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Conventional X-ray diffraction approaches to the study of enzyme mechanism: serine proteinases, aminoacyl-tRNA synthetases and xylose isomerase

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Techniques that have been used to study enzyme mechanism by conventional steady-state crystallographic techniques are reviewed. Substrates and substrate analogues can often be diffused into crystals, but occasionally co-crystallization is necessary. The poor solubility of substrates and inhibitors may pose a problem. Even if a substrate is present at adequate concentration, it may not be observed by X-ray diffraction. To observe a substrate, special measures may be needed to stop enzyme action, but sometimes this is not necessary because an equilibrium is established. Inhibitors may usefully model a particular reaction state, but one must always question whether the inhibitor provides a correct model. Stabilization of a transition state is often discussed, but rarely achieved. Where practicable, protein engineering can provide a powerful tool to test proposals about the catalytic mechanism. Molecular mechanics calculations can also be useful. These themes are developed in relation to enzymes studied in the authors' laboratory. Many of the same problems are encountered in the application of time-resolved techniques to the study of enzyme mechanism.

1. Introduction

The Laue technique provides a method to follow the time course of structural change in a crystalline material, which might be used to visualize the progress of an enzyme-catalysed reaction. Other papers in this volume consider the technical difficulties of this approach, and means of surmounting them. The aim of the present paper is to consider other crystallographic methods that have been used for structural analysis of enzyme action, their strengths and weaknesses, and the extent to which Laue techniques might supplement them. It is concerned purely with structural approaches, and does not include any review of the methods used by chemists to 'prove' the mechanism of a chemical reaction by isotopic transfers, kinetic isotope effects, stopped and quenched-flow techniques, etc. Other relevant kinetic spectroscopic techniques are considered elsewhere in this volume.

It is a fundamental limitation of diffraction techniques that they depend on an ensemble of diffracting objects. Normally the image is an average over space and time, covering the size of the diffracting object and the length of the observation

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period. Useful images are only obtained if the structure is approximately static over the time of observation, which until now has necessarily been long in comparison with the lifetimes of intermediate states of an enzyme-catalysed reaction.

Therefore it is necessary to use methods that bring a majority of the diffracting units to the same chemical state, and for this chemical state to be stable over the observation period. In reviewing the techniques used to achieve this, examples will be drawn from structural studies relevant to the mechanisms of action of some classes of enzymes studied by the authors, namely serine proteinases, aminoacyl-tRNA synthetases and xylose isomerase.

2. Chymotrypsin and the serine proteinases

The hydrolytic substrates of chymotrypsin are amides and esters, but isotopic studies show that for the free acid, the enzyme catalyses hydroxyl exchange with the solvent. Such a free acid derivative of a good substrate is known as a pseudo-substrate, because it is continuously taken through the cycle of the enzyme-catalysed reaction, with no net chemical effect. In the case of chymotrypsin it was possible to realise this simplest case of a static enzyme–substrate complex. In order to saturate the active sites of a crystal, it is necessary that the concentration of pseudo-substrate exceed its dissociation constant from the enzyme K_S.

The best chymotrypsin substrates have a tyrosine residue at P1 (nomenclature of Schechter & Berger 1967), but free tyrosine is an unsuitable substrate, not only because of its low solubility, but also because it has a free α-amino group. The classic substrate of kinetic studies is N-acetyl-tyrosine ethyl ester, but the corresponding free acid would not bind to enzyme crystals.

We conjectured that this was due to overcrowding between the pairs of closely approximated active sites in the crystal, and hoped to avoid the insolubility problems of tyrosine by using tryptophan instead. Crystals soaked with N-formyl-L-tryptophan provided a clear difference electron-density map, giving experimental electron density for a pseudo-substrate bound to a crystal, and also showing movements at the ends of chains due to the additional crowding (Steitz *et al.* 1969).

It is worth recalling that when this experiment was done there were no adequate refinement procedures which allowed the determination of calculated phases, so the difference maps were based on phases obtained from isomorphous replacement. A direct difference map was successful because of the low level of structural change in the enzyme, when the pseudo-substrate was bound.

Crystallographic data for an enzyme–substrate complex may sometimes be obtained by creating conditions where the turnover of the complex is slow compared to the observation time. In the early 1970s, using precession photography and a rotating-anode X-ray source, the minimum observation time was several hours. In chymotrypsin, two simple methods of reducing the rate are available: to lower the pH, or to lower the temperature. Primitive attempts in our laboratory to stabilize an acyl-enzyme by these methods failed in 1972. Fink (1976) carried out a careful investigation of the required conditions, setting up the conditions for a crystallographic analysis of the acyl–enzyme intermediate formed by an elastase substrate (Alber *et al.* 1976).

