Design, Synthesis, and Characterization of a Potent Xylose Isomerase Inhibitor, D-Threonohydroxamic Acid, and High-Resolution X-ray Crystallographic Structure of the Enzyme−Inhibitor Complex[†,‡]

Karen N. Allen,[§] Arnon Lavie, Gregory A. Petsko, and Dagmar Ringe*

Departments of Biochemistry and Chemistry, Rosenstiel Basic Medical Sciences Research Center, Brandeis University, 415 South Street, Waltham, Massachusetts 02254-9110

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ABSTRACT: The binding of a potent inhibitor to the enzyme D-xylose isomerase from *Streptomyces olivochromogenes* was examined by kinetics and X-ray crystallography. The inhibitor D-threonohydroxamic acid (THA) was designed to mimic the putative transition state of the isomerization step catalyzed by the enzyme on the substrate xylose. THA was synthesized and found to be a slow-binding competitive inhibitor with the substrate glucose. The $K_i \leq 100$ nM was at least one million-fold less than the K_M for glucose. The X-ray crystallographic structure of xylose isomerase with THA soaked into the crystals (concentration $= 1000K_i$) was obtained to 1.6-Å resolution and refined to an R factor of 21.6%. The free enzyme and the enzyme in the xylose isomerase−THA complex show no significant structural differences. THA binds in an analogous fashion to glucose, in a linear conformation, forming ligands with Mg-1 and Mg-2 and hydrogen bonds with His53 and Lys182. On the basis of these similarities to glucose binding and its potent inhibition, we propose that THA resembles the transition state for the enzyme-catalyzed hydride transfer reaction. The THA C2 hydroxyl forms a bridging ligand between Mg-1 and Mg-2; it must be deprotonated to do so. By analogy, we propose that, during the catalytic reaction, C2 of the substrate glucose is deprotonated, and that this proton can be moved to the C1 hydroxyl concomitant with hydride transfer. We find evidence for metal movement during catalysis upon deprotonation of the C2 hydroxyl, to allow formation of a bridging ligand. In addition, the observation in the xylose isomerase−THA complex of a water molecule bound to Mg-2 instead of a hydroxide (as seen in the native structure) suggests that protonation of the hydroxyl occurs in a step following ring opening. The metal-bound hydroxide ion may act as a general base to deprotonate the C2 hydroxyl of the substrate. Due to their ability to act as metal ligands, hydroxamic acids may be general inhibitors of dimetallic enzymes.

D-Xylose isomerase (EC 5.3.1.5), one of the most widely used industrial enzymes, catalyzes the interconversion of aldose and ketose sugars. The enzyme possesses a broad substrate specificity. Although the physiological reaction catalyzed by xylose isomerase is the interconversion between the five carbon sugars xylose and xylulose, it is used industrially to convert the six-carbon sugar glucose to fructose. The enzyme belongs to the family of metal-activated enzymes, utilizing two divalent metal cations per subunit for catalysis (Carrell et al., 1989; Collyer et al., 1990; Whitlow et al., 1991; Allen et al., 1994b; Lavie et al., 1994).

In contrast to the proton-transfer mechanism, which involves the formation of an ene-diol or -diolate intermediate, utilized by phospho−sugar isomerases such as triosephosphate isomerase (Reider & Rose, 1959; Albery & Knowles, 1976), a hydride transfer mechanism has been proposed for xylose isomerase (Farber et al., 1989; Collyer et al., 1990; Lee et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992; van Tilbeurgh et al., 1992; Lavie et al., 1994). This enzyme-catalyzed isomerization follows a less well studied enzyme-catalyzed ring-opening step (Schray & Rose, 1971). Kinetic results are inconsistent with a proton-transfer mechanism: there is a lack of observable proton−solvent exchange from the C2 position of glucose (Rose et al., 1969; Rose, 1981; Allen et al., 1994a) as well as a lack of elimination of fluoride from the three position of fluorinated analogs of glucose (Allen et al., 1994a). High-resolution crystallographic studies (<2.2 Å) have been carried out on xylose isomerase from *Streptomyces olivochromogenes* in this laboratory in order to elucidate the role of the active-site residues and metals in catalysis (Lavie et al., 1994). The X-ray crystallographic structures of xylose isomerases from other bacterial strains have been solved and found to be nearly identical (Collyer et al., 1990; Dauter et al., 1990; Meng et al., 1991; Whitlow et al., 1991; Jenkins et al., 1992). In all substrate-soaked crystal structures (Collyer et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992; Lavie et al., 1994) the linear form of glucose or xylose is observed bound to the two metals in the active site. These structures are inconsistent with a proton-transfer mechanism since there is no enzymic general base in position to abstract the substrate proton, as required by such a mechanism. However, the ligation of the substrate to the metals through the carbonyl and adjacent hydroxyl

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[‡] The coordinates of the structure have been deposited in the Brookhaven Protein Data Bank under file name 1GYI.

