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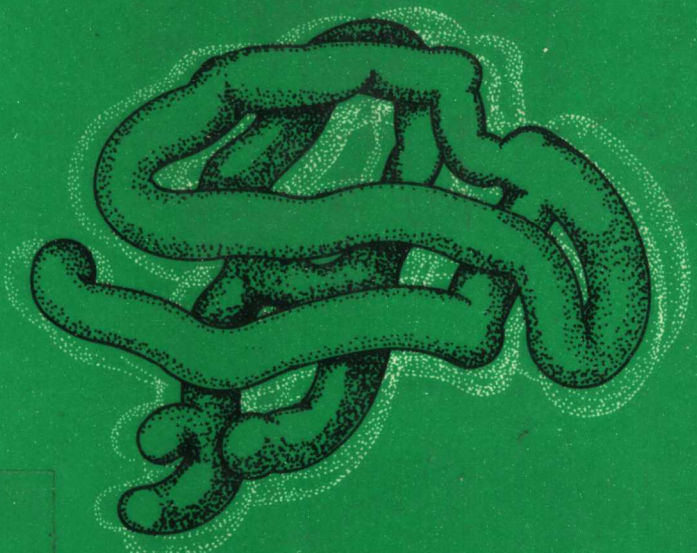
Mobility and Migration of Biological Molecules

Edited by **P.B. Garland** and **R.J.P. Williams**



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Mobility and Migration of Biological Molecules

ORGANIZED AND EDITED BY
P.B. GARLAND AND R.J.P. WILLIAMS

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Preface

This Symposium is the last of the five that I have organised for the Biochemical Society, and this completes my term of office as the Society's Symposium Organiser. To organise such symposia offers a little work and many privileges, not least of which is to meet the many distinguished contributors who make the symposia possible. The topic of Molecular Mobility and Migration arose from my own interests in the field, and from discussion with Professor R.J.P. Williams whom I was fortunate to have as co-organiser. There were areas that we had to neglect, not only because of our bias towards proteins and membranes but because there just was not time available to also include contractile systems, DNA unwinding, flagellar motion and so on: all excellent examples of the biological importance of molecular movement.

The Symposium Meeting was held at St. George's Hospital Medical School in London, in December 1980. We enjoyed a large and lively audience, with good discussion. I am grateful to the speakers for their excellent presentations, their prompt submission of manuscripts, and for making the organisers' job so simple.

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Contents

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Crystallographic Studies of Movement within Proteins

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Synopsis

The primary results of X-ray diffraction studies of crystalline proteins are maps, showing the distribution of electron density within the crystals, from which the structures of the protein molecules can be determined. Improved descriptions of the molecular structures are obtained in a second stage of the analysis in which the molecular parameters are refined in such a way that the X-ray data predicted from the structure agree as closely as possible with those observed. The refined molecular model obtained in this way takes account of the mobility of the structure by attributing to each atom a parameter defining its apparent mean-square amplitude of vibration. This apparent motion of the atoms may be real or may arise because different molecules in the crystals adopt slightly different conformations or orientations. Studies at different temperatures help to resolve this ambiguity. The parameters describing the apparent motion of the atoms are also sensitive to errors in the experimental data. Parallel studies of lysozymes from two different species have suggested strongly, however, that the X-ray data describe a characteristic property of the lysozyme molecule which is related to its activity. Preliminary studies of triose phosphate isomerase suggest that intramolecular motion and conformational variability play an important part in the activity of this enzyme.

Introduction

X-ray crystal structure analysis is essentially a form of microscopy in which an image of the distribution of electrons within a crystal is calculated from measurements of the X-rays diffracted by the crystal. The electron density, $\rho(xyz)$, and the amplitudes of the diffracted X-rays are related by the Fourier inversion:

$$\rho(xyz) = \frac{1}{V} \sum_h \sum_k \sum_l F(hkl) \exp i\alpha(hkl) \exp\{-2\pi i(hx + ky + lz)\} \quad (1)$$

where $F(hkl)$ is the observable structure amplitude of the diffraction maximum hkl and $\alpha(hkl)$ is its phase, which cannot be observed directly. V is the volume of the unit cell of the crystal and the summations are over all the diffraction maxima within a determined limit of resolution.