It is always possible to criticise the results of such an experiment. Because of the altered conditions, the predominant form in the crystal may be a non-productive complex, not on the direct reaction pathway, and perhaps a misleading artefact.

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An approach which offers an even more risky line of interpretation is to use a substrate analogue which chemically blocks the normal series of reaction steps at a specific point. For chymotrypsin a stable acyl-enzyme for crystallographic study was obtained by using indoleacryloyl imidazole as a substrate. Henderson (1970) provided a persuasive theory for the inability of this acylenzyme to de-acylate, providing by implication further insight into the stereochemistry of the 'true' deacylation step. Since the substrate is clearly different from a real substrate, any conclusion about the mechanism of the normal action of the enzyme must be hypothetical. In the context of this symposium, it is relevant that studies using 'caged' reagents are inevitably subject to similar criticism.

Another approach to detailed analysis of enzyme action is to study the effects of small chemical changes to the enzyme. In the case of chymotrypsin, the first candidate for such study was methyl-His57 chymotrypsin (Nakagawa & Bender 1970). The reactivity of methyl-chymotrypsin is reduced by 4–5 orders of magnitude (Henderson 1971), but the presence of the bulky methyl group causes a major realignment of the histidine side-chain, emphasizing the flexibility of this side chain, but confounding any attempt to contribute a quantitative study of its role (Wright *et al.* 1972).

A more detailed picture of the binding of a polypeptide substrate to trypsin and chymotrypsin was obtained by studies of complexes with peptide inhibitors. This was done first by a successful model-building study (Blow *et al.* 1972), and then by crystallographic studies both in Huber's group (Rühlmann *et al.* 1973) and by ourselves (Sweet *et al.* 1974) on complexes of trypsin with basic pancreatic trypsin inhibitor (Kunitz) and soybean trypsin inhibitor (Kunitz). These inhibitors function by binding so tightly to the active site of the enzyme that they make the enzyme unavailable for any reaction. The inhibitors are believed to bind exactly like a good substrate. In the case of soybean trypsin inhibitor, the inhibitor when released is predominantly in a form which has been hydrolytically cleaved. The crystal structures of these inhibitor complexes probably provide the most precise model for the binding of a substrate to the enzyme in a productive mode. There has been a long controversy about the chemical nature of the stabilized enzyme–inhibitor complex which is observed in crystals. The very close packing of the substrate against the active site led to initial claims that the inhibitor was covalently bound to the substrate as a tetrahedral intermediate. Huber's group were able to study a number of complexes which diffracted to high resolution, and could be carefully refined without assumptions about the chemical state of the bonds at the active site. These showed the situation to be that of a 'crowded van der Waals' interaction, in which the bond length (2.7 Å†) between the inhibitor carbonyl carbon and O^{γ}Ser195 is too long for a covalent bond, but too short for a normal non-bonded contact (Marquart *et al.* 1983; Huber & Bode 1991), as originally hypothesized by Fersht *et al.* (1973).

Marquart *et al.* (1983) also found a trypsin ligand which forms a genuine tetrahedral complex in crystals, with an unexpected geometry in which $N^{\epsilon 2}$His57 forms a bifurcated hydrogen bond to the serine oxygen and the carbonyl oxygen.

Huber's group also showed that formation of an enzyme-substrate complex or an enzyme-inhibitor complex is a potent means to stabilize the enzyme in its active conformation, even when the enzyme is chemically in its zymogen form, or when it has lost the α-amino group which stabilizes the active conformation.

† 1 Å = 10^{-10} m = 10^{-1} nm.

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Table 1. *Crystallographic studies on complexes in serine proteinases*

(Features of enzyme activity relevant to structural studies on mechanism: (*a*) hydrolytic (i.e. one substrate + water); (*b*) enzyme functions as rigid framework; (*c*) electron and proton transfers at active site, small movements of atoms.)

