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Using neutron protein crystallography to understand enzyme mechanisms

Jenny P. Glusker,[a]* H. L. Carrell,[a] Andrey Y. Kovalevsky,[b] Leif Hanson,[c] S. Zoe Fisher,[b] Marat Mustyakimov,[b] Sax Mason,[d] Trevor Forsyth[d] and Paul Langan[b]*

[a]Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111-2497, USA, [b]Bioscience Division, MS M888, Los Alamos National Laboratory, Los Alamos, NM 87545, USA, [c]Chemistry Department, University of Toledo, Toledo, OH 43606, USA, and [d]Institut Laue–Langevin, 6 Rue Jules Horowitz, BP 156, 38042 Grenoble CEDEX 9, France

Correspondence e-mail: jenny.glusker@fccc.edu, langan_paul@lanl.gov

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A description is given of the results of neutron diffraction studies of the structures of four different metal-ion complexes of deuterated D-xylose isomerase. These represent four stages in the progression of the biochemical catalytic action of this enzyme. Analyses of the structural changes observed between the various three-dimensional structures lead to some insight into the mechanism of action of this enzyme.

1. Introduction

Enzymes catalyze reactions in the body so that the processes of life can proceed. The manner in which this occurs is the subject of many types of research: studies of the rates of enzyme reactions and the effects of isotopic substitution in the substrates and the replacement of side chains in the active site by other side chains, and structural studies using X-ray and, more recently, neutron diffraction techniques. These last two techniques, which utilize the diffraction of short-wavelength radiation by a crystalline enzyme or other macromolecule, give a three-dimensional representation of the arrangement of atoms in the crystal. The precise details of the representation depend on which type of radiation was used and therefore which component of the material in the crystal has scattered the particular radiation. X-rays are scattered by the electron density around atoms and therefore the result of an analysis is an electron-density map; the scattering power of each atom is directly related to its atomic number, *i.e.* how many electrons surround the atomic nucleus. In contrast, neutrons are scattered by atomic nuclei and therefore the locations of atomic nuclei are obtained by diffraction studies. The scattering power of atomic nuclei for neutrons does not depend on their atomic number: for example, deuterium, which like its isotope hydrogen has a minimal power to scatter X-rays, scatters neutrons to the same extent as carbon and oxygen and somewhat less than nitrogen, while hydrogen has a negative scattering factor for neutrons.

The differences between the information obtained from crystal diffraction using these two types of radiation, one more wave-like and the other more particle-like, can be used constructively to obtain additional information on the three-dimensional structure and chemical bonding of molecules in the crystal. X-rays are available as high-flux beams that are well diffracted by a small crystal, but the intensity of the scattered radiation from an atom decreases with increased scattering angle because of the relatively large spatial extent of the atomic electron cloud. Neutrons, in contrast, are available as relatively low-flux beams which require larger crystals. This is a problem that is currently often impossible to

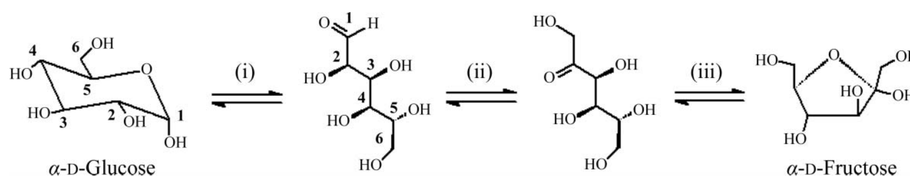

Figure 1
A chemical diagram of the three-stage sugar-interconversion reaction catalyzed by XI, involving (i) ring-opening, (ii) isomerization and (iii) ring-closure steps.

