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On the three-dimensional structure and catalytic mechanism of triose phosphate isomerase

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Triose phosphate isomerase is a dimeric enzyme of molecular mass 56000 which catalyses the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate. The crystal structure of the enzyme from chicken muscle has been determined at a resolution of 2.5 Å, and an independent determination of the structure of the yeast enzyme has just been completed at 3 Å resolution. The conformation of the polypeptide chain is essentially identical in the two structures, and consists of an inner cylinder of eight strands of parallel β-pleated sheet, with mostly helical segments connecting each strand. The active site is a pocket containing glutamic acid 165, which is believed to act as a base in the reaction. Crystallographic studies of the binding of DHAP to both the chicken and the yeast enzymes reveal a common mode of binding and suggest a mechanism for catalysis involving polarization of the substrate carbonyl group.

Introduction

The enzyme that allows the utilization of dihydroxyacetone phosphate (DHAP) in glycolysis is triose phosphate isomerase (TIM), which catalyses the interconversion of this substrate and D-glyceraldehyde-3-phosphate (D-GAP). The enzyme from many sources is a dimer of two identical subunits, each of molecular mass about 27000, and utilizes no cofactors or metal ions. The two subunits appear to be kinetically independent; there is no evidence for any metabolic control by activators or inhibitors at this step in glycolysis. The reaction catalysed by TIM is conceptually one of the simplest in biology, and has been studied extensively by a variety of physical and chemical methods. It is an attractive candidate for high-resolution structural studies by X-ray diffraction because the nature of the reaction, which involves a single substrate and product, makes possible a direct analysis of the structure of a productive enzyme–substrate complex. In this paper we summarize the results so far obtained in crystallographic studies of this enzyme and show how they may be combined with the biochemical data on the enzyme in solution to suggest a mechanism for the catalytic reaction.

Triose phosphate isomerase catalyses the transfer of the pro-*R* hydrogen on the C_1 carbon of DHAP to the C_2 carbon of D-GAP, as shown in figure 1. Principally on the basis of isotopic

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labelling experiments, Rose and his coworkers (Rieder & Rose 1959; Rose 1962) proposed a mechanism involving proton abstraction by a single base on the enzyme followed by formation of a *cis*-enediol intermediate. Recent experiments by Rose (this symposium) have suggested that the intermediate is, in fact, the diol rather than the diolate and this point will be discussed further below. Chemical labelling studies in a number of laboratories (Hartman 1968, 1971; Coulson *et al.* 1970; Miller & Waley 1971; de la Mare *et al.* 1972) led to the conclusion that the base concerned in the reaction is the side chain of glutamic acid residue 165, which is conserved in the four species for which amino-acid sequences are known (Corran

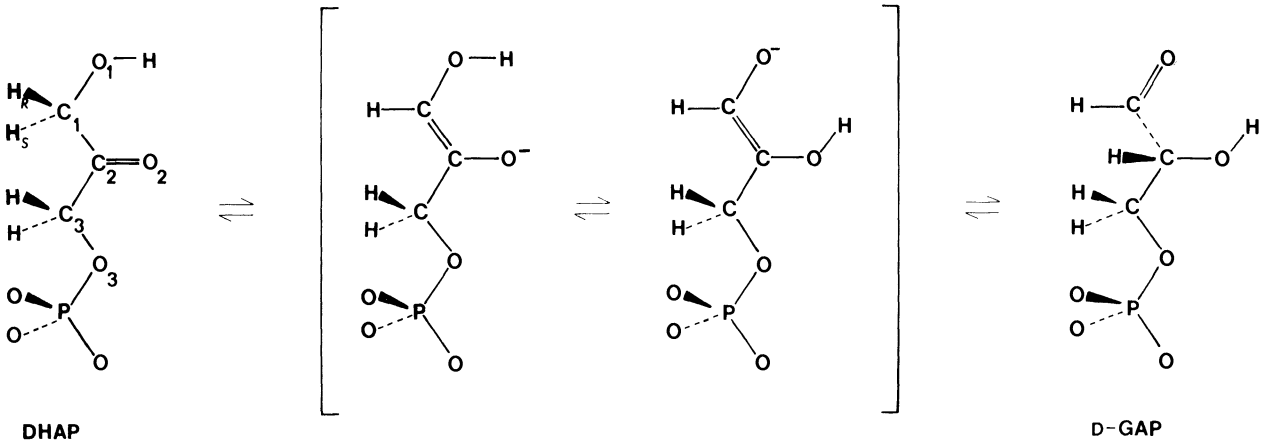

FIGURE 1. The isomerization of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde phosphate (D-GAP) catalysed by triose phosphate isomerase with enediolate intermediates (after Rieder & Rose 1959).