type of complex	example	reference
pseudo-substrate	formyl-L-tryptophan: chymotrypsin	Steitz *et al.* (1969)
true substrate, slow reaction	N-carbobenzoxy-L-alanyl-*p*-nitrophenyl ester: elastase low pH, low temperature	Alber *et al.* (1976)
stable intermediate	indole-acryloyl-chymotrypsin	Henderson (1970)
inhibitor complex, similar to true substrate complex	BPTI (Kunitz), STI (Kunitz) not tetrahedral intermediate 'compressed v.d.W. complex'	Rühlmann *et al.* (1973) Sweet *et al.* (1974)
tetrahedral adduct	amidinophenyl pyruvate: trypsin	Marquart *et al.* (1983)
chemically modified enzyme	methyl (His57) chymotrypsin	Wright *et al.* (1972)

The early crystallographic work on substrate and inhibitor binding to the serine proteases (table 1), was able to suggest the mode of binding a true substrate, as well as giving strong hints about the chemical and physical roles of the various components of the active site in promoting catalysis. Many of the simple complexes could be prepared by simple soaking of crystals, but others such as the complexes with protein inhibitors required co-crystallization. In much of this work, the conformation of the enzyme active site could be usefully considered as essentially rigid (though the close approach to an adjacent molecule of the chain termini which separate the B and C chains in α-chymotrypsin crystals caused these chain-ends to be displaced in the substrate complexes). Later work, particularly that which concentrated on the relation between the enzyme and its zymogen, emphasized the importance of conformational change and correct orientation in creating an effective enzyme–substrate interaction.

3. Tyrosyl-tRNA synthetase and other aminoacyl-tRNA synthetases

The serine proteinases have two substrates (amide or ester and water), but water is so ubiquitous that crystallographic studies without it have not so far been possible. New factors arise in crystallographic analysis when studies can be made with only some of the substrates present. Crystallographic studies exploiting this effect were first carried out on lactate dehydrogenase, whose binary complex (LDH : NAD) and an abortive ternary complex (LDH : NAD : lactate) could be isolated crystallographically (Adams *et al.* 1969; Leberman *et al.* 1969).

Tyrosyl-tRNA synthetase (TyrRS) has three substrates: tyrosine, ATP and the specific transfer RNA for tyrosine ($tRNA^{Tyr}$), but no crystalline complexes have been made which include tRNA. Subsequent work on other aminoacyl-tRNA synthetases (aaRS) have filled this gap, first by the crystallographic work of Rould *et al.* (1989) on GlnRS : $tRNA^{Gln}$ (table 2). In the work on TyrRS it proved easy to prepare an enzyme : amino acid complex (TyrRS : Tyr), and there seems little doubt that this complex represents the mode of binding tyrosine in the normal enzyme-catalysed reaction. There is, however, a curious inconsistency. Kinetic analysis demonstrates clearly that in solution the dimeric TyrRS molecule binds only one tyrosine molecule, while crystallographic studies invariably show enzyme with tyrosine fully bound at both sites of the dimer (Monteilhet & Blow 1978).

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Table 2. *Complexes of aminoacyl-tRNA synthetases used for structural study*

enzyme and one substrate		
enzyme–amino acid	TyrRS–Tyr	Monteihet & Blow (1978)
enzyme–tRNA	GlnRS–$tRNA^{Gln}$	Rould *et al.* (1989)
enzyme–ATP	MetRS–ATP	Brunie *et al.* (1990)
enzyme–intermediate using two substrates		
enzyme–aminoacyladenylate	TyrRS–TyrAMP	Monteilhet & Blow (1978)

The various aaRS always follow a specific order of reaction (not always the same), which means that a stable intermediate can be made by incubating the enzyme with one or two substrates. Incubation of TyrRS with the two substrates, tyrosine and ATP, readily produces tyrosyl adenylate and this product is quite stable. The crystalline enzyme–tyrosyl-adenylate complex is readily prepared, and seems to represent the correct mode of binding for the normal reaction pathway. In crystals, both active sites of the dimer are found to contain tyrosyl adenylate at full occupancy (Monteilhet & Blow 1978).

A more stable analogue of the tyrosyl adenylate intermediate is provided by tyrosinyl adenylate. The tyrosinyl moiety cannot be transferred to $tRNA^{Tyr}$ by the enzyme, and is very resistant to hydrolysis. This substrate analogue produces electron density which is almost indistinguishable from the tyrosyl adenylate complex, so it is an example of an enzyme–substrate-analogue complex, whose structure closely mimics the role of a true substrate.