According to W.L. Bragg's (1913) original interpretation of the interaction of X-rays with a crystal, the diffraction maxima *hkl* can be regarded as reflexions of the X-rays by lattice planes with these Miller indices. The relationship between the interplanar spacing, d(*hkl*), the angle 2θ(*hkl*) through which the incident X-rays are deflected and λ, the wavelength of the X-rays, is given by Bragg's familiar equation:

$$\lambda = 2d\ (hkl)\ \sin\theta\ (hkl) \tag{2}$$

The detail to be expected in an image calculated from the amplitudes of these reflexions clearly depends upon the number and range of reflexions included in the calculations. If d_m is the minimum interplanar spacing for which *hkl* and the corresponding values of F and α are included in eqn. (1), then detail on a smaller scale than about:

$$r \approx 0.71\ d_m = 0.71\ \lambda/2\sin\theta_{max.} \tag{3}$$

cannot be resolved in a three-dimensional image. The close correspondence between this formula and Abbe's formula for the resolving power of a microscope is apparent. In crystallographic studies of proteins, however, the resolution of the image is usually defined simply by a statement of the value of d_m used in the analysis.

The fundamental problem of crystal-structure analysis is that the phase angles α(*hkl*) associated with the structure amplitudes F(*hkl*) of the X-ray reflexions cannot be measured directly. The amplitudes themselves can be derived in a straightforward way from the intensities of the X-ray reflexions – subject, of course, to a variety of experimental errors – but the phase angles are derived much less directly by the study of isomorphous crystals containing additional heavy-atoms (e.g. mercury or uranium). The method of isomorphous replacement was first shown to be practicable in the study of proteins by Green *et al.* (1954); Blow (1958) demonstrated later that the anomalous scattering of the X-rays by the heavy atoms in the derivative crystals provides additional phase information. The methods of protein crystallography have been described by Blundell & Johnson (1976).

The first image of a protein molecule produced by the method of multiple isomorphous replacement showed the structure of sperm-whale myoglobin at 6Å resolution (Kendrew *et al.*, 1958) (distances are given throughout this paper in Å; 1Å = 0.1 nm). At this low resolution, which was chosen mainly in order to limit the measurements to a manageable number, the molecule could be seen to be made of eight α-helices of different lengths joined by less regular arrangements of the polypeptide chain to form a compact structure surrounding the haem group. The interpretability of this image depended to a great extent upon the fact that myoglobin is a predominantly α-helical structure and later studies of more complex molecules at this low resolution were not very informative. Accordingly, after the successful extension of the myoglobin study to 2Å resolution (Kendrew *et al.*, 1960), which showed the conformation of the polypeptide chain in detail, protein crystallographers have concentrated in the main on producing images of proteins with resolutions (expressed in terms of d_m values) in the range 3 – 2Å.

In the study of hen egg-white lysozyme at 2Å resolution (Blake *et al.*, 1965) and in many subsequent analyses, such images have been found to be sufficiently clear to allow the achievement of the crystallographers first objective, interpretation of the image in terms of a detailed molecular structure. It must be stressed, however, that the successful completion of this process has depended upon knowledge of the amino-acid sequence of the protein, and that it has often been made difficult by seeming inadequacies of the X-ray images, which are often less clear in some regions than in others. This variation of clarity in the image clearly arises to some extent from errors in the measurements and derived phases that have been used in the calculation, but it may also reflect real properties of the protein molecule, some parts of which may, for example, be more mobile than others. It is increasingly important, therefore, to consider by what means these images of protein structures may be improved and to what extent the descriptions of protein molecules derived from them may be made more precise. In particular, these improved descriptions of the molecules must take into account the X-ray evidence for their mobility.