* Author to whom correspondence should be addressed.

[§] Present address: Department of Physiology, Structural Biology Group, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118-2394.

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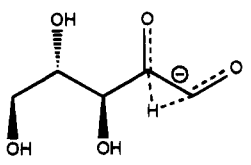
group may be favorable for promoting a hydride shift.

In the X-ray crystallographic structures of the enzyme–substrate complexes the interpretation of the substrate electron density was not straightforward (Allen at al., 1994b; Lavie et al., 1994). Since the crystals are catalytically active, the substrate electron density represents a mixture of substrate and product present at equilibrium. Moreover, as a result of the low affinity of substrates for the enzyme [K_M of 5 and 290 mM for xylose and glucose, respectively (Lambeir et al., 1992)], the observed electron density also represents an average of bound and unbound active sites. Lastly, in comparing the xylose isomerase structure in the native form to that with substrate bound, the elliptical shape of the electron density for one metal ion suggests a metal movement during catalysis. It seemed likely that the questions raised by these phenomena would be answered by the use of a strong-binding, stable inhibitor.

There are no known tight-binding inhibitors for xylose isomerase. The available X-ray crystallographic structures of xylose isomerase with inhibitors bound utilize compounds with affinities similar to that of substrate [e.g., xylitol and sorbitol with K_i values of 0.3 mM and 6.5 mM, respectively (Collyer et al., 1990)]. These structures address the problems of metal movement and turnover of substrate during catalysis but are still plagued by the problem of their low affinity. Moreover, the structure determinations were not performed at high resolution. Low-affinity inhibitors would more likely resemble the ground state represented by enzyme–substrate complexes, while high-affinity inhibitors would more likely resemble the transition state (Haldane, 1930; Jencks, 1969; Wolfenden, 1969) or a strongly bound intermediate (Schloss, 1988).

We designed a transition-state analog for xylose isomerase assuming a hydride-shift mechanism. The structure of the postulated transition state for the hydride-transfer step in such a mechanism for the substrate xylose (**1**) will have partial



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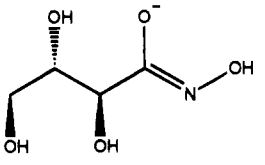


(**2**)

negative charges on the C1 carbonyl oxygen and the C2 oxygen and partial double-bond character between C1 and C2. D-Threonohydroxamic acid (**2**) was chosen to best mimic these desired steric and electronic features. The compounds phosphoglycolohydroxamic acid and phosphoglycolic acid, with double-bond character mimicking that of an ene-diol, have previously been shown to be potent inhibitors of triosephosphate isomerase (Wolfenden, 1969; Collins, 1974).

In this paper we report the synthesis of THA,[1] its kinetic characterization with xylose isomerase, and determination of the enzyme–inhibitor complex structure by X-ray crystallography to 1.6-Å resolution. THA is a potent inhibitor of xylose isomerase with K_i of $\leq$100 nM. The inhibitor is observed to bind to the active site in a manner similar to that of linear glucose. The structure shows a position of one of the two metals that previously could only be inferred from the enzyme–substrate complex structures. Such metal movement has been proposed as a part of the hydride-shift mechanism (Collyer et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992; Lavie et al., 1994).

[1] Abbreviations: THA, D-threonohydroxamic acid; XyI-THA, native xylose isomerase–THA complex.

MATERIALS AND METHODS

Enzyme, Substrate, Metal. The recombinant, overexpressed D-xylose isomerase enzyme from *S. olivochromogenes* was purified as previously described (Allen et al., 1994a) and was shown by SDS–PAGE stained with Coomassie blue to be of >90% purity. The substrate α-D-glucose was obtained from Fisher Scientific and used without further purification. The cofactor magnesium was purchased as the magnesium chloride hexahydrate at the highest purity available from Aldrich Chemical Co. All other reagents were of technical grade or better.