Attempts to visualize the localization of ATP in TyrRS crystals, before formation of the adenylate, have totally failed. Incubation of enzyme crystals with ATP alone causes it to bind in a disordered manner in the tyrosine-binding site, which is certainly not a productive binding mode; incubation with ATP and tyrosine analogues such as tyrosinol or tyramine (at concentrations far in excess of K_M) shows no density for bound ATP. Although model-binding exercises suggested a favourable mode of binding for ATP (Leatherbarrow *et al.* 1985), the conclusion was drawn that ATP does not become tightly localized until a chemical bond to tyrosine begins to develop (Brick *et al.* 1989). This conclusion could perhaps be directly tested in a Laue experiment using a caged ATP analogue, if crystals which diffract strongly enough were available.

There are important differences between the various aaRS in this respect. A crystalline MetRS:ATP complex was prepared showing clear ATP density (Brunie *et al.* 1990), but attempts to prepare crystals of a MetRS–methionine complex have not succeeded.

In the 1980s it became possible to study the effects of chemical alterations to an enzyme by direct manipulation of the corresponding gene. TyrRS became the first enzyme used for protein engineering experiments (Wilkinson *et al.* 1983), and provided many new variants for kinetic and crystallographic study (Fersht *et al.* 1984). It was possible to demonstrate crystallographically, in many cases, that such variants fold indistinguishably from the wild-type (Brown *et al.* 1986). In these cases, it might be reasonable to consider the engineered variant of the enzyme to be the original enzyme with a few atoms removed or added. The relatively large proportion of variants with unaltered structure may be due to the use of extremely conservative

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mutants, but not all fall into this category. In particular, attempts to change Asp176, at the bottom of the tyrosine binding pocket, to another amino acid, led to no expression of the enzyme.

A great deal of effort was devoted to mutants of Thr51 which is close to the adenine ring of the tyrosyl adenylate enzyme. Several variants, with significantly altered activity, exhibited no structural change to the main chain (Brown *et al.* 1987; Brown *et al.* 1992), while the insertion of cysteine at this point permitted formation of a sulphur bridge to cysteine 35 which contacts the ribose of the adenylate (J. Fothergill, personal communication). An interesting case was the Gly51 variant, which caused only small structural changes to the enzyme, but destabilized the bend and helix adjacent to position 51, leading to severe disorder in this part of the molecule. Here is another example where conformational rigidity or flexibility have important roles in enzyme function.

4. Xylose isomerase

The final enzyme to be discussed, xylose isomerase, has provided us with a more detailed crystallographic study of a reaction pathway than any of the others. This is partly because the different steps of the reaction require significant conformation changes in both enzyme and substrate. Another important factor is that the equilibrium catalysed by this enzyme is one in which the substrate and product are in equilibrium at comparable concentrations. A further feature, which proved very helpful in analysis of the reaction pathway, is that this enzyme is not a Michaelis enzyme: that is to say, the initial enzyme–substrate complex is not the lowest point on the free energy profile of the reaction pathway. The following features of the enzyme activity relevant to structural studies on mechanism are:

(*a*) metallo-enzyme: two cation-binding sites;

(*b*) equilibrium between substrate and product at approximately equal concentrations:

(*c*) conformationally variable substrate;

(*d*) significant enzyme conformation changes during reaction;

(*e*) non-Michaelis enzyme: stable intermediate form;

(*f*) possibility to stabilize transition-state analogue?

The conformational variants of substrate and product are: closed-ring pyranose; open-chain; and closed-ring furanose. The conditions affecting enzyme conformation are: pH; temperature; bound cations; and state of bound substrate.

This enzyme catalyses the isomerization between aldose sugars and the corresponding ketose. The natural substrate of the enzyme is the pentose D-xylose, which is isomerized to D-xylulose, but many bacterial enzymes, particularly from *Actinomycetes*, work efficiently on hexose sugars. They are used industrially to convert the glucose in corn syrup to its sweeter ketose isomer, fructose. The equilibria between the aldose and ketose forms are reached under normal conditions with similar concentrations of each isomer: in the industrial process only about 60% of glucose is converted to fructose.

The activity and stability of the enzyme depends on the presence of divalent cations at the active site. Co^{2+} is the most effective cation, but in food applications Mg^{2+} is usually used. Ca^{2+} acts as an inhibitor.

X-ray crystallography (Carrell *et al.* 1984) shows the enzyme to be based on the eight stranded α/β structure first observed in triose-phosphate isomerase. The

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molecule has a long carboxyl-terminal extension on the α/β barrel, and associates into tetramers in which the extensions on one pair of monomers embrace the other pair. The tightly associating pair of monomer units associate with their α/β barrels almost coaxial, and with the carboxyl-terminal ends of the β strands facing each other. The active site lies at this end of the barrel axis, so that the tight dimer brings two active sites close together. In fact Phe25 from one subunit forms an important component of the active site facing it on another subunit.