Crystal-Structure Refinement

The object of crystal-structure refinement is to improve the description of the structural model in such a way as to give the best set of atomic parameters consistent with the experimental observations. This involves calculation of the structure amplitudes from the atomic parameters by use of the equation:

$$\begin{aligned}\mathbf{F}(hkl) &= F(hkl)\exp i\alpha(hkl) \\ &= \sum_{j=1}^{N} f_j \exp\{-B_j \sin^2\theta\,(hkl)/\lambda^2\} \exp\{2\pi i\,(hx_j + ky_j + lz_j)\} \qquad (4)\end{aligned}$$

where $f_j(hkl)$ is the atomic scattering factor, that is the ratio of the amplitude of X-rays scattered by the atom j in the direction of the hkl reflexion to the amplitude scattered in that direction by a single electron at the same position $(x_j\, y_j\, z_j)$; and the term $\exp\{-B_j \sin^2\theta\,(hkl)/\lambda^2\}$ takes into account the fact that the effective value of f_j is reduced if the atom is moving with respect to its mean position. If this motion is assumed to be harmonic and isotropic, the constant B_j is related to the mean square amplitude of vibration, $\overline{U_j^2}$, by the expression

$$B_j = 8\pi^2\, \overline{U_j^2} \qquad (5)$$

Clearly $\mathbf{F}(hkl)$ is complex and embodies the phase information needed for the calculation of (1). It comprises the vector addition of the scattering, with amplitude $f_j \exp\{-B_j \sin^2\theta\,(hkl)/\lambda^2\}$ and phase $2\pi(hx_j + ky_j + lz_j)$, from each individual atom in the structure.

Two approaches to the problem of refinement are briefly described, both of which have been long used in studies of small molecules. The first is generally known as Fourier refinement. In its simplest form this is an iterative process in which structure amplitudes and phases are calculated from the current model

of the structure by the use of eqn. (4) and the calculated phases are then combined with the observed structure amplitudes in the calculation of a new image of the structure (eqn. 1). Interpretation of this image leads to a new model and the process is repeated until no further improvement in the agreement between observed and calculated structure amplitudes is achieved. The agreement between $F_{obs.}$ and $F_{calc.}$ is usually expressed by the index

$$R = \sum | F_{obs.} - F_{calc.} | / \sum | F_{obs.} | \qquad (6)$$

Many modifications of this simple approach have been made to increase its efficiency, three of which are particularly worthy of note. First, instead of calculating a new image of the whole structure it is common practice, following Cochran (1951), to calculate the difference between the true and the current model structure by using amplitudes $F_{obs.} - F_{calc.}$ and calculated phases in eqn. (1). Interpretation of such 'difference' maps makes possible the correction of known atomic positions, the assessment of temperature factors and the improvement of the model by the addition of new atoms representing, for example, water molecules. But a word of caution is necessary: it will be clear that the use of calculated phases biases the new image towards the current model and great care must be taken to avoid the retention of incorrect features. Particularly when the problem is to establish the positions and properties (occupancies and motions) of water molecules or disordered side chains, this is more easily said than done.

The second improvement in Fourier refinement is to use not simply the phase angles calculated from the current model but the best phases consistent with all of the evidence. The phases determined experimentally are usually expressed in terms of a probability function (Blow & Crick, 1959). Calculated phases can be represented in the same way, and Fourier refinement appears to converge most rapidly when the two probability distributions, appropriately weighted, are multiplied together to give combined phases for use in the next round of image formation (Hendrickson & Lattman, 1970; Rice, 1981).

The third important aspect of Fourier refinement that needs to be mentioned is peculiarly relevant to the refinement of protein structures. Except in studies at very high resolution [$d_m < 1.5$Å] the individual atoms in the molecules are not resolved, and it is unreasonable to suppose that bond lengths and bond angles will be determined as precisely in these studies as they have been determined already in analyses of simpler structures. Accordingly it is customary in the Fourier refinement of protein structures to include an additional stage in each cycle during which the current model of the molecular structure is constrained to satisfy well-established stereochemical criteria (Diamond, 1966; Dodson *et al.*, 1976). In this stage, for example, bond lengths and bond angles are made to agree as well as possible with standard values, and planar groups of atoms are made planar. In effect the incorporation of this stage may be regarded as reducing the number of independent variables to be determined, since the individual atomic positions must be consistent with the known stereochemistry of protein molecules.

The incorporation of stereochemical information in the refinement process

has been achieved more systematically in the least-squares refinement of protein structures. In the most recent development of this method (Hendrickson & Konnert, 1980), the function minimized is

$$\theta = \sum_h \sum_k \sum_l W_F (F_{obs.} - F_{calc.})^2 + \sum_m W_x (d_l - d_C)^2 \qquad (7)$$

where W_F is the weight for $F_{obs.}$; d_l and d_C are ideal and observed distances between atoms; m is a summation over all restraints; and W_x is the weight on distance d_l. Here the stereochemical information is treated as providing additional observations with which the refined model of the protein structure must agree. This is proving a very powerful method of refinement especially in association with the use of the most powerful modern computers such as the CRAY-1 computer at the Daresbury Laboratory of the S.R.C.

