Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 1362–1366, February 1990
Biochemistry

# Observations of reaction intermediates and the mechanism of aldose–ketose interconversion by D-xylose isomerase

(enzyme mechanism/non-Michaelis/hydride shift/bound intermediate/glucose isomerase)

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*Communicated by M. F. Perutz, November 14, 1989 (received for review August 25, 1989)*

**ABSTRACT** **Crystallographic studies of D-xylose isomerase (D-xylose ketol-isomerase, EC 5.3.1.5) incubated to equilibrium with substrate/product mixtures of xylose and xylulose show electron density for a bound intermediate. The accumulation of this bound intermediate shows that the mechanism is a non-Michaelis type. Carrell *et al.* [Carrell, H. L., Glusker, J. P., Burger, V., Manfre, F., Tritsch, D. & Biellmann, J.-F. (1989) *Proc. Natl. Acad. Sci. USA* 86, 4440–4444] and the present authors studied crystals of the enzyme–substrate complex under different conditions and made different interpretations of the substrate density, leading to different conclusions about the enzyme mechanism. All authors agree that the bound intermediate of the sugar is in an open-chain form. It is suggested that the higher-temperature study of Carrell *et al.* may have produced an equilibrium of multiple states, whose density fits poorly to the open-chain substrate, and led to incorrect interpretation. The two groups also bound different closed-ring sugar analogues to the enzyme, but these analogues bind differently. A possible explanation consistent with all the data is that the enzyme operates by a hydride shift mechanism.**

The crystal structure of D-xylose isomerase (XI; D-xylose ketol-isomerase, EC 5.3.1.5), important in the food industry as glucose isomerase because it catalyzes the conversion of glucose to fructose, has been determined recently in several laboratories (1–5). The enzyme is a symmetrical tetramer, with two cation sites in each subunit. Its catalytic action requires the transfer of two hydrogen atoms, to interconvert an aldose and a ketose. Two types of mechanism have been discussed (6) to explain the stereospecific hydrogen transfer (7, 8), one going through an ene-diol intermediate (Fig. 1, Scheme I), and the other including the transfer of a hydride ion (Fig. 1, Scheme II). The ene-diol mechanism has been demonstrated for triose phosphate isomerase, and the aldose ketose isomerases are often considered as a single class of enzymes (9).

Unlike some other isomerases, XI has an absolute requirement for divalent cations (14) and the isomerization displays no solvent exchange at the $C^1$ or $C^2$ of the ketose or aldose, respectively (7, 15). The sugar substrates of XI exist in solution predominantly in closed-ring forms, and XI acts and reacts specifically with the α-D-pyranose forms of xylose and glucose (15, 16) [and in the reverse reaction on α-D-fructofuranose (17)].

Collyer *et al.* (18) have observed complexes of the metal-activated enzyme with thio-α-D-glucose, D-xylose/xylulose, and D-xylose/xylulose in the presence of $Al^{3+}$ and have concluded that these complexes are analogous to steps of the aldose to ketose reaction. This proposal is shown to be consistent with the metal requirement, the substrate specificity, the stereospecificity and the lack of solvent exchange.

The crystallographic observations of the electron densities for the D-xylose/xylulose–metal–enzyme complex have all been interpreted as extended-chain sugars (18, 19, †, ‡). Collyer *et al.* (18) postulate that the extended-chain intermediate is an aldehyde or ketone, whereas Carrell *et al.* (19) interpret their electron density for the sugar as representing a *cis*-ene-diol transition state. The relevant experimental data are summarized in Table 1. Studies reported from our laboratory are on the *Arthrobacter* B3278 enzyme (5), while Carrell *et al.* use material from *Streptomyces rubiginosus* (1).

