementary Publication No. SUP 37057 (as microfiche). Free copies may be obtained through The Technical Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England.

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