solve, but if an appropriate crystal can be prepared then the intensity of scattered neutrons from a nucleus does not fall off with scattering angle because of its small size. The resolution of the resulting electron-density or nuclear maps will also depend on the quality of the crystal (the extent to which each unit cell has identical contents) and the wavelength of the radiation used [with shorter wavelengths giving better (*i.e.* higher) resolution]. We have studied the enzyme D-xylose isomerase by X-ray diffraction methods for many years (Carrell *et al.*, 1984, 1989). Crystals can be grown that are 1 or 2 mm along each edge. Therefore, we decided to investigate the neutron diffraction of this enzyme because the powerful neutron scattering power of deuterium makes it possible, if the crystalline enzyme is soaked in deuterium oxide (heavy water), to obtain information on the ionization states of the various enzyme side chains. This type of experiment was performed in 1981 by Kossiakoff and Spencer for deuterated bovine serine protease, an enzyme that has a catalytic triad composed of His57, Asp102 and Ser195 (Kossiakoff & Spencer, 1981). They were able to show that in this enzyme His57 is protonated with a deuterium on both ring N atoms, whereas Asp102 is not protonated on either of its carboxylate O atoms. They therefore inferred that the function of Asp102 is to stabilize the doubly protonated state of His57. The enzyme that we are studying, D-xylose isomerase, has two hydrogen-utilizing steps and the catalytic triad just described for the serine proteases is similar to an arrangement in D-xylose isomerase that is utilized in the first of its reaction steps.

The enzyme D-xylose isomerase converts aldo-sugars such as D-xylose and D-glucose to keto-sugars such as D-xylulose and D-fructose. It does this by opening the sugar ring to obtain an open-chain form of the substrate sugar (Fig. 1, step i) and then effecting an isomerization of this substrate by transferring a carbon-bound H atom from one C atom to the adjacent C atom (Fig. 1, step ii). Proposed mechanisms of action have included a *cis*-ene diol intermediate, a hydride shift and a metal-assisted hydride shift. The protein folds as a $(\beta\alpha)_8$ barrel. It binds two divalent metal ions, as shown in Fig. 2, and holds them firmly. Both metal ions bind six O and/or N atoms. One, M1, binds four carboxylate groups (Glu181, Glu217, Asp245 and Asp287) and two water molecules. The other, M2, binds three carboxylate groups (Glu217, Asp257 and bidentate Asp255), one water molecule and a histidine residue (His220). The carboxylate group of Glu217 is shared between the two metal ions.

Here, we report comparisons of the crystal structures of four complexes of this enzyme that represent four stages in its biochemical catalytic cycle. This was made possible by the introduction of different metal ions, which each interrupted the enzymatic reaction at a different stage. For crystallization studies we used D-xylose isomerase that had been soaked in deuterium oxide so that all labile H atoms were replaced by

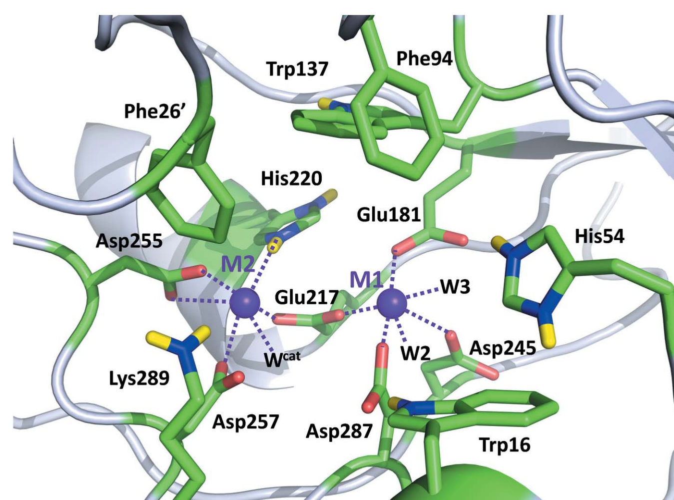

Figure 2
The active site of native XI, showing polar residues binding the metal sites M1 and M2 and hydrophobic residues lining the sites. The catalytic water is labeled W^{cat}, while the two water molecules coordinated to M1 are labeled W2 and W3. The coordinates are from 2gve.

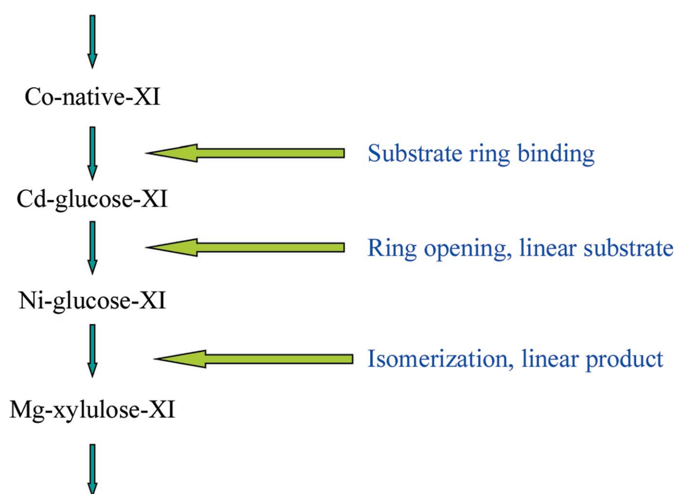

Figure 3
A chart of the neutron structures obtained for XI. Each complex represents an intermediate state on the sugar-interconversion reaction pathway.