& Waley 1973; Kolb *et al.* 1974; Furth *et al.* 1974; Artavanis-Tsakonas & Harris 1980). The proton transfer between carbons C_1 and C_2 of the triose phosphate occurs on the *re-re* face of the proposed *cis*-enediol intermediate (Rose 1958), and Webb & Knowles (1975) have shown that bound DHAP can be reduced stereoselectively at its C_2 carbonyl from the *si* face by sodium borohydride. The rate of reduction was found to be eight times faster than that of unbound DHAP, a finding that led to the proposal that an electrophile at the active site of the enzyme plays a part in the mechanism of this reduction reaction, by polarizing the carbonyl group, and hence by implication in the proton abstraction from C_1 in the enzyme-catalysed isomerization (Webb & Knowles 1975). In this proposal, however, no distinction was made between the C_1-OH of the substrate and an enzyme-derived group as the electrophile in question (Rose 1975).

Albery & Knowles and their colleagues (see Albery & Knowles 1977) have studied the energetics of the TIM-catalysed reaction by an elegant series of isotope-exchange experiments from which they have derived the free-energy profile. This work also showed that the enzyme-catalysed reaction, which is almost 10^{10} times faster than the uncatalysed one, is so efficient that no further evolutionary pressure can be exerted to increase the effectiveness of the enzyme: TIM is an almost perfect catalyst.

Both DHAP and D-GAP exist in solution as a mixture of hydrated and free species, but TIM uses only the free triose phosphates as substrates. When correction is made for the relative proportions of free and hydrated DHAP and GAP, the overall equilibrium constant

for the reaction DHAP $\rightleftharpoons$ GAP is found to be 340 at pH 7, 25 °C (Trentham *et al.* 1969; Reynolds *et al.* 1971). Recent work by Rose (this symposium) demonstrates, however, that the equilibrium constant for these same species *on the enzyme*, i.e. E · DHAP/E · GAP, is close to unity, which is in general accord with the conclusions of Albery & Knowles (1976) and implies that a crystallographic study of the enzyme–substrate complex would show a superposition of these two species in roughly equal proportions, with an additional contribution from the intermediate structure, which Rose (this symposium) has shown to be the diol.

X-ray crystallographic studies of the structure of triose phosphate isomerase

Our knowledge of the molecular structure of TIM is based upon the results of two crystal-structure studies which are, at present, at different stages of development. The structure of the chicken-muscle enzyme has been determined at 2.5 Å resolution by the method of multiple isomorphous replacement with anomalous scattering (Banner *et al.* 1975, 1976), and the resultant description of the molecule is being improved by restrained least-squares refinement (Hendrickson & Konnert 1980). The current index of agreement between observed and calculated structure factors is 0.25 (W. Taylor, unpublished). The crystals used in this analysis were prepared by salting out with ammonium sulphate (to 60 % saturation) and are orthorhombic, space group $P2_12_12_1$, with unit cell dimensions $a = 106.01$ Å, $b = 74.76$ Å, $c = 61.74$ Å. There is one dimeric molecule in the asymmetric unit of the crystal structure so that the chemically identical subunits have different environments in these crystals and may have different conformations. No assumptions about molecular symmetry were made during the analysis.

Yeast TIM was crystallized in the presence of ammonium sulphate (100 g/l) by the addition of polyethylene glycol (Alber *et al.* 1981). Before data collection, however, the crystals were transferred to sulphate-free mother liquor, with no effect on the unit cell dimensions or the quality of the diffraction pattern. The crystals are monoclinic, space group $P2_1$, with unit cell dimensions $a = 61.3$ Å, $b = 98.2$ Å, $c = 49.5$ Å; $\beta = 90.9°$. In these crystals also there is one dimeric molecule in the asymmetric unit but the intermolecular contacts are different from those in the chicken TIM crystals. A detailed comparison of the two structures at a later stage should shed light on the influence of crystal environment on the structure and behaviour of the molecule. The structure of yeast TIM has also been solved at 3 Å resolution by the method of isomorphous replacement with anomalous scattering (Alber & Petsko, unpublished) but, in the absence of amino acid sequence data, the electron density map has not yet been interpreted in detail. Preliminary comparison with the structure of the chicken-muscle enzyme shows clearly, however, that despite the differences in crystal packing and amino acid composition the overall conformations of chicken and yeast TIM are similar. In the following account, the structural details are taken from the analysis of chicken-muscle TIM, and additional evidence derived from the studies of yeast TIM is identified explicitly.

The molecular structure of triose phosphate isomerase

The triose phosphate isomerase subunit comprises a strikingly regular arrangement of eight strands of parallel β-pleated sheet with mainly α-helical connections of the standard

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right-handed kind. The pleated sheet is twisted into a cylinder to form the core of the molecule with the helices packed around the outside (figure 2). In addition there are a number of loops of less-regularly arranged polypeptide chain, the most important of which are formed by residues 70–80 and 169–176 in the sequence of the chicken enzyme. The latter region is severely disordered in one subunit in the structures of both the chicken and the yeast enzymes. In both structures the more ordered loops in the other subunits interact with neighbouring molecules but, presumably because the intermolecular contacts are different in the two structures, these relatively well ordered loops appear to have somewhat different conformations. Thus, there is evidence in the native structures for conformational variability in this part of the enzyme molecule.