Synthesis of D-Threonohydroxamic Acid. The barium salt of D-threonic acid was prepared from D-threose as previously described (Moore & Link, 1940) by the method in which barium iodide is added before the oxidation (this compound is now commercially available from Aldrich Chemical Co.). To the barium salt of D-threonic acid, 0.07 g (0.26 mmol), suspended in 20 mL of methanol, was added 0.5 g (10 equiv) of Dowex AG50 × 4 (H^+ form) which had previously been dried in methanol by washing on a Buchner funnel. The suspension was swirled on a gel rocker for 1 h in which time the barium threonate completely dissolved. The sample was filtered and the filtrate evaporated to obtain 0.03 g (0.25 mmol, 96% yield) of a clear oil, D-threonolactone: ^{13}C NMR (D_2O) δ 171.9 (s,C1), 69.5 (s,C4), 68.3 (s,C2), 59.1 (s,C3).

To 0.03 g (0.25 mmol) of D-threonolactone, dissolved in 10 mL dry methanol, was added 2.57 mL of a 1 M solution (2.57 mmol) of sodium methoxide. To this solution, 0.18 g (2.57 mmol) of hydroxylamine hydrochloride was added. After a few minutes, a white precipitate was observed. The white precipitate was removed by gravity filtration, and the filtrate was evaporated to obtain a yellow oil. The oil was dissolved in 2 mL of H_2O and run over a 1 × 25 cm Sephadex G-10 gel filtration column at 25 °C. Fractions of 5 mL were collected and evaporated. Fractions containing a light yellow oil were pooled to afford 0.037 g (0.25 mmol, 100% yield) of D-threonohydroxamic acid: ^{13}C NMR (D_2O) δ 171.8 (s,C2), 72.7 (s,C3), 71.6 (s,C4), 63.0 (s,C5).

Enzyme Assays, Analytical Procedures. Xylose isomerase was assayed using α-D-glucose as substrate by detecting the amount of fructose produced over time by the method of Dische and Borenfreund (1951) as previously described (Allen et al., 1994a). For all enzyme assays and crystallization procedures the buffer utilized was 0.025 M HEPES-KOH with 0.01 M $MgCl_2$, pH 7.0. Protein determination was performed by the method of Bradford (1976). All NMR data were acquired on a Varian XL300, 300-MHz NMR spectrometer. The standard to which ^{13}C NMR signals were referred was methanol (50.0 ppm). The ^{13}C NMR spectra were proton decoupled. All experiments were performed at 25 °C unless otherwise stated.

Kinetic Parameters. The value of K_i for THA was determined from Lineweaver–Burk plots using initial velocities with α-D-glucose as substrate. Since the inhibitor was found to be slow-binding, xylose isomerase at 2.5 ×

Table 1: Data Collection and Refinement Statistics for the X-ray Crystallographic Structure of D-Xylose Isomerase Complexed with the Inhibitor THA

Data Collection	
space group	$P2_12_12$
unit cell	$a = 86.90$, $b = 99.05$, $c = 93.65$, $\alpha = 90.0$, $\beta = 90.0$, $\gamma = 90.0$
molecules per asymmetric unit	2
resolution (Å)	44.72−1.60
reflections: total	190 809
unique	83 616
R-merge (% on *I*)	5.1
Refinement	
resolution range (Å)	10.0−1.6
number of unique reflections	83 228
completeness (%)	78
final *R*-factor (%)	21.6
rms deviation	
bond lengths (Å)	0.016
bond angles (deg)	3.0
dihedral angles (deg)	23
improper angles (deg)	1.3
average *B* factor (Å^2)	
main chain	16
side chain	18
water	27
total number of protein atoms	6048
total number of waters	636

10^{-10} M and THA at $(0.5-5) \times 10^{-7}$ M were incubated for 1 h in the assay buffer (after which time no further development of inhibition was observed) before substrate was added to determine the extent of enzyme activity remaining. The substrate was added in a minimal volume, so as not to disturb the equilibrium reached between enzyme and THA. The concentration of THA was based on weight of the compound.