deuterium. In addition, all of the substrate or product ligands were prepared in a perdeuterated form so that all of the H atoms in them were replaced by D. These complexes are listed in Fig. 3. The first is a ligand-free enzyme with divalent cobalt ions in the metal sites (PDB code 2gve; 1.8 Å resolution; Katz *et al.*, 2006). This represents the enzyme prior to any reaction. The second is a structure with bound perdeuterated cyclic D-glucose and with cadmium ions in the metal sites (PDB code 3kcl; 2.0 Å resolution; Kovalevsky *et al.*, 2010). This demonstrates the initial stage of sugar binding before ring opening. Apparently, the cadmium prevents ring opening from taking place, so that the first step in the reaction is frozen in time. The third is a complex with open-chain perdeuterated D-glucose and with nickel ions in both metal sites (PDB code 3kco; 1.8 Å resolution; Kovalevsky *et al.*, 2010). In this structure the sugar ring has opened but the isomerization that is normally expected to follow has not occurred. Finally, the fourth structure contains perdeuterated xylulose, the reaction product, with magnesium ions in the metal site (PDB code 3cwh; 2.2 Å resolution; Kovalevsky *et al.*, 2008). This manipulation of metal content has allowed us to study these various stages of the catalytic mechanism, presumably because of the different sizes and chemistries of divalent Mg, Co, Cd and Ni.

2. The enzyme structure prior to substrate binding

The active site of D-xylose isomerase before substrate binding is shown in Fig. 2. This was determined at 1.8 Å resolution from the crystal structure 2gve, which has bound divalent cobalt ions in its metal sites. In Fig. 2 the two metal ions, shown in purple, are surrounded by carboxylate side chains and His220, as listed above. There are two tryptophan residues, Trp16 and Trp137, that serve to provide hydrophobic surfaces that help to align the open-chain sugar substrate in the active site ready for reaction. The two histidine residues, His54 and His220, play significant roles in the mechanism of action of the enzyme. The orientation of His54 is firmly controlled by Asp57, which forms three hydrogen bonds to it. As already mentioned, the system Asp57, His54 and the substrate ring O