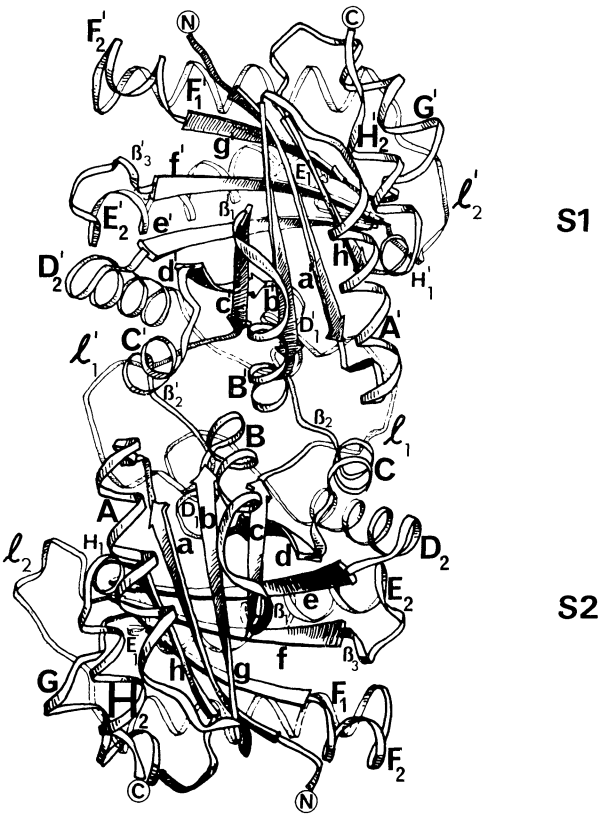

FIGURE 2. Schematic drawing of the triose phosphate isomerase molecule. Elements of secondary structure are labelled a, b ,..., h for strands of β-structure; A, B, ..., H for α-helices; β 1... for β-bends; and l_1 and l_2 for the loops comprising residues 70–80 and 169–176. Features in subunit 1 are shown with a prime: *a′*, *A′*, etc.

Apart from these and some other less impressive surface variations, the two monomer subunits have closely similar conformations. They are related by a twofold axis and interact closely with one another. The most striking elements in this interaction involve the loops formed by residues 70–80, which encircle the methionine 14 residues in the adjacent subunits. Between these isologous contacts there is a polar pocket in which a number of salt bridges, main-chain and side-chain hydrogen bonds, and solvent molecules also play a part in the subunit interaction.

The active site of triose phosphate isomerase

The active site in each subunit is easily identified as a pocket that contains the putative essential base, Glu 165. This pocket, shown in figure 3, is at the carboxyl-end of the parallel β-pleated sheet, as is often the case for substrate binding sites in the glycolytic enzymes. In the chicken TIM structure, where the crystal mother liquor is greater than 3 M in sulphate (which is a competitive inhibitor of the enzyme activity) a sulphate ion is found at the mouth of the pocket. It appears to be bound to the amide nitrogens of residues Gly 232 and Gly 233, at