## OBSERVATIONS

**Ring Sugars.** The two groups (18, 19) have both reported structures of complexes containing one closed-ring sugar analogue molecule and two divalent cations in each subunit of the tetrameric enzyme. Collyer *et al.* (18) studied 5-thio-α-D-glucopyranose (TGP), for which an electron-density difference map is shown in Fig. 2*a*, unambiguously defining the orientation of the ring. It binds with $O^1$ and $S^5$ close to His-53 and $O^6$ close to Thr-89. Cation site 1 coordinates $O^3$ and $O^4$. Cation site 2 is not in direct contact and is remote from $C^1$, $C^2$, and $C^5$, in agreement with NMR evidence (20). Carrell *et al.* (19) incubated the enzyme with 3-deoxy-$C^3$-fluoromethylene-α-D-glucose (DFMG), a chemically reactive sugar derivative that is observed bound in a β-D-fructofuranose form and has therefore already undergone isomerization. It is covalently bound to the enzyme. It alkylates His-53* at $N^2$, leaving $C^3$ close to His-53* and the ring oxygen $O^5$ liganded to $Mn^{2+}$ in cation site 1 (ref. 19; Fig. 2).§

**Extended Chain Sugars.** Henrick, Collyer and Blow have studied several complexes of the enzyme containing the open-chain inhibitors xylitol and D-sorbitol (5), and the substrate/product D-xylose/xylulose, which are always observed bound in the same extended open-chain form (18) (Fig. 2*c*). The resolution of this study is limited because these crystals do not diffract strongly beyond 2.5 Å. The electron density for D-xylose/xylulose appears almost symmetrical, but several indications exist to distinguish the two ends of the molecule: (*i*) when xylitol is bound, it appears at a site almost identical to that of D-xylose/xylulose, but when the 6-carbon inhibitor sorbitol is bound additional density is observed at

Abbreviations: XI, D-xylose isomerase; TGP, 5-thio-α-D-glucopyranose; DFMG, 3-deoxy-$C^3$-fluoromethylene-α-D-glucose; ES, enzyme–substrate complex; EP, enzyme–product complex.

†Jenkins, J. A., Rey, F., Chlamdl, M., Janin, J., Lasters, I., Pio, F. & Wodak, S., Abstracts of the First European Workshop on Crystallography of Biological Macromolecules, May 15–19, 1989, Como, Italy.

‡Whitlow, M. & Howard, A. J., Abstracts of the Annual Meeting of the American Crystallographic Association, July 23–29, 1989, Seattle.

§For clarity amino acid residues in the *S. rubiginosus* enzyme are assigned a sequence number with an asterisk, indicating the homologous amino acid of the *Arthrobacter* enzyme (21).

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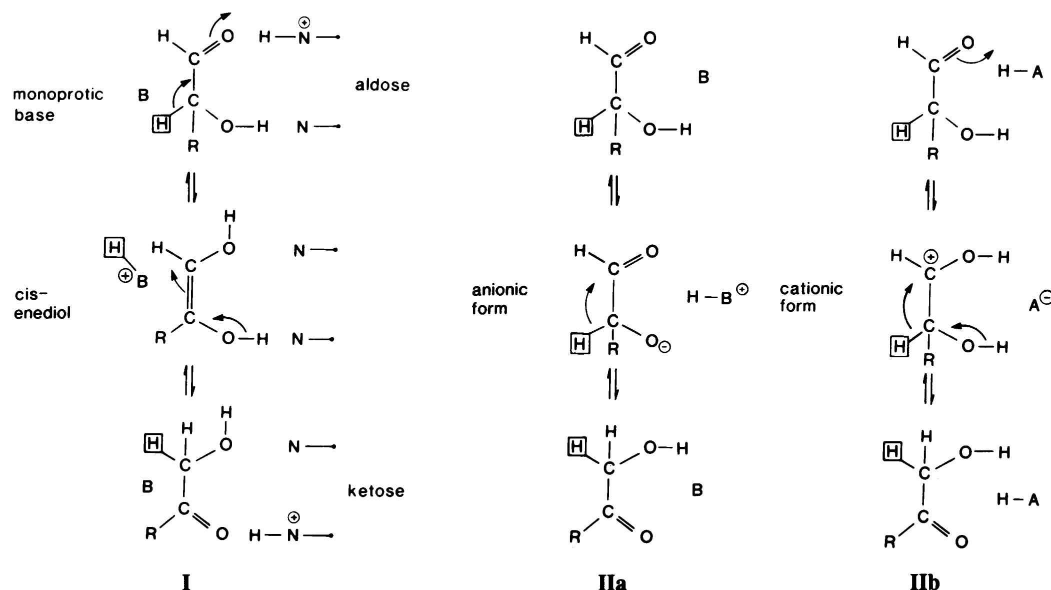