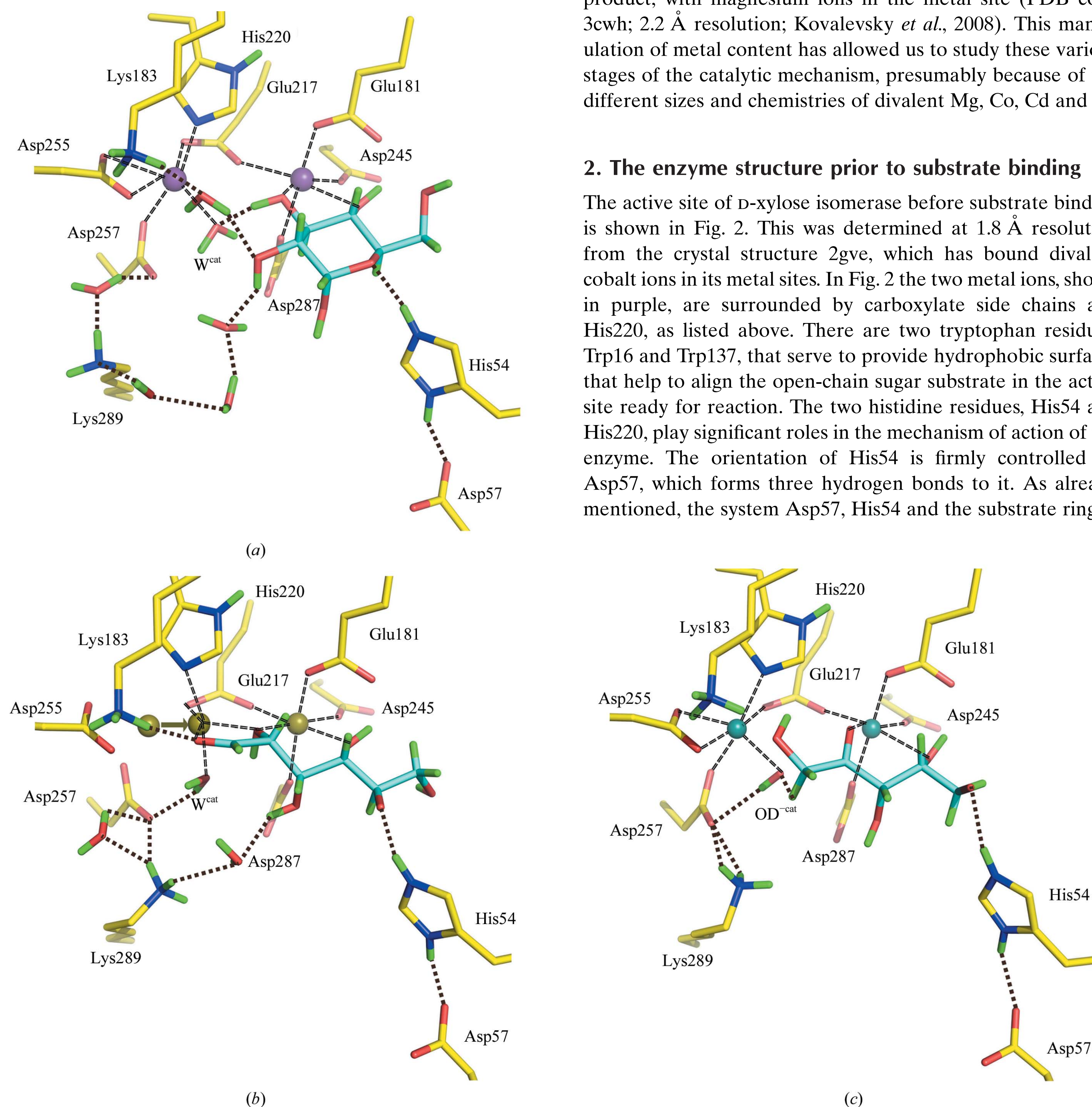

Figure 4
The intermediate states along the sugar-interconversion reaction as observed in the neutron structures. (*a*) Cyclic substrate glucose bound, (*b*) linear substrate glucose bound after ring opening has occurred and (*c*) linear product xylulose bound after the ring-opening and isomerization steps have occurred. Metal coordination is represented by black dashed lines, while hydrogen bonding is represented by dotted brown lines. D atoms are colored green. Note the interaction of the catalytic OD^- with the C1 methylene group of the sugar.

atom are arranged in a similar fashion in the catalytic triad in the serine proteases. There are also two important lysine residues, Lys183 and Lys289. To our surprise, the neutron diffraction study showed that while Lys183 has three D atoms on its terminal N atom and hence could be described as protonated, Lys289 only had two bound D atoms and therefore was neutral (or deprotonated). We considered that the water molecule near C1 and C2 of the substrate (the isomerization site) might donate hydrogen (deuterium) to the substrate. This neutron analysis of the enzyme without bound ligand clearly showed that this was a water molecule with two attached D atoms, not a hydroxide ion with only one. We refer to this water molecule as the 'catalytic water'.

3. The enzyme after cyclic substrate binds but before ring opening occurs

The three-dimensional structure of the enzyme with bound cadmium ions and the cyclic substrate D-glucose (3kcl; 2.0 Å resolution) is shown in Fig. 4(*a*). The main events that occur at this stage are the binding of the substrate in the required orientation and the detection by distant groups in the enzyme that this has taken place, presumably so that they can then commence their own roles in the mechanism. During substrate binding, His54, which was shown to be doubly protonated, binds the ring O atom of the cyclic glucose, while the other end of the glucose molecule coordinates to the metal ion M1 *via* O3 and O4. The D atom on the hydroxyl group O3 forms a strong hydrogen bond to the catalytic water, while the D atom on O4 forms a hydrogen bond to Glu181 to give a metal ion–hydroxyl–carboxylate motif. The fact that substrate binding has occurred is noted by His220 *via* an interaction of O3 of the substrate with the histidine $C^{\varepsilon 2}$ H atom. O1 of the cyclic glucose points towards the aromatic face of Trp16. The status of the two lysines (Lys183 protonated and Lys289 neutral) is found to be unchanged upon ring binding. The binding of His220 to M2 is weak in this structure, with a distance of 2.7 Å from the ring N to the metal ion. This is interesting and suggests that His220 may break its coordination to M2, possibly when it detects substrate binding.