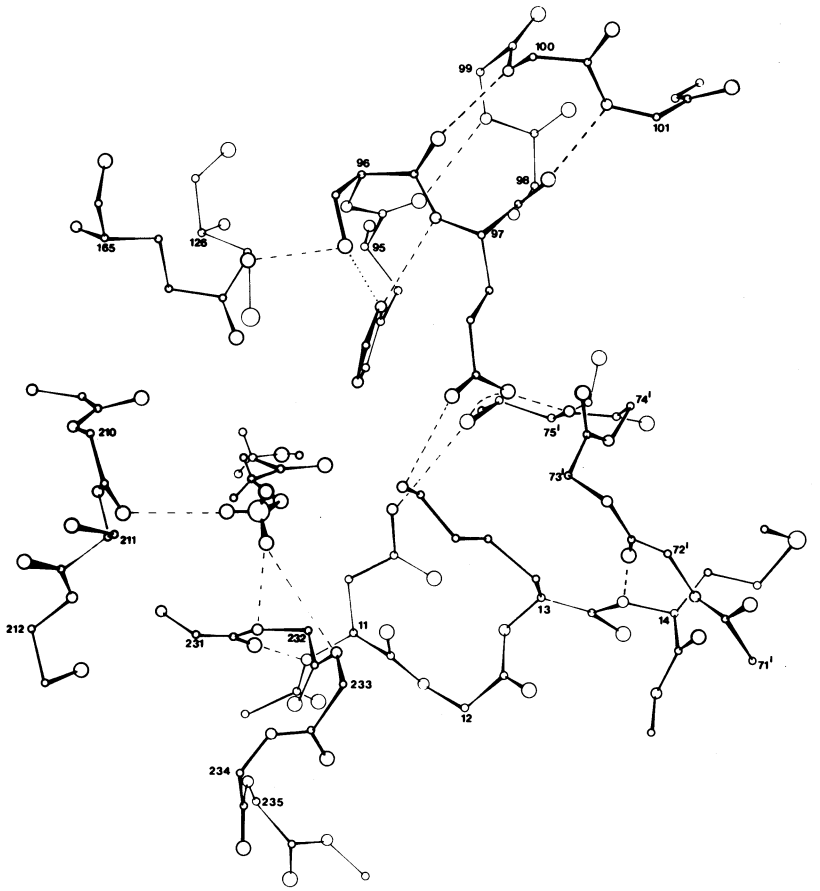

Figure 3. The active site of triose phosphate isomerase as seen in the native structure of the chicken enzyme, subunit 1. Possible hydrogen bonds are shown by broken lines. A molecule of DHAP is also shown in the position indicated by the electron-density map of the difference between the enzyme–substrate complex and the native enzyme (cf. figure 5).

the amino terminus of a short 3_{10} helix comprising residues 232–237. The sulphate ion interacts with the positive end of the electric dipole of the helix in the manner described by Hol *et al.* (1978). The anion is also bound by the side-chain hydroxyl group of serine 211. The mother liquor of the yeast TIM crystals contains no dianions, so this feature is absent there.

In addition to the side chain of Glu 165, the active site pocket includes a number of interesting functional groups. The side chain of Lys 13 projects from the side of the pocket opposite to Glu 165, and its ε-amino group interacts closely with the carboxyl oxygen $O_{\varepsilon 1}$ of

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Glu 97, presumably forming a salt bridge. The other carboxyl oxygen of Glu 97 appears to be hydrogen bonded to the main-chain nitrogen atoms of Phe 74 and Thr 75 from the adjacent subunit in the dimer. Thus each active site in the enzyme molecule involves residues from both subunits.

The oxygen atom $O_{\varepsilon 1}$ of Glu 97 is also in van der Waals contact with the imidazole side chain of His 95, which is located at the end of the pocket furthest from the sulphate binding site. The orientations of the two groups are not appropriate, however, for the formation of a hydrogen bond between them. The imidazole of His 95 appears instead to be hydrogen bonded through its $N_{\delta 1}$ atom to the main-chain NH of residue 97. In this arrangement, $N_{\delta 1}$ acts as a hydrogen bond acceptor so that the protonation of the imidazole would require a change in conformation to take place – an observation that is consistent with the apparent titration behaviour of this residue (Browne *et al.* 1976). However, $N_{\delta 1}$ of His 95 is also in contact with O_{γ} of Ser 96, though not so well oriented with respect to it for hydrogen-bond formation in the crystal structure. Clearly the possibility of a rearrangement of these groups during the catalytic process cannot be excluded.

Finally it must be noted that the O_{γ} of Ser 96 appears also to be in contact with the $O_{\varepsilon 1}$ of Glu 165.

Inhibitor and substrate binding to crystalline triose phosphate isomerase

Early observations on the diffusion of inhibitors into chicken TIM crystals (Johnson & Wolfenden 1970) showed that changes in unit cell dimensions were caused that were so large as to prevent study of the complexes by the usual method of calculating difference maps. This phenomenon was explored in some detail (Banner *et al.* 1971) and it was shown eventually (Petsko 1973) to depend critically upon the salt concentration of the crystal mother liquor. Crystals stabilized in ammonium sulphate solutions with concentrations greater than 3.1 M showed cell-dimension changes on complex formation while crystals held at lower salt concentrations did not. This finding opened the way to the direct study of complexes between chicken TIM and inhibitors and substrates, and it also suggested a preliminary study of the effects produced by the phosphate ion, which is a component of the substrates.

Phosphate binding to chicken TIM

Crystals of chicken TIM were transferred from 3.1 M ammonium sulphate solution to 3 M ammonium phosphate and soaked for 24 h. X-ray diffraction data to 6 Å resolution were collected on a five-counter, five-circle diffractometer and a difference electron-density map calculated with coefficients $(F_{phos}-F_{sulp})$ and native phases (Rivers 1977). The map showed only one significant feature (figure 4) in one of the two subunits (hereafter called subunit 1). A large region of negative density in the position of residues 169–176 in subunit 1 with an adjacent large positive feature suggested a movement of this external loop towards the active site of this subunit. There was no change in density at the position of the sulphate ion as would be expected if the phosphate merely replaced the sulphate, and the positive density could be interpreted as bringing the main chain of residue Thr 172 close enough to the active site to interact with one of the phosphate oxygens. As judged from the low-resolution difference

map this involves a movement of Thr 172 through a distance of about 8 Å, which is clearly a large and interesting conformational change to be brought about by the exchange of sulphate for phosphate.