The apparent thermal motion of protein molecules

Interpretation of the B-values (eqn. 4 and 5) obtained in these refinements of protein structures is not straightforward because of a fundamental difficulty. The thermal vibrations of the atoms, which have a frequency of about $10^{13}s^{-1}$, are slow compared with the X-ray frequency which is about $10^{18}s^{-1}$. Consequently, to the X-rays, the atoms appear to be stationary but displaced from their mean positions. But such displacements may be due not only to thermal motion but also to static disorder if corresponding atoms in different unit cells throughout the crystal take up slightly different mean positions. In a protein crystal such disordering may arise because corresponding molecules in different unit cells adopt different positions or orientations (due, for example, to the inclusion of impurities) but it may arise also because different molecules take up different conformations. Provided that the resultant atomic displacements are less than the nominal resolution (eqn. 3) achieved in the electron-density map of the structure their effect in a study at one temperature will be indistinguishable from the effects of true thermal motion.

In practice, matters are even more complicated. It has been known since the earliest studies of myoglobin (Kendrew *et al.*, 1961) that individual side chains may take up radically different conformations in different molecules so that separate images of them can be seen in an electron-density map. Such conformational variability, which in some structures extends to more extensive regions than individual chains (e.g. Huber, 1979), cannot be modelled adequately by allocating to each atom a single position and an apparent amplitude of vibration. It is necessary to refine the atomic positions, apparent amplitudes of vibration and occupancies corresponding to each distinguishable conformation of the protein molecule. Given that the X-ray data correspond to a structure that is averaged over the time of the experiment and all the molecules in the crystal, this is clearly a formidable challenge.

One further difficulty of a different kind must be mentioned. Studies of small molecules at high resolution have shown clearly that errors in the observed structure amplitudes have a more serious effect upon the B-values than upon atomic positions. Systematic errors, such as those arising from

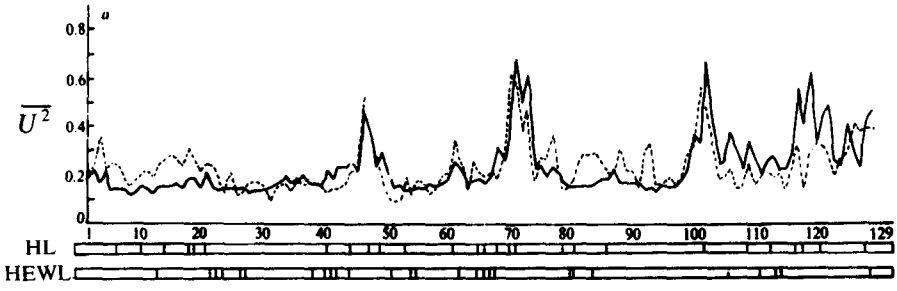

Fig. 1. *Plot of the mean* $\overline{U^2}$ *values against residue number for main-chain atoms in human lysozyme (HL, full line) and hen egg-white lysozyme (HEWL, broken line)*

The numbering scheme is for HEWL: the extra residue in HL is inserted between residues 47 and 48. Residues involved in intermolecular contacts in the crystals of the two molecules are indicated in the lower rectangle.

absorption of the X-rays in the crystal or its mounting or from irradiation damage, have particularly serious effects. In addition, defects in the molecular model, such as the omission of hydrogen atoms from the calculations, also affect the apparent temperature factors — especially in analyses in which the atoms are not resolved.

Despite all these difficulties, however, encouraging results are being obtained in studies of the apparent thermal motion and conformational variability of protein molecules.