FIG. 1. Mechanisms for the isomerization of an aldose to a ketose have been reviewed by Rose (9). A square indicates the hydrogen atom that is transferred stereospecifically. The ene-diol mechanism of Scheme **I** is the accepted mechanism of triose phosphate isomerase, and other enzymatic aldose to ketose conversions are often discussed in terms of this mechanism. The hydride shift mechanisms of Schemes **IIa** and **IIb** are believed to operate in the alkali- or acid-catalyzed conversions of some sugars (10, 11). In Scheme **IIa**, a proton is withdrawn before the hydride shift, which then operates on an anionic form (12, 13); in Scheme **IIb**, the hydride transfer operates on a protonated form (11).

one end of the molecule likely to represent $C^6$; (*ii*) when the cyclic 6-carbon sugar analogue TGP is bound (Fig. 2*a*), the bulb of density representing the 6-methylhydroxyl group is observed at the site associated with $C^6$ of sorbitol; (*iii*) the refined conformations for D-xylose/xylulose retain the same torsion angles about the $C^4$—$C^5$ bond, and the same interactions between $O^5$ and His-53, $O^4$ and cation site 1, as are observed for TGP, although the remainder of the sugar is bound in an extended conformation. With this assignment of D-xylose/xylulose, there is a simple route, without serious steric hindrance, between a closed-ring sugar conformation as observed for TGP and the observed open-chain form. Cation site 1 coordinates $O^2$ and $O^4$, and cation site 2 is near $O^1$ and $O^2$ of the open-chain sugar but not in direct contact.

Carrell *et al.* (19) also studied substrate binding but at higher resolution. Despite the higher resolution of this study, the electron density in the published figures fits poorly to an extended D-xylose/xylulose molecule. The difference in electron density is interpreted as an open-chain sugar, but the interpretation is different. The carbon chain of the sugar is considered to be running in the opposite direction and is slightly displaced in the two interpretations (Fig. 2*b*; cf. Fig. 2*c*). Thus, Carrell *et al.* (figure 1*e* in ref. 19) have $O^3$ and $O^5$ interacting with the cation in site 1, while $C^1$ is near His-53*.

Table 1. Comparison of data for metal–substrate–enzyme complex

| | Carrell *et al.* (19) | Collyer *et al.* (18) |
|---|---|---|
| Soak | | |
| $(NH_4)_2SO_4$ | 0.76 M | 3.2 M |
| Buffer | 10 mM Pipes | 50 mM triethanolamine |
| pH | 7.4 | 8 |
| Activating cation | 2 mM $Mn^{2+}$ | 100 mM $Mn^{2+}$ |
| D-Xylose/xylulose | 1 M | 1 M |
| Temperature | 4°C | 18°C |
| Time | 4 days | 2 days |
| Diffraction data | | |
| Temperature | 12°C–15°C | −10°C |
| Resolution | 1.9 Å | 2.5 Å |
| *R* factor | 14.1% | 14.8% |
| Fraction of possible data | 78% | 91% |
| Bond length rms deviation | 0.022 Å | 0.018 Å |

## DISCUSSION

There are three conflicts to be resolved between the interpretation of Carrell *et al.* (19) and Collyer *et al.* (18): (*i*) the differences in the observed binding of the closed ring sugars; (*ii*) the differences of electron density observed when a xylose substrate is bound; (*iii*) the differences of interpretation about the mode of binding of the xylose substrate in the open-chain form, and the consequential different conclusions about the enzyme mechanism.

**Inconsistencies.** The first conflict is relatively simple to resolve because it is questionable whether the "mechanism-based inactivator" DFMG is a good model for the binding of a closed-ring substrate. DFMG is a competitive inhibitor, but because of its chemical reactivity it is not very likely to mimic substrate binding. His-53* is a favorable target for an alkylating compound, and if $C^3$ of DFMG is bound to it for this reason, then this observation gives no information about the position of $C^3$ when an ordinary xylose substrate is bound in the ring form. Confusingly, an analogue of the unreactive $\beta$ anomer of D-fructofuranose (17) is observed bound to the enzyme.

There are inconsistencies in the explanation given for the observed mode of binding of this inhibitor. Carrell *et al.* (19) suggest that "inactivation occurs with the open chain form" of DFMG and "cyclization follows the isomerization reaction." Since Carrell *et al.* (19) propose that His-53 is the base required for the isomerization of the ene-diol, their mechanism requires that His-53 cannot be alkylated until after the isomerization has taken place. If DFMG binds in a productive conformation, then it must bind in a manner similar to their proposed D-xylose/xylulose conformation with $O^3$ bound to the cation at site 1. This is not possible because DFMG has a fluoromethylene group at $C^3$.