The most abundant forms of xylose, xylulose, glucose and fructose in solution are closed-ring forms. The most abundant forms of the aldose sugars contain a six-membered pyranose ring, while the ketoses contain predominantly the five-membered furanose ring.

Our first crystal-structure determination for the *Arthrobacter* enzyme was of crystals containing the polyol inhibitor, xylitol. The structure is extremely similar to that previously determined for the *Streptomyces* enzyme, showing an octahedrally-coordinated Mg^{2+} at the active site. Two xylitol hydroxyls, O2 and O4, coordinate Mg^{2+}, which also coordinates four carboxylate groups on the enzyme. The xylitol molecule is in an extended conformation, which was also found when the six-carbon polyol inhibitor, sorbitol, was introduced into crystals (Henrick *et al.* 1989).

When crystals are incubated with an equilibrium mixture of xylose and xylulose, in the presence of either Mg^{2+} or Mn^{2+}, the electron density again shows the sugar to be in an extended chain form, with O2 and O4 coordinated to the cation site. These experiments were done at pH 8, and under these conditions Mn^{2+} is also bound at a second site [2] where it is octahedrally coordinated to four carboxylate oxygens, a histidine, and a water molecule. The cation at site [2] is not coordinated to the substrate, but is in indirect contact with oxygens O1 and O2 through this water molecule (Collyer *et al.* 1990).

Since this complex is observed under equilibrium conditions where the enzyme is active and saturated with substrate and product, it suggests that this is the lowest energy complex under these conditions. The resolution of our study (2.5 Å) does not permit us to distinguish whether the bound ligand is xylose or xylulose. Whitlow *et al.* (1991), working with crystals of the *Streptomyces rubiginosus* enzyme which diffract to considerably better resolution, observed density indicative of the ketose form (though the type of cation was not controlled in their experiment). There is some evidence (from our own work and from H. L. Carrell, personal communication) that the sugar conformation depends on the precise conditions of cation, pH and temperature. It might well be rewarding to do Laue experiments under these conditions, using temperature jump or perhaps pH jump to trigger a change of conformational equilibrium.

The rate of reaction of the enzyme is faster than the spontaneous ring-opening rate, leading to the conclusion that the enzyme catalyses ring-opening of the aldose form. To test this, we studied two sugar analogues which have a more stable pyranose-like ring. This was achieved by replacing the ring oxygen by sulphur (5-thio-α-D-glucose) (Collyer & Blow 1990) or by nitrogen (nojirimycin, 5-imino-α-D-glucose) (Collyer *et al.* 1992). These showed density for a cyclised molecule, implying a position for the ring oxygen within hydrogen bonding distance of His53 $N^{\epsilon 2}$.

This histidine is polarized by a partly buried aspartate (Asp 56), indicating a mechanism for ring opening which uses the polarized histidine to assist proton transfer from O1 to O5. In these inhibitor complexes, Mn^{2+} was present as a cation, and was bound at both sites. Site [1] coordinates O3 and O4, while site [2] coordinates

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a water molecule as usual, this water molecule being also hydrogen-bonded to O3 (Collyer *et al.* 1990).

The type of difference map used for the interpretation of chymotrypsin would be quite useless in this case. Although the xylose isomerase tetramer remains similar in the different crystal forms, it rotates and translates about a crystallographic 2-fold axis which runs through the centre of the tetramer. The difference maps which we use here are phased on the refined structure, without any sugar. There must be a range of conformations accessible to the sugar ligands, and several other complexes we have studied cannot be clearly interpreted, showing evidence of several different binding modes.

The conformation change required for the transition to the open-chain form of the bound sugar requires the removal of O3 from cation site [1], while O2 approaches it. The water molecule adjacent to site [2] remains in place. It loses its hydrogen bond to O3, but forms hydrogen bonds to O1 and O2. The active site is just large enough to accommodate these changes. Conventional crystallography offers no prospect of observing the pathway of the conformation change, but molecular mechanics calculations can suggest a likely route (Smart *et al.* 1992).

The model for the cyclized sugar substrate is open to the usual objections that the inhibitors are not exactly the same molecule, and it is not proved that xylose or glucose in the α-pyranose form would bind in the same way. A Laue experiment could provide further information if it proved possible to synchronize the change in the crystal.