The absence of changes in subunit 2 may be explained by the different intermolecular contacts involving this subunit. Loop movement in subunit 2 is hindered by a strong interaction between residues 169–176 and a neighbouring molecule. Presumably this contact prevents the loop from moving when phosphate binds.

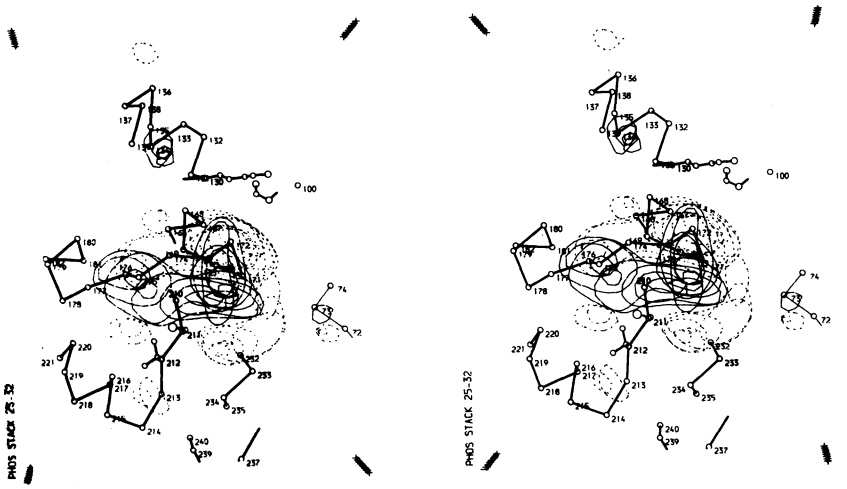

FIGURE 4. Sections $Z = 25/40$ to $32/40$ of the electron-density map at 6 Å resolution, showing the difference between chicken TIM crystals in 3 M ammonium phosphate and similar crystals in 3 M ammonium sulphate. The atomic positions are as determined in the analysis of the chicken TIM structure at 2.5 Å resolution (Banner *et al.* 1976).

SUBSTRATE BINDING TO CHICKEN TIM

As mentioned earlier, TIM is a favourable enzyme for direct crystallographic observation of a productive enzyme–substrate complex. Diffusion of substrate into crystals of a hydrolytic enzyme would give two products that would simply diffuse away. Single substrate – single product reactions can be adjusted by choosing a suitably high substrate concentration so that the lowest-energy species present at equilibrium is the enzyme–substrate complex. Substrate diffusion into TIM crystals will yield product, but product is the substrate for the back reaction and the system will settle to equilibrium.

The complex formed when DHAP is diffused into chicken TIM crystals has been studied at 6 Å resolution (Rivers 1977; Phillips *et al.* 1977). Crystals of chicken TIM were soaked in 200 mM DHAP in 3.05 M $(NH_4)_2SO_4$ for 24 h. Both the soaking and data collection were carried out at 4 °C to minimize decomposition of the substrate to inorganic phosphate and methyl glyoxal. Increased crystal disorder restricted data collection to 6 Å resolution. The differences electron density map between (TIM + DHAP) and the native enzyme showed extensive conformational changes in both subunits 1 and 2, but only in subunit 1 was new density observed in the active site, indicative of substrate binding. There was also a paired negative–positive feature corresponding to the same movement of the 169–176 loop observed in the phosphate–sulphate difference map, but, again, this feature occurred only in subunit 1.

The new density in the active site of this subunit (figure 5) extended from the phosphate/sulphate position into the pocket towards Glu 165 and His 95, and was consistent in extent with the triose moieties of the substrates in fully extended conformations (figure 1).

Substrate binding to yeast TIM

To determine the effects of crystal packing on the observed conformational changes, and in the hope that higher-resolution data on the substrate complex could be obtained, substrate binding studies are being performed on yeast TIM crystals (Alber & Petsko, unpublished).

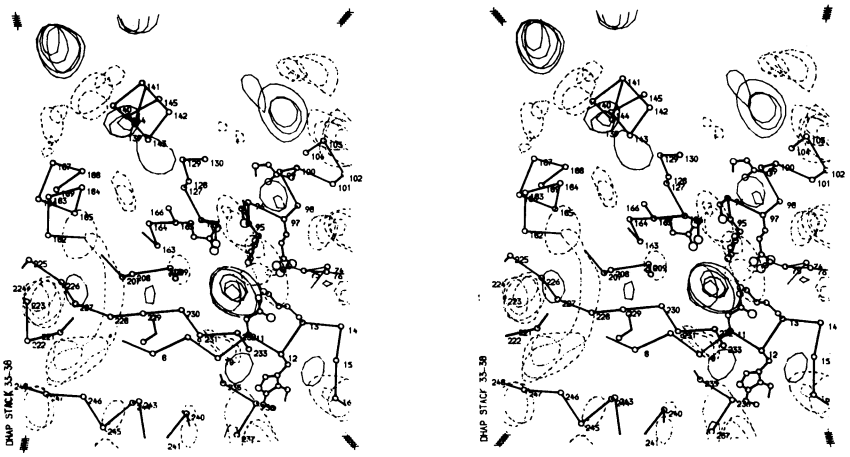

Figure 5. Sections $Z = 33/40$ to $38/40$ of the electron-density map at 6 Å resolution showing the difference between chicken TIM crystals soaked in a 200 mM solution of DHAP and the native crystals. The atomic positions are as determined in the analysis of the chicken TIM structure at 2.5 Å resolution (Banner *et al.* 1976).