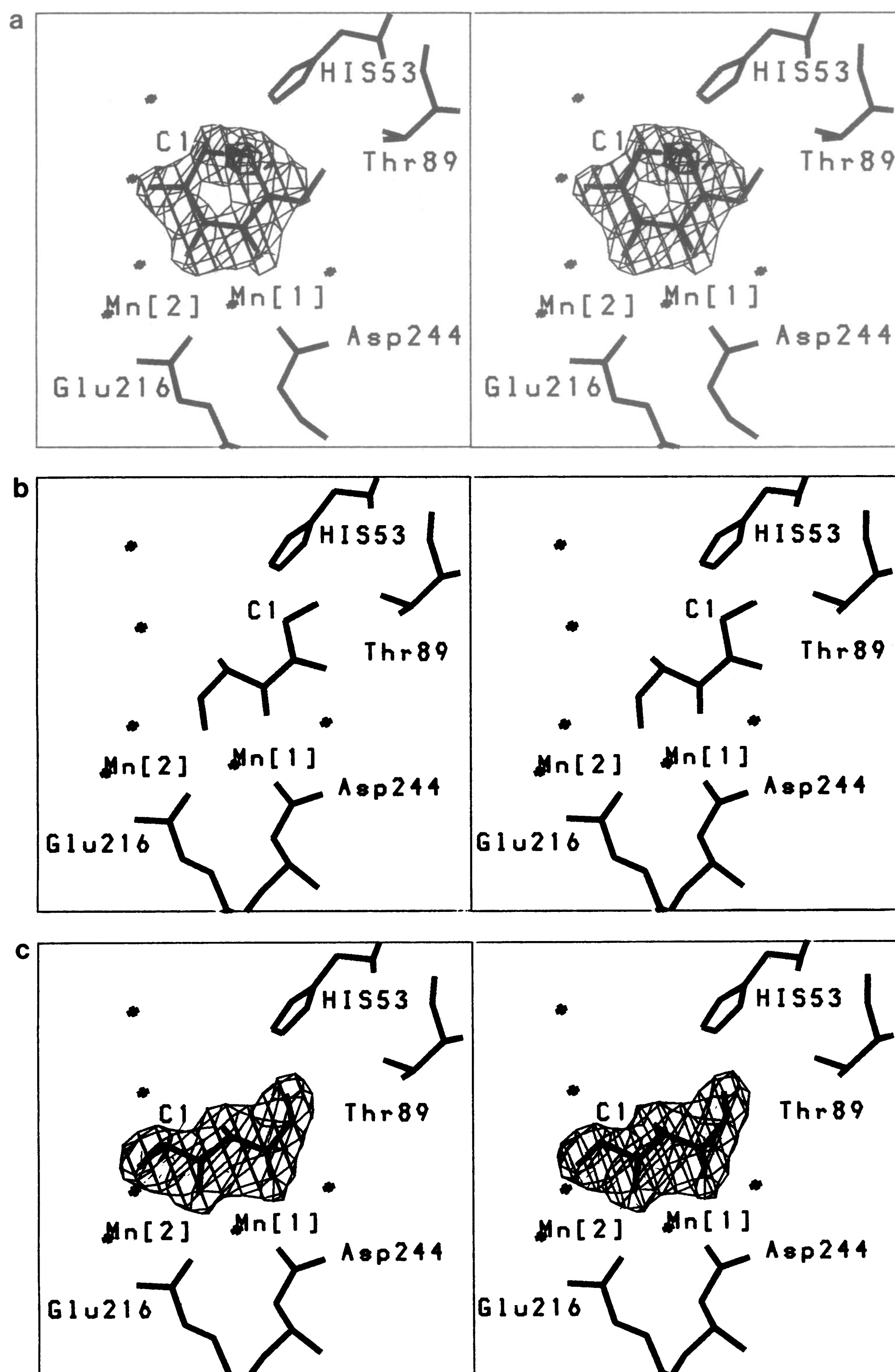


FIG. 2. (*a*) Electron-density difference map in stereo at 2.5 Å resolution with Fourier coefficients $F_o - F_c$ ($F_c$, calculated from a refined model, including metal and solvent sites) for TGP bound at the active site of XI. The contour levels are $5\sigma$ and $10\sigma$. The higher electron-density level (thicker lines) indicates the sulfur position, while the lower contour shows the shape of the sugar ring. Density at $O^6$ is weaker ($>3\sigma$). The positions of adjacent ligands are identified. Crystals were grown in metal-free conditions at 18°C and soaked with 50 mM $Mn^{2+}$/1 M TGP. Diffraction was measured at −8°C: full experimental details are given elsewhere (18). We have also studied 1-deoxynojirimycin, a glucose analogue with an imino group replacing the ring oxygen, which binds with the ring in just the same orientation (data not shown). (*b*) Interpretation of the model presented by Carrell *et al.* (19) of the binding of the substrate D-xylose/xylulose. (*c*) Electron-density difference map in stereo at 2.5 Å resolution with Fourier coefficients $F_o - F_c$ for an equilibrium mixture of D-xylose and D-xylulose bound at the active site of XI. Contour level, $5\sigma$. The assignment of the $C^1$ and $C^5$ positions follows considerations presented in the text. The positions of adjacent ligands are identified. Small crosses not identified as Mn represent solvent sites. Crystals were grown in metal-free conditions at 18°C and soaked with 100 mM $Mn^{2+}$/1 M xylose. ($Pb^{2+}$ was subsequently added to these crystals but evidently did not bind.) Diffraction was measured at −10°C. Full experimental details are given elsewhere (18).

Evidently DFMG does not bind like a closed-ring substrate.

**Hypothesis.** The second conflict cannot be resolved so simply, and only a hypothetical solution can be offered.

Crystallographic data from a number of laboratories have been reported as showing a bound substrate in an open-chain form (18, 19, †, ‡). These observations provide clear evidence of a non-Michaelis type of mechanism.

The Michaelis–Menten analysis assumes an enzyme that binds substrate rapidly to form a "Michaelis complex" ES (enzyme–substrate complex), which then passes more slowly through a transition state to form an enzyme–product complex, EP. There is no appreciable accumulation of intermediate forms. The energy profile for catalysis by an ideal Michaelis–Menten enzyme is shown at the top of Fig. 3, which also shows an energy profile for a more typical enzyme, whose kinetics will provide satisfactory agreement with Michaelis kinetics. For an ideal Michaelis enzyme, the Michaelis constant $K_M = K_S$, the dissociation constant of the ES complex, and this relation remains approximately true for many real enzymes.