4. The enzyme structure after ring opening but before isomerization

Now we come to the stage at which many changes occur. This is shown by the structure of the enzyme with bound perdeuterated glucose and with nickel ions in its metal sites (3kco; 1.8 Å resolution; see Fig. 4*b*). The metal ion in the M2 site appears to populate two positions that are separated by 1.9 Å, the new position being nearer than before to the now linear substrate and causing Asp255 to lose its coordination to M2. Thus, in about half of the unit cells the metal ion has moved away from Asp255 towards the aldehyde terminus of the linear sugar to bind to O1 and O2 of the substrate and thus facilitate isomerization. X-ray studies showed that if no linear substrate is present then only one M2 site (the usual one) is occupied by nickel, so that this metal-ion movement is the result of linear substrate binding, not nickel-ion binding. There is a 60° rotation difference about the C3—C4 bond of the D-glucose substrate between its cyclic and linear forms when bound to the enzyme. This rotation (in the nickel complex) causes O3 to lose the coordination it had with M1 in the cadmium complex, but allows it to donate a hydrogen bond to a water molecule, which then accepts a hydrogen bond from Lys289. The planar configuration around C1 of the substrate indicates that the C1—O1 linkage is an aldehyde and that isomerization has not taken pace.

Our key findings here are that during ring opening of the cyclic sugar the catalytic water is still D_2O, but Lys289, which was neutral before ring opening, is now protonated and O5 of the open-chain substrate has become deprotonated. His54 remains doubly protonated on its ring N atoms and donates a D atom to the negatively charged O5. Lys289 now interacts with Asp257. At the other end of the open-chain substrate O1 coordinates to the second (new) M2 site and also accepts a hydrogen bond from Lys183 (see Fig. 4*b*).

5. The enzyme structure after isomerization when product has been formed

The final stage of the reaction of D-xylose isomerase with D-xylose is the formation of D-xylulose. The result of this is seen in the structure of the enzyme with bound magnesium ions (as in the native enzyme) and perdeuterated D-xylulose (see Fig. 4*c*). In this structure the catalytic water has clearly lost a proton so that it has become a hydroxyl ion. OD^- forms a symmetric bifurcated hydrogen bond with the carboxylate of Asp257 and makes a C—H···O contact with the CD_2 methylene group at C1 in D-xylulose. We interpret this to mean that the catalytic water molecule probably donates a proton during the isomerization reaction. Furthermore, during isomerization C2 loses a proton and C1 gains a proton, the O1 aldehyde becomes protonated to a hydroxyl group and O2 is deprotonated. Again O5 is negatively charged and remains firmly bound to the protonated His54.

6. Conclusions

We have described here the ways in which the enzyme side chains adjust during the catalytic reaction and suggest the possible significance of their ionization states and general chemistry to these movements. In the four complexes representing four stages in the biochemical mechanism, Lys289 is deprotonated (neutral) in the first two stages and becomes protonated in the last two. In addition, O5 is deprotonated (becoming negatively charged) in the last two steps, while His54 is doubly protonated in all four stages. Probably the most significant event is the deprotonation of the catalytic water in the last stage. The variable charges on O5 of the substrate (to aid the enzyme in holding it in place) and on Lys289 (to help in some way the flow of protons to and from the isomerization site) are important components of the mechanism, as well as the movement of the metal ion M2 so

that substrate can be bound near the catalytic water ready for isomerization.

It was our aim to present the experimental data and possible explanations for the events as we saw them. Further scientific studies, however, are needed to help us refine a possible three-dimensional mechanism for this enzyme. Our hope is that the results of our neutron diffraction studies will contribute to the general understanding of the mechanism of substrate isomerization in biological systems.

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