Rapid Reports

Hydrogen Location in Stages of an Enzyme-Catalyzed Reaction: Time-of-Flight Neutron Structure of D-Xylose Isomerase with Bound D-Xylulose[†,‡]

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ABSTRACT: The time-of-flight neutron Laue technique has been used to determine the location of hydrogen atoms in the enzyme D-xylose isomerase (XI). The neutron structure of crystalline XI with bound product, D-xylulose, shows, unexpectedly, that O5 of D-xylulose is not protonated but is hydrogen-bonded to doubly protonated His54. Also, Lys289, which is neutral in native XI, is protonated (positively charged), while the catalytic water in native XI has become activated to a hydroxyl anion which is in the proximity of C1 and C2, the molecular site of isomerization of xylose. These findings impact our understanding of the reaction mechanism.

D-Xylose isomerase (XI)[1] from *Streptomyces rubiginosus* binds two divalent metal ions and catalyzes the conversion of aldo sugars D-xylose and D-glucose to keto sugars D-xylulose and D-fructose, respectively. During the reaction, hydrogen (H) transfer occurs. The ring of the cyclic substrate (D-xylose or D-glucose) is opened catalytically to give a linear-chain molecule on which the enzyme can further act. Isomerization from aldose to ketose involves the removal of H from C2 of the substrate and its transfer to C1 (*1–3*). Three possible mechanisms for this movement, involving a *cis*-ene diol intermediate (*4, 5*), a hydride shift (*1, 2, 6, 7*), and a metal-mediated hydride shift (*3, 8*), have been suggested.

XI is a crucial enzyme in sugar metabolism, with important commercial applications of topical interest, notably in the production of biofuels and high-fructose corn syrup. Understanding the transfer of H or protons during the catalytic reaction will aid in protein engineering efforts to improve the industrial performance of XI. Since we first determined the structure of XI (*9*), structures relevant to its reaction mechanism have been studied extensively by X-ray crystallographic techniques. However, H atom positions have been difficult to locate by X-ray diffraction in complexes of XI with various substrate and product analogues, even at the high resolution of 0.94 Å (*10*). This problem of locating H atoms in proteins has been successfully addressed using neutron crystallography, but rarely with proteins as large as XI (which is a 172 kDa homotetramer) because of difficulties in obtaining sufficiently large diffraction-quality crystals (*11*).

We have exploited the recently developed time-of-flight neutron Laue diffraction method (*12, 13*) to make neutron diffraction studies of XI a possibility, since large crystals had been obtained. Initially, we determined the 1.8 Å neutron structure of the native enzyme XI (*10*). We now present results of the 2.2 Å neutron structure of XI in complex with the reaction product D-xylulose; we designate this complex as "XI-xylulose". In this study, the enzyme was deuterated so that accessible and exchangeable H atoms (bound to O or N) were replaced with D, and the D-xylulose that was bound to the enzyme was perdeuterated (all H atoms replaced with D during synthesis).

Neutron diffraction studies of this combination of a deuterated enzyme with a perdeuterated product, described here, were conducted to reveal exactly where H atoms are located in the complex so that their transfer during the catalyzed reaction could be inferred. We stress that the ligand in the complex that we report here (D-xylulose) corresponds to that of the product of the reaction.

The great advantage of neutron crystallography is that D atoms (neutron scattering length of 6.67×10^{-15} m) appear as strong positive peaks in neutron scattering density maps, thereby revealing the locations of isotopically exchanged H atoms, while H atoms (neutron scattering length of -3.74×10^{-15} m) appear as negative troughs (and therefore can be distinguished from D). Both C and O (6.65 and 5.80×10^{-15} m, respectively) scatter about the same as D ($6.67 \times$

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[‡] Coordinates and structure factors have been deposited in the Protein Data Bank as entry 3CWH.

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[1] Abbreviations: XI, D-xylose isomerase; PCS, protein crystallography station; LANSCE, Los Alamos Neutron Science Center; PDB, Protein Data Bank.

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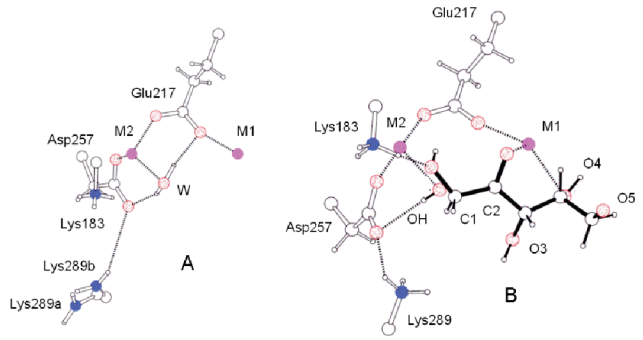

FIGURE 1: Environment of the catalytic, metal ion-bound, water molecule: D_2O in native XI (A) and OD^- in XI-xylulose (B).

10^{-15} m), while N (9.36×10^{-15} m) scatters more (*14*). The neutron scattering length of Mg^{2+} (5.37×10^{-15} m) and Mn^{2+} (-3.75×10^{-15} m) are of opposite sign, so a mixture of these, commonly found in XI, can give no apparent scattering.