The apparent mobility of lysozyme

Refinements of the structures of two different species of lysozyme have been undertaken and the results compared in an attempt to resolve the difficulties of interpretation discussed above (Artymiuk *et al.*, 1979). The structure of hen egg-white lysozyme (Blake *et al.*, 1965) was refined by the method of Fourier refinement (Grace, 1980) to an *R*-value (eqn. 6) of 0.22 for the 9600 reflexions to 2Å resolution and a structural model comprising 1001 protein atoms (excluding hydrogen) and 250 water molecules. The structure of human lysozyme (Osserman *et al.*, 1969) has been refined principally by the method of least squares with stereochemical restraints at 1.5Å resolution using 19 000 X-ray reflexions and a model comprising 1026 protein atoms and 130 water molecules: the current *R*-value is 0.18 (Artymiuk, 1979; P.J. Artymiuk & C.C.F. Blake, unpublished work). Fortunately, though not by design, the X-ray measurements were made by different methods in the two analyses and the data were corrected differently for the various systematic errors to which they are subject. Finally it must be noted that the two species of lysozyme crystallize in unrelated crystal forms with the molecules quite differently arranged in their respective unit cells.

The apparent mean square amplitudes of vibration (eqn. 5) of the main-chain atoms in the two molecules are shown, averaged over individual residues, in Fig. 1. Clearly, to a first approximation at least, the two plots have the same characteristics, a fairly constant background on which are superposed

several peaks corresponding to residues 47, 70–73 and 101–102. Although there are some differences the general agreement is good, as is shown by the correlation coefficient of 0.61. The distribution of $\overline{U^2}$ is much smoother, however, for human lysozyme almost certainly because this structure has been refined more completely at higher resolution than has that of hen egg-white lysozyme. Nevertheless the comparison is valuable because it suggests strongly that the observed variations in $\overline{U^2}$ cannot be due either to the effects of systematic errors in the data (which must be different in the two analyses) or to interactions between molecules in the crystals (which are different in the two structures). It seems, rather, that the parallel variation of the $\overline{U^2}$ values obtained in the two studies represents some property of the lysozyme molecules themselves.

It is to be expected that, to some extent at least, the variation in $\overline{U^2}$ values along the polypeptide chain arises from motion of the whole molecule as a rigid body. Careful consideration of this possibility for the two lysozymes (Sternberg *et al.*, 1979; Artymiuk *et al.*, 1979) shows that some part of the apparent motion may arise from uniform displacements of the whole molecules from their mean positions together with independent librational displacements about three principal axes intersecting at their centres. Here another ambiguity arises. In addition to the problem of distinguishing between real motion and static disorder (which is covered in the foregoing account by use of the word displacements instead of vibrations), there is a difficulty in distinguishing between librational and breathing motion with respect to molecular axes. If the apparent motion of the molecule is a rigid-body libration about a particular axis an individual atom will appear to move predominantly along the arc of a circle about that axis. If, on the other hand, the molecular motion can be described as a breathing motion in which the molecule expands and contracts, an individual atom will appear to move radially with respect to the axis. Given only the values of $\overline{U^2}$ averaged over all directions that are derived from isotropic B-values (eqn. 5) these two types of motion cannot be distinguished. But they could be distinguished if anisotropic B-values were available. Such an analysis would require the substitution of a new temperature-factor term in eqn. (4) of the form:

$$\exp\{-(\beta_{11}h^2 + \beta_{22}k^2 + \beta_{33}l^2 + 2\beta_{12}hk + 2\beta_{23}hl + 2\beta_{31}lh)\} \quad (8)$$

where the six independent parameters define the orientation of an ellipsoid and the amplitudes of vibration along its principal axes. Only an analysis at very high resolution would provide enough observations to determine so many additional parameters reliably.

Even when apparent rigid-body motions are taken into account, however, the $\overline{U^2}$ values shown in Fig. 1 are not fully explained and the residual variations imply the existence of intramolecular motion (or conformational variability). Close examination of these variations shows that they correlate well with the three-dimensional structure of the lysozyme molecule. For example, there are cyclic variations in the helical residues 4–16 and 108–116 that correspond to greater apparent motions of residues located on the exposed surfaces of these