These crystals do not require divalent anions in their mother liquor, so the DHAP concentration needed to saturate the enzyme was much lower than it was in the crystals of chicken TIM. To minimize the effect of the phosphate elimination reaction on the experiment, fresh DHAP solution at 3 mM concentration was continuously flowed over the crystal at -5 °C during the entire period of data collection. The flow rate was set at 0.5–1.0 ml/day, and the diffraction pattern was monitored to determine when saturation occurred. After 1 h 20 min of substrate flow, the crystal underwent an abrupt change in mosaic spread; the full width of reflexions, measured on the ω-circle at 4 Å resolution, increased anisotropically from 0.5 to 1.5–2.0°. Intensity measurements were therefore made by scanning over the entire reflexion

Figure 6. Difference map showing the binding of substrate in the active site of yeast TIM. The density added on formation of the enzyme–substrate complex is shown in solid contours; electron density present in the native enzyme but absent in the enzyme–substrate complex is shown in broken contours. The coordinates of the chicken enzyme were rotated into the yeast TIM unit cell and are shown superimposed on the difference map.

(*a*) The peak on the left-hand side of the largest positive feature can be interpreted in terms of substrate, which extends into the plane of the sections and is bound by the loop of residues 168–177 represented by the density on the right of the feature. Parts of the side chains of His 95 and Glu 165 are shown below the substrate electron density to the left and right, respectively.

(*b*) 7 Å further into the difference map, the large negative feature (top right) corresponds to the average position of the loop 168–177 in the native structure. The loop has moved from this position to that indicated in (*a*) above.

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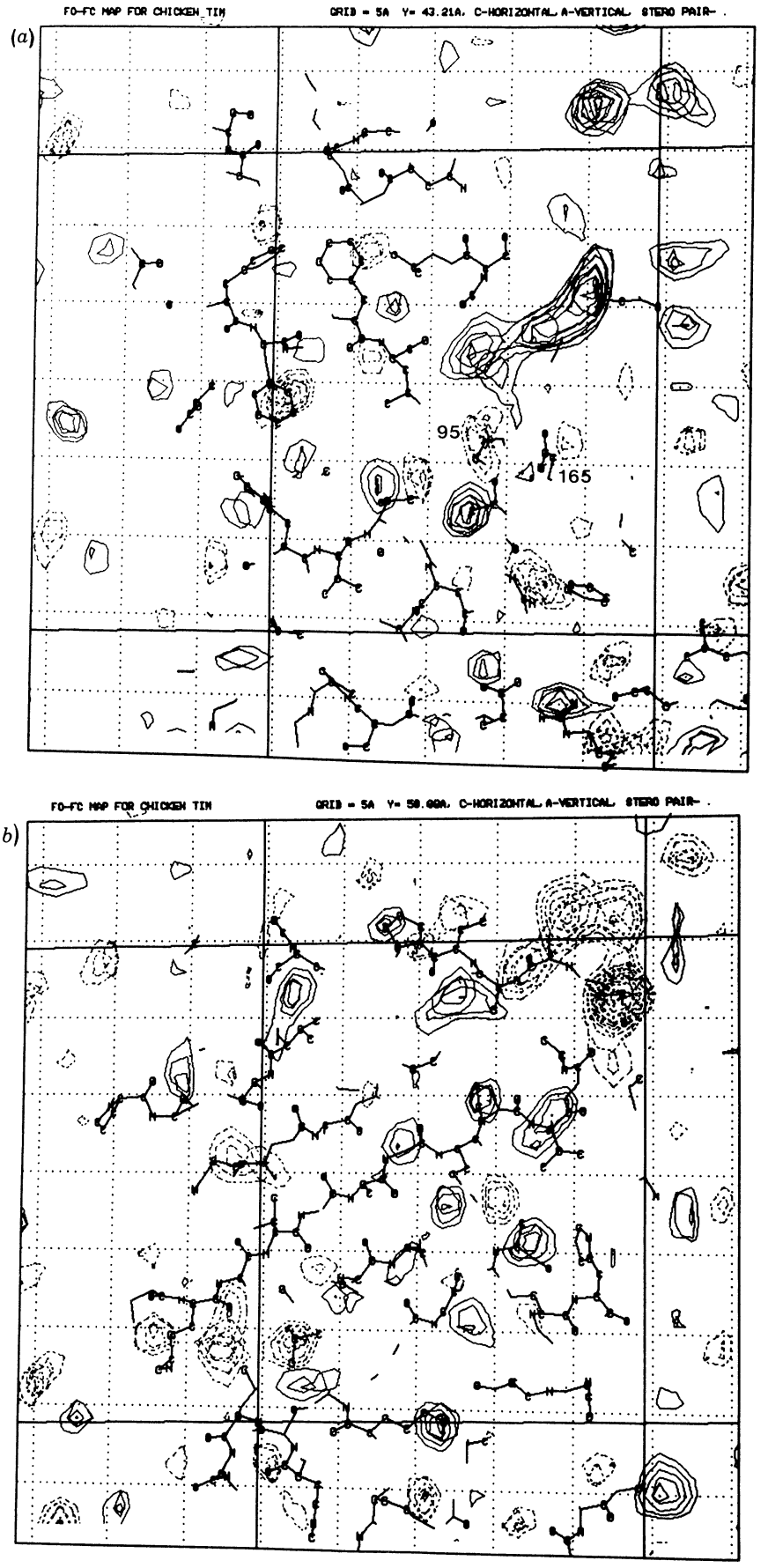