Crystals of XI were grown and then soaked with buffers containing D_2O rather than H_2O (*15*). In this way, all labile and accessible H atoms in the enzyme were replaced with D. The crystals were then soaked with D_2O solutions of perdeuterated D-xylose. This D-xylose became bound in the active site where it was enzymatically converted to D-xylulose. These crystals (grown at pH 7.6 or pD 8.0) were used for the room-temperature neutron data collection for XI-xylulose on the PCS at LANSCE (*11*).

The structure, for which O, N, and C atomic coordinates had already been reported (*8–10, 15*), was determined with the software suite CNS (*16*), modified for neutron refinement (nCNS) (*17*). The metal ions were fixed in the same octahedral holes at positions M1 (called the structural metal) and M2 (called the catalytic metal) as found in other XI X-ray crystal structures [coordinates from PDB entry 4XIS (*8*) were used]. Mg^{2+}, while not a relatively strong scatterer of neutrons, is a relatively strong scatterer of X-rays and is therefore more accurately located using X-ray crystallographic techniques. Omit and difference nuclear density Fourier maps confirmed the exact location of a metal ion at the M1 position. However, there was little density in those maps at the M2 position, possibly indicating a mixture of Mn^{2+} and Mg^{2+} or greater mobility of the metal ion at this position. Experimental details are given in the Supporting Information.

Several interesting differences are observed when the neutron structures of XI-xylulose and native XI are compared. First, the catalytic metal ion-bound water molecule is D_2O in native XI but a hydroxyl anion, OD^-, in XI-xylulose (Figure 1A,B). Second, Lys289 has only two D atoms in native XI, while it is positively charged, having three D atoms, in XI-xylulose (Figure 2). Third, in both the neutron structures of native XI and XI-xylulose, His54 is doubly protonated on N with the D on $N_{\delta 1}$ bound to deprotonated Asp57. Fourthy, in XI-xylulose, His220 is only singly protonated on N and therefore can bind more firmly to the metal ion than it does in native XI where His220 is doubly protonated on N.

The terminal hydroxyl group, O5 of the xylulose, is, to our surprise, deprotonated and binds to doubly protonated His54 in an $N_{\varepsilon 2}-H^+\cdots O5^-$ interaction (Figure 3). We carefully verified this negatively charged state of O5. Neither

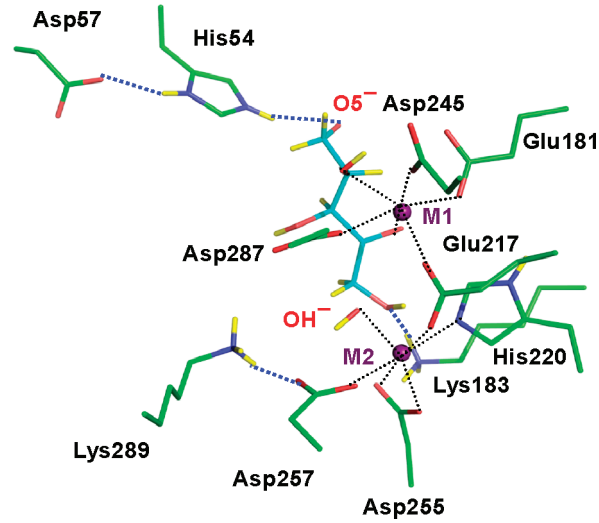

FIGURE 2: Active site of XI-xylulose. C, O, N, and D atoms are colored green, red, blue, and yellow, respectively. H atoms have been left out for the sake of clarity.

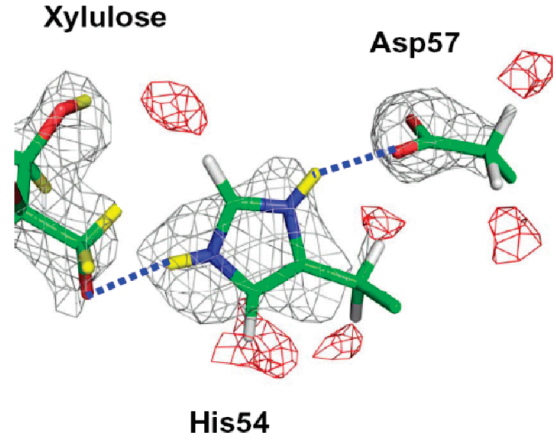

FIGURE 3: $2F_O - F_C$ nuclear density, positive in gray ($+1.2\sigma$) and negative in red (-2.0σ), showing His54 doubly protonated. The slight displacement of the negative density troughs from the actual H atom positions is typical for nuclear density maps and is due to the superposition of positive and negative density features of different magnitudes.

omit nor difference Fourier maps indicated the presence of a D atom on O5. Adjacent C4 has clear density for covalently bound D atoms in the Fourier maps, eliminating the possibility that O5 is disordered; the *B* factor of O5 is found to be similar to that found for other atoms in D-xylulose. Asp57 is not protonated and is bound to His54 via protonated $N_{\delta 1}$, stabilizing the position of the latter. O5 accepts an H-bond from His54 and from a water molecule, but there are no acceptors nearby to which it can donate an H.