The mechanism of the isomerization step, has been approached through a stable crystal analogue which mimics the transition state of the isomerization. Collyer followed up experiments of Bungard (1973) on the low activity shown by xylose isomerase in the presence of Al^{3+}. Using Al^{3+} as well as 5 mM Mg^{2+}, with an equilibrium xylose/xylulose mixture, an interestingly different conformation was observed. The cation has left site [2], and is observed at site [2′], about 1.0 Å away, where it is directly ligated to the substrate O1 and O2. As well as the water molecule, two of the carboxylate ligands have been displaced, with significant torsional changes of Glu216 and Asp254. In this position the cation at [2′] is strongly polarizing the C–O1 and C–O2 bonds towards C^{+}–O^{-}. This polarization is assisted at O2 by ligation to the cation at [1], and at O1 by a hydrogen bond from a lysine. This polarization is considered strong enough to drive off the hydroxyl proton, leaving the substrate as a transition state analogue between the aldose and the ketose form. The isomerization step is the transfer of a hydride ion between O1 and O2. The hydride ion is protected from attack by water by several hydrophobic side-chains of the enzyme, notably the aromatic rings of Phe93, Trp136 and Phe25. In this experiment, several pieces of evidence indicate that the cation at site [2′] is Mg^{2+}. In the presence of Al^{3+} without Mg^{2+} sites [2] and [2′] are unoccupied, and no substrate is bound (Collyer *et al.* 1990).

This model is also open to criticism, in that a less active trivalent cation has been used to realize it experimentally. It could therefore be an artefact. This is the only experiment so far reported which shows a cation in direct contact with O1 and O2, which are adjacent to the site of isomerization.

It has proved difficult to model adequately the ring-closure of the ketose to a furanose ring. No satisfactory model analogue has been found, and the arguments about the mechanism of ring closure, and about the probable conformation of furanose bound to the enzyme, are indirect. They will not be pursued here.

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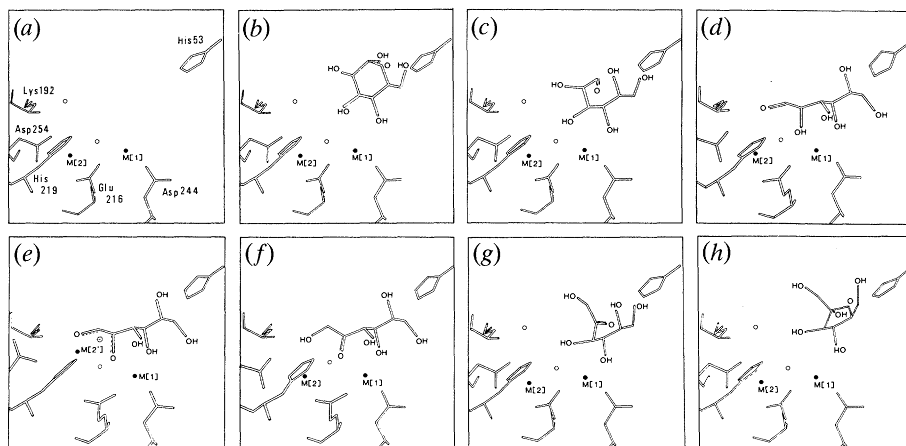

Figure 1. Stepwise description of a proposed sequence of conformational changes at the active site accompanying glucose-fructose conversion in xylose isomerase (modified from Collyer *et al.* 1990). The panels show (*a*) the active site of the enzyme with cations bound at both sites but without substrate; (*b*) binding of glucose as a cyclic α-pyranose; (*c*) open chain glucose in a pseudo-cyclic conformation; (*d*) glucose in an extended-chain conformation; (*e*) proposed transition-state with the cation in site [2] displaced to [2′], accompanied by conformational changes of Glu 216 and Asp 254; (*f*) fructose in an extended-chain conformation; (*g*) open chain fructose in a pseudo-cyclic conformation; (*h*) hypothetical conformation for bound cyclic fructofuranose, which is unlikely to be correct. The structures shown in (*a*), (*b*), (*d*) and (*f*), (*e*), are based on crystal structure refinements (Collyer *et al.* 1990).

Thus in the eight-panel schematic drawing which was used to illustrate the proposed mechanism (Collyer *et al.* 1990) (figure 1), (*h*) has still not been observed, and is probably not correct. But it contains four panels that illustrate structures which have been observed crystallographically: (*a*), (*b*), (*e*) and (*f*). (In fact (*d*) and (*f*) are essentially identical at our resolution.)