FIGURE 6. For legend see opposite.

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profile and measuring individual backgrounds on either side of the peak. Data were measured to 3.5 Å resolution and a difference map was calculated between this structure and that of the native enzyme. From this map (figure 6) it was immediately obvious that conformational changes similar to those seen in chicken TIM on binding of DHAP occur in the yeast crystals, but in *both* subunits in the dimer. In particular, the movement of residues 168–177 was seen in both, as was extended positive density in the active site pocket. At 3.5 Å resolution the differences on substrate binding are somewhat easier to interpret than at 6 Å, and they confirm what was observed in the chicken TIM experiments. In these crystals the main chain of Thr 172 has moved by 10.4 Å from its position in the native structure to a position consistent with a direct interaction with one of the phosphate oxygen atoms in the substrate. The new electron density representing the substrate is itself clear, with stronger density at the phosphate position, and it confirms the substrate position observed in subunit 1 of the chicken TIM molecule. Detailed analysis of the yeast TIM maps is still in progress, but we can conclude at this stage that substrate binds in the same way to yeast and to chicken TIM though only one subunit is available for substrate binding in the chicken TIM crystals described here.

The enzyme–substrate complex and the catalytic mechanism

At 6 Å resolution the electron density representing bound substrate is not well enough resolved to make possible an unambiguous determination of the position, conformation and orientation of the substrate. However, some progress is possible when other evidence is taken into account.

The absence of any feature at the sulphate position in the chicken TIM–DHAP difference map suggests that this position is occupied by the phosphate group in the substrate; this conclusion is supported by the evidence from yeast TIM–DHAP. Accordingly, one point in the substrate can be fixed with some confidence and this appears to depend for its location principally upon an interaction with a helix dipole, as noted above.

The electron density representing the triose part of the substrate is roughly cylindrical and extends from the sulphate/phosphate position into the active site pocket towards His 95 and Glu 165. The form of this electron density suggests that DHAP is bound in a fully extended conformation; this is in accord with expectation in two respects. First, in the light of the proposal (Rieder & Rose 1959) that the catalysed reaction involves a *cis*-enediol intermediate, it might be expected that DHAP would bind to the enzyme in a cisoid conformation favouring the subsequent formation of the *cis*-enediol. Secondly, as was suggested to us first by Dr A. Eschenmoser, the binding of the substrate to the enzyme would be expected to disfavour unwanted side reactions, in this case the decomposition of DHAP by the elimination of inorganic phosphate. Stereoelectronic considerations (cf. Deslongchamps *et al.* 1972) suggest that this object would be achieved if DHAP were bound with oxygen atom O_3 in the plane of the developing ene-diol. Accordingly, a model of DHAP in the extended conformation shown in figure 1 has been located in the difference electron density as shown in figure 3. This is not, of course, the minimum-energy conformation of DHAP in isolation, and its choice certainly reflects a prejudice in favour of the idea (Pauling 1946) that an enzyme binds substrate in a conformation related to that of the transition state of the catalysed reaction. If this mode of binding is substantiated by more detailed studies, it will represent in itself an important part of the catalytic mechanism.

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Even if this conformation of DHAP is that adopted in the complex with the enzyme, the present low-resolution X-ray result does not provide unambiguous evidence for the orientation of this substrate in the active site of the enzyme. Rotation of the DHAP model about its length (roughly the line joining the C_1 carbon and phosphorus atoms) has only a small effect on the fit to the electron density. The orientation shown in figure 3. therefore, takes into account the proposal that the carboxylate side chain of Glu 165 provides the base responsible for abstracting the pro-*R* proton from the C_1 carbon atom of DHAP and inserting a proton at C_2 in the formation of D-GAP.