If in the course of the enzyme reaction an intermediate is formed whose free energy is less than the free energies of EP or ES (or is only a few $kT$ greater than them) a significant population of this intermediate will accumulate and the Michaelis–Menten mechanism does not apply (Fig. 3) (22). Crystallographic observations are a weighted average of all the states populated in the crystal. The observations of an extended-chain intermediate form of the substrate prove that this situation exists in XI. In many enzyme reactions, the final equilibrium is overwhelmingly in favor of the reaction product, so that permanent accumulation of an intermediate cannot occur. But the D-xylose/xylulose equilibrium is 80:20 (at 65°C) (8), and in this situation a permanent accumulation of enzyme/intermediate is possible.

The observations reported by Collyer *et al.* (18) were certainly under conditions in which the crystal had brought the drop of mother liquor in contact with it to equilibrium between D-xylose and D-xylulose. In each case, the crystal was mounted in contact with a drop of mother liquor containing 1 M D-xylose, whose volume was of the same order as the crystal volume. The mother liquor also contained 100 mM $Mn^{2+}$ and was buffered at pH 8. The mounted crystal was incubated at room temperature for at least 1 day before being x-rayed. After mounting on the diffraction apparatus, the crystal was rapidly chilled to −10°C and then used for data collection from a synchrotron source for up to 8 hr. The variation of $k_{cat}$ with temperature reported from two laboratories (23, 24) indicates a constant activation energy for the reaction over a wide range of temperatures. Extrapolating these rates to the temperature of the diffraction experiment (−10°C) leads to the expectation that the crystal would have equilibrated with its small drop of mother liquor. Under these conditions, 15–20% of the unbound sugar is expected to be present as the ketose product D-xylulose (8). Carrell *et al.* (19) incubated their crystals in a small droplet at 4°C for 2 days in 2 mM $Mg^{2+}$/2 mM $Mn^{2+}$ before measuring x-ray intensities on an area detector at 12°C–15°C. It seems almost certain that equilibrium would have been reached at this temperature.