This has been achieved by classical, steady-state crystallography. Although it would be thrilling to see the structures of (*c*) and (*g*) by Laue photography, this still appears a remote hope because of the technical difficulty of triggering the appropriate change. We have therefore had to rely on molecular mechanics to simulate these states.

5. Conclusion

X-ray crystallography of enzyme:substrate and enzyme:inhibitor complexes provides an accurate description of a wide variety of stable structures, which help us in defining enzyme mechanism. They demonstrate conformations which are energetically stable, and which are far less hypothetical than structures generated by model-building. They show that overall enzyme conformation is often very invariant to the different steps of enzyme reactions (but there are major exceptions, like adenylate kinase). These conformations can often help us in identifying sensible pathways for enzyme-catalysed processes, but sometimes they give confusing leads. They certainly do not prove that any observed conformation lies on the pathway of a real enzyme process.

They have an advantage over the states which can be observed in time-resolved processes because they do not need to be triggered at a specific time. The disadvantage is that only stable states can be observed: stable, that is to say, on a

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time scale of minutes. Laue methods can observe transient states, but the whole diffracting object must be precisely synchronized, and must remain ordered. Both the conventional crystallographic method and the Laue method are subject to similar criticism, in that the situation inside a crystal is somewhat artificial, and that the particular reactants which are used may not lead to conformations which characterize the 'true' enzymatic reaction. Therefore, great judgment is required in interpreting results; in particular, other types of chemical data may give important information about the relevance of a particular structural model.

References

Adams, M. J., Haas, D. J., Jeffery, B. A., McPherson, A., Mermall, H. L., Rossmann, M. G., Schevitz, R. W. & Wonacott, A. J. 1969 Low resolution study of crystalline lactate dehydrogenase. *J. molec. Biol.* **41**, 159–188.

Alber, T., Petsko, G. A. & Tsernoglou, D. 1976 Crystal structure of elastase-substrate complex at −55 °C. *Nature, Lond.* **262**, 297–300.

Blow, D. M., Wright, C. S., Kukla, D., Rühlmann, A., Steigemann, W. & Huber, R. 1972 A model for the association of bovine pancreatic trypsin inhibitor with chymotrypsin and trypsin. *J. molec. Biol.* **63**, 137–144.

Bode, W. & Huber, R. 1991 Ligand binding: proteinase–protein inhibitor interactions. *Curr. Opin. Struc. Biol.* **1**, 45–52.

Brick, P., Bhat, T. N. & Blow, D. M. 1989 Structure of tyrosyl-tRNA synthetase refined at 2.3 A resolution. Interaction of the enzyme with the tyrosyl adenylate intermediate. *J. molec. Biol.* **208**, 83–98.

Brown, K. A., Vrielink, A. & Blow, D. M. 1986 Crystal structures of two factitious mutants of tyrosyl-tRNA synthetase. *Biochem. Soc. Trans.* **14**, 1228–1229.

Brown, K. A., Brick, P. & Blow, D. M. 1987 Structure of a mutant of tyrosyl-tRNA synthetase with enhanced catalytic properties. *Nature, Lond.* **326**, 416–418.

Brown, K. A., de Meester, P., Fersht, A. R. & Blow, D. M. 1992 Structural analysis of a series of mutants of tyrosyl-tRNA synthetase: enhancement of catalysis by hydrophobic interactions. (Submitted.)

Brunie, S., Zelwer, C. & Risler, J. L. 1990 Crystallographic study at 2.5 A resolution of the interaction of methionyl-tRNA synthetase from *Escherichia coli* with ATP. *J. molec. Biol.* **216**, 411–424.

Carrell, H. L., Rubin, B. H., Hurley, T. J. & Glusker, J. P. 1984 X-ray crystal structure of D-xylose isomerase at 4-A resolution. *J. biol. Chem.* **259**, 3230–3236.

Collyer, C. A. & Blow, D. M. 1990 Observations of reaction intermediates and the mechanism of aldose-ketose conversion by D-xylose isomerase. *Proc. natn. Acad. Sci. U.S.A.* **87**, 1362–1366.

Collyer, C. A., Henrick, K. & Blow, D. M. 1990 Mechanism for aldose-ketose conversion by D-xylose isomerase involving ring-opening by a 1,2-hydride shift. *J. molec. Biol.* **212**, 211–235.

Collyer, C. A., Goldberg, J. D., Viehmann, H., Blow, D. M., Ramsden, N. G., Fleet, G. W. J., Montgomery, F. J. & Grice, P. 1992 Anomeric specificity of D-xylose isomerase. (Submitted.)