At the present stage of the analysis, in keeping with our earlier expectations about the nature of the predominant enzyme–substrate complex, relatively little consideration has been given to the binding of D-GAP to the enzyme. It is clear, however, that a model of D-GAP in the conformation shown in figure 1 fits the low-resolution electron density tolerably well with its phosphate group close to the sulphate position but with the C_2 atom moved closer to Glu 165 so that the best line through the molecule lies close to the axis of the roughly cylindrical difference density.

Given that the model of the enzyme–DHAP complex shown in figure 3 was constructed in the way described above, it is interesting to note what other interactions between enzyme and substrate emerge. Before they are detailed, however, a further qualification must be emphasized. The electron-density map showing the difference between the enzyme–DHAP complex and the native enzyme structure at 6 Å resolution (figure 5) shows significant features at the positions of amino acid side chains in the active site. At this low resolution these features in the difference map cannot be interpreted in detail, but they warn against any assumption that the positions of such side chains are the same in the complex and in the native structure of the chicken enzyme. The structure of the active site in the native enzyme is the natural starting point for discussion of the present model of the enzyme–substrate complex, but the likelihood of conformational changes on formation of the complex, and during the catalysed reaction, must be kept in mind.

The model of the enzyme–DHAP complex built from the 6 Å difference map is shown in figure 3. It suggests a number of interesting interactions between the substrate and the enzyme. The most striking are: (1) a close contact (*ca.* 2.9 Å) between the ε-amino group of Lys 13 and the carbonyl oxygen atom O_2; (2) a slightly longer contact (*ca.* 3.3 Å) between this ε-amino group and the oxygen atom O_3; (3) a good contact (*ca.* 3.2 Å) between the N_ϵ atom of His 95 and the O_1 oxygen atom; and (4) a contact (*ca.* 3.5 Å) between this imidazole nitrogen and the O_2 oxygen. How may these interactions be involved in the catalytic mechanism?

It is attractive to suppose that, in the reaction proceeding from DHAP, the abstraction of the pro-*R* proton from the C_1 carbon atom by Glu 165 is facilitated by polarization of the O_2 carbonyl oxygen by Lys 13 and/or His 95. A general acid–base mechanism would involve Lys 13 (or His 95) donating a proton to the carbonyl oxygen, but the native structure observed in the chicken TIM crystals does not favour this idea. The amino group of Lys 13 seems unlikely to lose a proton because that would leave Glu 97 with a buried and uncompensated negative charge: a highly unfavourable state. Similarly, the imidazole ring of His 95 is at the amino end of the D_1 helix, hydrogen bonded to the main chain NH of Glu 97 through its N_δ and lying at the positive end of the helix dipole. In this situation the ε-NH of His 95 might be expected to carry a larger partial positive charge than usual – that is, to have somewhat enhanced electrophilicity – but it could not easily act as a proton donor without a reorientation that would

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allow a compensating protonation at N_8. The most straightforward suggestion at present, therefore, is that Lys 13 and His 95 act as electrophiles. Furthermore the current model indicates, albeit weakly, that Lys 13 may play the dominant part in polarizing the carbonyl group of O_2 while His 95 acts principally to polarize the O_1 oxygen in the reaction proceeding from D-GAP. The necessity to promote partial negative charges on the C_1 carbon atom of DHAP and the C_2 carbon atom of D-GAP, in this view of the formation of the two transition states, underlines the fact, however, that a proper understanding of the enzyme requires a much more detailed analysis of the enzyme–substrate interactions, based upon firmer structural data.

The results presented by Rose (this symposium) show that the two transition states formed from DHAP and D-GAP collapse to a common *cis*-enediol intermediate, which requires the protonation of the O_1 and O_2 oxygen atoms. If the arguments about the properties of Lys 13 and His 95 that have been given in outline above have led to the correct conclusions, identification of the acids needed to protonate the substrate oxygens is not obvious from the present structural evidence. Water may be involved, however, either directly or in supplying protons to Lys 13 or His 95 to replace those transferred by one or other of these residues. Conformational changes and the interaction between His 95 and Ser 96 may also play a part.

Clearly, further evidence is needed, some of which may be provided by the structural studies now in progress. Among other things, we need to know what conformations are accessible to His 95 and which of them are important in catalysis; to what extent His 95, Lys 13 and the substrate oxygen atoms O_1 and O_2 in the bound complex are accessible to solvent; whether there is any evidence for direct participation by water as a general acid; whether Lys 13 and His 95 (and other residues) change their conformations and relative positions as the reaction proceeds from DHAP through the intermediate to D-GAP (with its necessarily different shape); and the significance of the complex network of interactions linking Lys 13, Glu 97, His 95, Ser 96 and Glu 165.

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