For a non-Michaelis-type enzyme, the equilibrium between intermediate bound states and EP and/or ES will be temperature dependent, and at higher temperatures the higher-energy states will be more populated. Significantly different appearances for the electron density of the substrate have been reported by Carrell *et al.* (19) at 12°C–15°C and by Collyer *et al.* (18) at −10°C, which may well be accounted for by the difference in temperature between the two studies. Another possibility would be that the bound intermediate is more stable in the *Arthrobacter* enzyme crystals.

It may therefore be suggested that the electron density observed by Carrell *et al.* (19) represents an average electron density of multiple states and is a mixture of the electron densities for closed ring and extended chain forms of the sugars.

**Reconciliation.** Once it is recognized that by good fortune Collyer *et al.* (18) conducted their experiment under conditions at which virtually all their bound substrate was in the extended-chain form, while Carrell *et al.* (19) were not so lucky, the resolution of their difference of interpretation is straightforward. Fig. 2*b* shows the substrate conformation proposed by Carrell *et al.* (19), giving the best fit for their electron-density map for *S. rubiginosus* XI; but it is illustrated in relation to the structure of the *Arthrobacter* active site observed by Collyer *et al.* (18). In Fig. 2*c* the difference density observed by Collyer *et al.* (18) is shown. It is clearly consistent with an open-chain substrate and clearly inconsistent with the substrate conformation of Fig. 2*b*.

The observed DFMG inactivation is consistent with the mode of binding of Fig. 2*c*. In this conformation, the replacement of $O^3$ by fluoromethylene as in DFMG would bring the alkylating group close to His-53; if the analogue were bound in this conformation, alkylation of the histidine seems almost certain to follow. The isomerization step does not involve His-53 and therefore may occur independently of the alkylation. Ring closure would not be catalyzed by the enzyme, but it could presumably occur spontaneously as it does in free solution.

the "ideal" Michaelis enzyme

a typical enzyme

a non-Michaelis enzyme

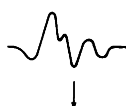

FIG. 3. A number of the possible free energy profiles for enzymatic catalysis. Arrow indicates a bound intermediate state, which may be observed in crystallographic experiments.

## CONCLUSION

The two interpretations of the orientation of the substrate D-xylose/xylulose have led to opposite conclusions concerning the isomerization mechanism. Carrell *et al.* (19) consider His-53*, polarized by Asp-56* behind it, as the monoprotic base accepting a proton from $C^2$ of D-xylose and transferring it to $C^1$ to form xylulose (Fig. 1, Scheme I). A water molecule between Thr-89* and Thr-90* polarizes the carbonyl group $CO^1$, delivering a proton to it, and subsequently accepts a proton from $O^2$. The role of the cations is to "maintain the structural integrity of the active site," and for site 1 to "stabilize the open-chain form of the sugar by binding the hydroxyl groups at the end of the molecule most distant from the site of the isomerization reaction" (19). It is suggested that the mode of binding of DFMG, alkylating His-53*, confirms its role as the monoprotic base (Fig. 1, Scheme I). In this proposed ene-diol mechanism $O^1$ and $O^2$ must be in a *cis* conformation, with a monoprotic base adjacent to $C^1$, and