The surroundings of the catalytic water molecule (in native XI) or hydroxyl group (in XI-xylulose) are of particular interest because both are near the C1−C2 bond of xylulose and therefore are presumed to play a significant role in the isomerization reaction. In native XI, the M2-bound water donates H-bonds to Asp257 and Glu217, forming two metal ion−carboxylate−water motifs. The O atom of the D_2O points one of its lone electron pairs toward M2 and the other in the direction where the C1−C2 bond of the linear substrate in a complex would be located. On the other hand, in the XI-xylulose structure, the M2-bound water is deprotonated to OD^-. The D of the hydroxyl group points toward a

carboxylate O of Asp257, which was found for D_2O in the native enzyme structure. The O of the hydroxyl group appears to remain bound to M2 and now lies close to C1 of the bound product xylulose. Lys289 is well-ordered unlike the structure in 4XIS and in the native XI structure, and its N_ζ has three D atoms. One of these is involved in an H-bond to a carboxylate O of Asp257 (Figure 2). Since Lys289 only has two protons on N_ζ in native XI but three in XI-xylulose, this might suggest that it is somehow involved in proton transfer during the reaction. N_ζ of Lys183 has three D atoms in both native XI and XI-xylulose. One of these D atoms is H-bonded to O1 of xylulose in the complex.

O2 of xylulose is deprotonated in XI-xylulose, confirming that the C2−O2 bond has become a carbonyl group. O2 also interacts with $C_{\varepsilon 1}$ of His220 by means of a C−H···O bond. The nuclear density and also the planar geometry of C2 indicate that C2 has lost, and C1 has gained, a D atom. O1, O3, and O4 of xylulose are protonated with their D atoms pointing toward $N_{\varepsilon 2}$ of His220, solvent, and an O of Glu181, respectively. In this neutron structure of XI-xylulose, M1 appears to bind six O atoms in Glu181, Glu217, Asp245, and Asp287 and O2 and O4 of xylulose (Figure 2), showing that xylulose has replaced two metal ion-bound water molecules that were found in the native unliganded structure. M2 also appears to bind six groups: His220, Glu217, Asp257, Asp255 (bidentate), and the hydroxyl group O atom.

Unlike the situation in native XI, His220 is only singly N-protonated in XI-xylulose, thereby making the nonprotonated N able to bind more firmly to M2. In native XI, $N_{\varepsilon 2}$ of the His54−Asp57 pair donates an H-bond to a water molecule, whereas in XI-xylulose, this water has been replaced with O5 of xylulose (Figure 3). This strong salt bridge-type $Ne_{\varepsilon 2}-H^+\cdots O5^-$ interaction assists in holding the substrate in the active site, ready for the isomerization reaction.

These observations have important implications for our understanding of the reaction mechanism of XI. The main aims of this enzyme are to open the cyclic sugar that has bound to the metal ion at M1 and then to move the H from C2 to C1 of the substrate. It would appear that the deprotonation of the catalytic water is important in this. The finding that O5 is deprotonated suggests that, if a ring-opening stage exists, it may differ in detail from the conventional Lewis acid-catalyzed reactions observed for other enzymes (*18*). Ionization of O5 is unlikely to have any significance with respect to the mechanism of isomerization because it is distant from the site of the chemical reaction, but it may be important, along with the metal cations, in stabilizing multiply charged intermediate ligands.

In XI-xylulose, the hydroxyl anion is only 2.8 Å from C1 and there is nothing close to C2. This is consistent with our hypothesis that this hydroxyl group is involved in an interaction with C1 and/or C2. It is possible that this interaction involves an H of the M2-bound water molecule protonating C1. This would be consistent with the formation of an ene-diol intermediate. However, there is biochemical evidence that the C1 and C2 H atoms do not exchange with solvent during the reaction, and this calls into question the ene-diol mechanism for this enzyme (*5*). The hydroxyl group is also only 2.9 Å from O1 (which is not bound to M2). Another possibility is that the M2-2 bound water molecule is involved in protonation of O1, and then that the resulting hydroxyl anion stabilizes the positive charge on C1 in the resulting carbocation intermediate.

In summary, we suggest that the linear sugar substrate (xylose or glucose), after a ring-opening stage involving the His54−Asp57 pair, is extended and tethered at either end by an H-bond from Lys183 and by an interaction with His54 $N_{\varepsilon 2}$ (to O5). At some stage, the M2-bound water is deprotonated and Lys289 is protonated. Meanwhile, O2 of xylulose is deprotonated, and the resulting unstable intermediate is stabilized by removing H from C2 and adding H to C1, either through a hydride shift or through an ene-diol intermediate. The precise role of the hydroxyl group in this proton transfer remains to be determined.

SUPPORTING INFORMATION AVAILABLE

Details of crystallization, neutron crystallographic data collection, and structure refinement. This material is available free of charge via the Internet at http://pubs.acs.org.

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