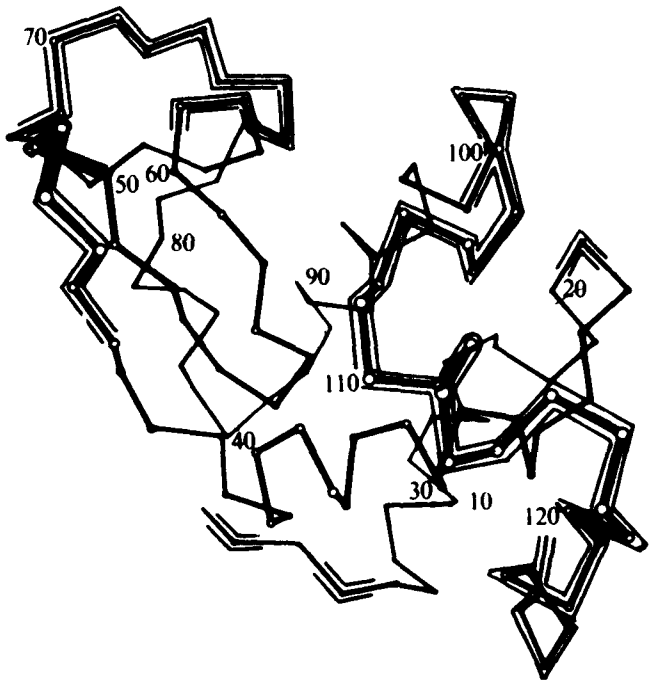

Fig. 2. *Perspective drawing of the main polypeptide chain in lysozyme with residues having apparent* $\overline{U^2} > 0.2\text{Å}^2$ *in HL emphasized*

helices, and the most exposed strand (residues 42–46) of the small three-stranded β-pleated sheet in lysozyme is subject to larger apparent motion than the other two strands. These, and other, close correlations of apparent motions with structure reinforce the conclusion that they represent a characteristic molecular property of lysozyme and not some artefact of the crystallography.

If the regions of highest apparent motion shown in Fig. 1 for human lysozyme are located on the lysozyme molecule (Fig. 2) they are found to form a continuous strip on the enzyme surface that begins at the exposed strand of β-sheet, runs around the loop at the top of the molecule, down the side of the active-site cleft and along to the carboxyl-terminus. This region includes both lips of the active site and most of the main-chain segments that undergo conformational change when inhibitors are bound (Blake *et al.*, 1967).

This last observation suggests that the apparent motion may play a part in the activity of the enzyme and it emphasizes again the importance of distinguishing between true motion and conformational variability. The information derived from studies of inhibitor binding could be explained by inhibitor binding involving the selection of a restricted number of conformations from those accessible to the enzyme that are observed superimposed upon one another in the structure of the native enzyme. Alternatively, inhibitor binding may diminish the motion of some parts of the enzyme molecule. To examine these possibilities further, a detailed study of enzyme–inhibitor complexes in which their structures are fully refined is

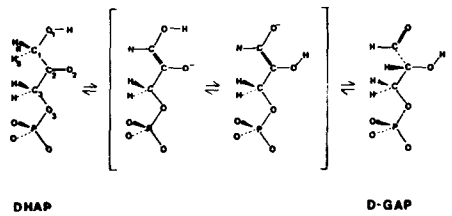

Fig. 3. *Isomerization of dihydroxyacetone phosphate (DHAP) and* D-*glyceraldehyde phosphate* (D-*GAP*) *catalysed by triosephosphate isomerase with enediolate intermediates as suggested by Rieder & Rose (1959)*

clearly needed. Furthermore, these studies should be designed to eliminate, so far as may be possible, the ambiguities inherent in the present analysis.

The way ahead in crystallographic studies of enzyme mobility is already clear to some extent from the work of Frauenfelder *et al.* (1979) on myoglobin. By studying the structure of this protein over a range of temperatures from 220 to 300K these authors have shown how true motion and static disorder, or conformational variability, may be distinguished. Their conclusions on the varying degrees of motion in different parts of the myoglobin are similar to those described here for lysozyme, but their use of low-temperature methods adds an important new dimension to the analysis that will be especially important as direct studies of enzyme–substrate complexes at low temperatures are further developed (Alber *et al.*, 1976).

Also important in the future will be the extension of these analyses to higher resolution. There is evidence that the use of high-intensity X-rays from synchrotron sources makes possible the recording of higher-angle diffraction data than has been accessible hitherto in most analyses. If this turns out to be true and the methods are used over a range of temperatures, many studies may be possible at near atomic resolution in which anisotropic *B*-values may be determined and the various types of motion and disorder distinguished to an extent that has not yet been possible.