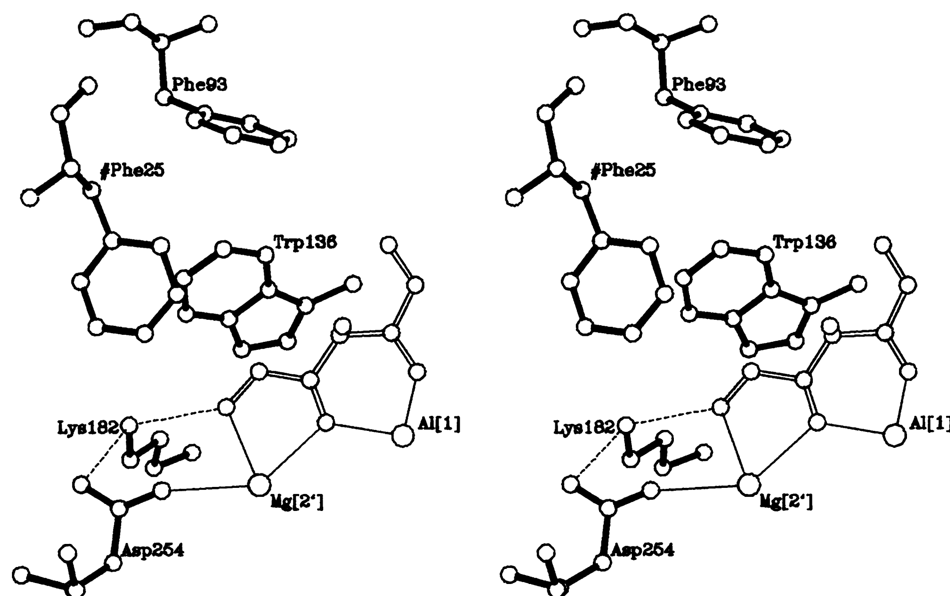


FIG. 4. Stereoview of the hydrophobic environment above the $C^1$—$C^2$ bond of the substrate molecule in a refined model (18), which is created by residues Trp-136, Phe-93, and Phe-25. Phe-25 is from a neighboring subunit in the tetramer. The electrophilic environment below the $C^1$—$C^2$ bond formed by both cations and Lys-182 is also shown. The substrate is shown by open lines. Only part of the coordination shell of the metal sites is shown and the bonds are indicated by thin lines. Dashed lines indicate possible hydrogen bonds. Full experimental details are given elsewhere (18).

this requirement will have biased the modeling of D-xylose/xylulose into the difference density.

Collyer *et al.* (18) also recognize His-53 as a base activated by the polarizing influence of Asp-56, but its role is to catalyze ring opening. The TGP complex, a model for the Michaelis complex with glucopyranose, shows His-53 adjacent to $S^5$ (Fig. 2*a*). The conformation proposed for the open-chain substrate (Fig. 4) is readily reached after ring opening, with little or no movement of $C^4$ and $C^5$ of D-xylose. With the substrate in the open-chain conformation, there is no base positioned above the $C^1$—$C^2$ bond, as required for the ene-diol mechanism. The isomerization site at $C^1$—$C^2$ is close to the cation binding sites, and it is proposed that cations facilitate the isomerization by ionization: there is some evidence (18) that the cation in site 2 moves slightly to site 2′ to coordinate $O^1$ and $O^2$ during the transition. In the presence of substrate, with $Al^{3+}$ at site 1, $Mg^{2+}$ is detected at a site 2′ 1 Å from site 2 (Fig. 3). Cation 2, assisted by Lys-182 and cation 1, is the electrophile that expels the proton from $O^2$ during the shift of the hydride ion. The substrate becomes anionic in the transition state (Fig. 1, Scheme **IIa**), and the proton acceptor is probably a water molecule situated between $O^1$ and $O^2$.

The side of the substrate where the hydride shift occurs is in a totally hydrophobic environment, surrounded by Trp-136, Phe-93, and also Phe-25 from an adjacent subunit (Fig. 3). Thus, as observed (15, 16), there can be no exchange of the hydride ion with solvent. Although there is no mechanistic requirement for a *syn* conformation at $C^1$—$C^2$, an approximate *syn* conformation is indicated by the electron density for this end of the substrate, bringing $O^1$ and $O^2$ close together in contact with the cation near site 2, and is consistent with the observed stereochemistry of hydride transfer (7, 8).

The observations and the interpretation of substrate binding of Collyer *et al.* (18) are consistent with the observations reported by Carrell *et al.* (19). The interpretation presented by Carrell *et al.* (19) is internally inconsistent and disagrees with biochemical data. Although x-ray crystallography of stable states cannot prove a kinetic mechanism, and although the interpretation of electron density must be equivocal when individual atomic positions are not resolved, the data presented here and elsewhere (18) suggest strongly that this enzyme operates by a hydride shift mechanism (Fig. 1, Scheme **IIa**).

We thank Bayer AG for a gift of 1-deoxynojirimycin, and Hans Viehmann for assistance with the 1-deoxynojirimycin complex structure determination. X-ray diffraction data were recorded at the Synchrotron Radiation Source, Daresbury, U.K. This research was supported by the Protein Engineering Initiative of the Science and Energy Research Council, U.K.

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