Fersht, A. R., Shi, J.-P., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y. & Winter, G. 1984 Analysis of enzyme structure and activity by protein engineering. *Angew. Chem.* **23**, 467–473.

Fink, A. L. 1976 Cryoenzymology of chymotrypsin: the detection of intermediates in the catalysis of a specific anilide substrate. *Biochem.* **15**, 1580–1586.

Henderson, R. 1970 Structure of crystalline α-chymotrypsin. IV. The structure of indoleacryloyl α-chymotrypsin and its relevance to the hydrolytic mechanism of the enzyme. *J. molec. Biol.* **54**, 341–354.

Henderson, R. 1971 Catalytic activity of α-chymotrypsin in which histidine-57 has been methylated. *Biochem. J.* **124**, 13–18.

Henrick, K., Collyer, C. A. & Blow, D. M. 1989 Structures of D-xylose isomerase from *Arthrobacter*

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strain B3278 containing the inhibitors xylitol and D-sorbitol at 2.5 Å and 2.3 Å resolution, respectively. *J. molec. Biol.* **208**, 129–157.

Leatherbarrow, R. J., Fersht, A. R. & Winter, G. 1985 Transition-state stabilization in the mechanism of tyrosyl-tRNA synthetase revealed by protein engineering. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7840–7844.

Leberman, R., Smiley, I. E., Haas, D. J. & Rossmann, M. G. 1969 Crystalline ternary complexes of lactate dehydrogenase. *J. molec. Biol.* **46**, 217–219.

Marquart, M., Walter, J., Deisenhofer, J., Bode, W. & Huber, R. 1983 The geometry of the reactive site of the peptide groups in trypsin, trypsinogen and its complexes with inhibitors. *Acta crystallogr.* **B39**, 480–490.

Monteilhet, C. & Blow, D. M. 1978 Binding of tyrosine, adenosine triphosphate and analogues to crystalline tyrosyl transfer RNA synthetase. *J. molec. Biol.* **122**, 407–417.

Nakagawa, Y. & Bender, M. L. 1970 Methylation of histidine 57 in α-chymotrypsin by methyl-*p*-nitrobenzene sulphonate. A new approach to enzyme modification. *Biochem.* **9**, 259–267.

Rould, M. A., Perona, J. J., Söll, D. & Steitz, T. A. 1989 Structure of *E. coli* glutaminyl-tRNA synthetase complexed with $tRNA^{Gln}$ and ATP at 2.8 Å resolution. *Science, Wash.* **246**, 1135–1142.

Rühlmann, A., Kukla, D., Schwager, P., Bartels, K. & Huber, R. 1973 Structure of the complex formed by bovine trypsin and bovine pancreatic trypsin inhibitor. Crystal structure determination and stereochemistry of the contact region. *J. molec. Biol.* **77**, 417–436.

Schechter, I. & Berger, A. 1967 On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Comm.* **27**, 157–162.

Smart, O., Akins, J. & Blow, D. M. 1992 Molecular mechanisms simulations of a confirmational rearrangement of D-xylose in the active site of D-xylose isomerase. *Proteins* **13**, 100–111.

Steitz, T. A., Henderson, R. & Blow, D. M. 1969 Structure of crystalline α-chymotrypsin. III. Crystallographic studies of substrates and inhibitors bound to the active site of α-chymotrypsin. *J. molec. Biol.* **46**, 337–348.

Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. & Blow, D. M. 1974 Crystal structure of the complex of pork trypsin with soybean trypsin inhibitor (Kunitz) at 2.6-Å resolution. *Biochem.* **13**, 4212–4228.

Whitlow, M., Howard, A. J., Finzel, B. C., Poulos, T. L., Winborne, E. & Gilliland, G. L. 1991 A metal-mediated hydride shift mechanism for xylose isomerase based on the 1.6 Å *Streptomyces rubiginosus* structures with xylitol and D-xylose. *Proteins* **9**, 153–173.

Wilkinson, A. J., Fersht, A. R., Blow, D. M. & Winter, G. 1983 Site-directed mutagenesis as a probe of enzyme structure and catalysis: tyrosyl-tRNA synthetase cysteine-35 to glycine-35 mutation. *Biochem.* **22**, 3581–3586.

Wright, C. S., Hess, G. P. & Blow, D. M. 1972 Structure of crystalline methyl-chymotrypsin. *J. molec. Biol.* **63**, 295–303.

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