Protein mobility and enzyme action

The potential importance of such detailed studies of the mobility of enzymes is apparent already from many crystallographic analyses: studies of triosephosphate isomerase provide an excellent example (Banner *et al.*, 1975).

This enzyme catalyses the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde phosphate (D-GAP) as shown in Fig. 3. The enzyme from chicken muscle is a dimer formed from two identical polypeptide chains each comprising 247 amino-acid residues. The crystal structure has been determined at 2.5Å resolution by the standard methods (Banner *et al.*, 1975) and it is now being refined by the method of least squares with stereochemical restraints. In advance of the results of this refinement, however, evidence of the mobility of the enzyme and the importance of this mobility to its activity has already been provided by a number of studies which will be described here very briefly.

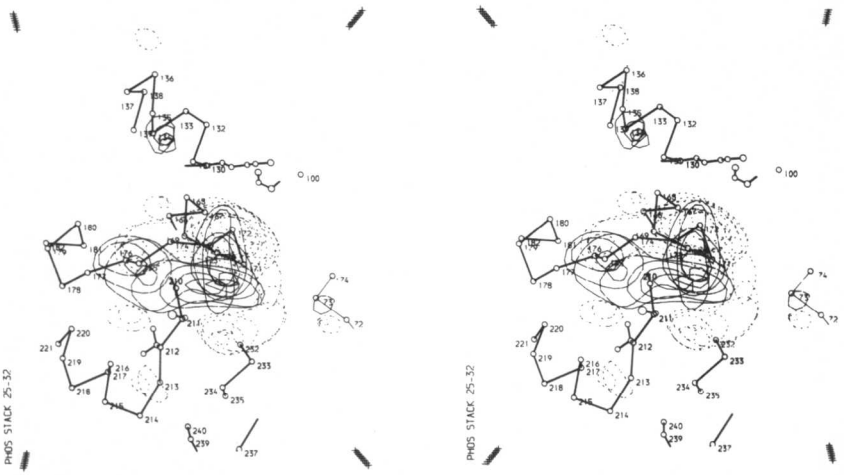

Fig. 4. *Part of sections z = 25/40–32/40 of the electron-density map at 6Å resolution showing the difference between chicken triosephosphate isomerase in 3.1* M*-ammonium sulphate and similar crystals in 3*M*-ammonium phosphate*

Broken contours show regions where the density decreased on the addition of phosphate. The atomic positions are as determined in the analysis of the enzyme structure at 2.5Å resolution.

The enzyme molecule has a remarkably regular structure in which each polypeptide chain is folded into alternate β strands and α-helical segments to form a central cylinder of parallel β-pleated sheet surrounded by a largely-helical surface. Two extended loops are also formed on the surface of each subunit. One of them comprising residues 70–80, forms an important part of the interface connecting the two subunits, in which it envelops residue methionine-14 from the other polypeptide chain. The second loop, residues 168–177, in each subunit forms part of the outer rim of the active site pocket. It is clear, however, in the unrefined map of the native enzyme that this loop can adopt alternative conformations. In one subunit it is seen relatively clearly, making a contact with a neighbouring molecule, while in the other subunit it is less clear but apparently in a somewhat different conformation.

These indications of conformational variability are confirmed by studies of the interactions of inhibitors and substrates with the enzyme (Rivers, 1977). The enzyme was crystallized in the presence of 3.1M-ammonium sulphate, which is itself an inhibitor of the enzyme activity. Transfer of the crystals to 3M-ammonium phosphate solution gave rise to intensity changes in the diffraction pattern that can be seen, in the following way, to correspond to a conformational change in the enzyme. An electron-density map was calculated from eqn. (1) with amplitudes $(F_{\text{phos.}} - F_{\text{sulp.}})$, derived from the X-ray intensities in phosphate and sulphate, and phases determined in the analysis of the native structure. It shows only one highly significant feature (Fig. 4). In the vicinity of residues 168–177 in one of the subunits there is a region of negative density in the position occupied by these residues in the native structure, and a region of positive density indicating that in the presence of phosphate this loop moves to a new position below the original one (as seen in Fig. 4). It must be noted that there is no change observed in the corresponding loop in the second