Crystallization and Data Collection. Crystals of D-xylose isomerase were grown by the sitting-drop vapor diffusion method as previously described (Farber et al., 1987), using a total drop size of 100 μL. The crystals were soaked in a solution of THA by the addition of 1 μL of a stock solution of inhibitor to the drop containing the crystal, such that the final concentration of THA was 1.0×10^{-4} M. The crystals were soaked for >48 h and mounted in a quartz capillary. The native xylose isomerase−THA complex (XyI−THA) data set was collected on a Rigaku R-Axis II image plate system mounted on a Rigaku RU200 X-ray generator using a copper anode. The data were reduced using the Rigaku IIc software package supplied by Molecular Structure Corporation (see Table 1), resulting in a total of 190 809 reflections with $I/\sigma(I) > 1$ from 44.7- to 1.6-Å resolution, of which 83 616 were unique reflections (78% of the theoretically possible unique data).

Refinement. The XyI−THA crystals were isomorphous with native xylose isomerase crystals, allowing the native phases to be used to solve the structure. The XyI−THA model was refined using the molecular-dynamics refinement program X-PLOR (Brünger, 1992) with manual rebuilding performed between refinement cycles on an Evans & Sutherland graphics station using FRODO (Jones, 1978). Each monomer in the dimeric asymmetric unit was independently rebuilt. An initial *R* factor of 24.1% was obtained after one cycle of refinement using the coordinates of native xylose isomerase (Lavie et al., 1994). The inhibitor was not included in the initial refinement to avoid phase bias. After the initial round of refinement, the two magnesium ions in the active site were located from an electron density map calculated with the coefficients $(F_{obs} - F_{calc})$ contoured at the 9σ level. Water molecules were added using the program Waterhunter (S. Sugio, unpublished results), refined, and checked individually for their fit into an electron density map calculated using the coefficients $(2F_{obs} - F_{calc})$ with a 1σ cutoff on density. In the second cycle of refinement the model of THA was fitted into an electron density map calculated with the coefficients $(F_{obs} - F_{calc})$ contoured at 3σ. The fit of THA into the electron density was unambiguous. Further refinement cycles were performed with the inhibitor present in the coordinate file. The final cycle of refinement consisted of a simulated annealing omit map (Hodel et al., 1992) where the inhibitor and protein side chains forming ligands to both magnesium ions were omitted from the coordinate file. The final electron-density maps agreed with the original model for the ligands, metals, and inhibitor. The final *R* factor was 21.6% with good geometry for the model (see Table 1).

RESULTS

Kinetics of Inhibition by THA. The compound THA (**2**) was synthesized as described in Materials and Methods and tested as an inhibitor for xylose isomerase in the presence of the substrate glucose. THA is a slow-binding, competitive inhibitor of xylose isomerase with a K_i of 100 nM. The on- and off-rates for slow binding were not determined. Since xylose isomerase is a metal-activated enzyme, 10 mM $MgCl_2$ was included in all assay buffers. The dependence of the inhibition constant upon the magnesium concentration was not determined. Since the hydroxamate group is expected to bind to divalent metal cations, the binding of THA to the enzyme active site would compete with the binding of THA to free metal ions in solution. Therefore, the measured inhibition constant represents a maximum value for K_i.

The Structure of the Xylose Isomerase-THA Complex. In order to assure full occupancy of inhibitor in the enzyme active site, the crystals were soaked in a solution of THA at a concentration of 1000 K_i for >48 h. Data were collected to a resolution of 1.6 Å, and the structure was refined to a final *R* factor of 21.6%. Only minor changes to the starting native xylose isomerase model were made during the refinement, with the final XyI−THA model possessing a rms deviation for Cα carbons of 0.27 Å compared to the native model. Figure 1 depicts THA in its electron density and the surrounding active-site ligands.

THA is bound to xylose isomerase in an extended, linear form. Metal−ligand and metal−inhibitor distances are shown in Figure 2. In the THA model the C2 hydroxyl forms a bridging ligand between Mg-1 and Mg-2, indicating that this hydroxyl is deprotonated (see Figure 2 for numbering). Each magnesium makes two ligands to the THA inhibitor: the C4 hydroxyl and C2 hydroxyl to Mg-1, and the C2 hydroxyl and N1 hydroxyl to Mg-2. All other ligands to Mg-1 are identical to those in the native structure. Mg-2, however, is observed 1.8 Å away from its position in the native structure, moved in the direction of Mg-1. Thus, the metals are separated by a distance of 4.2 Å, 0.9 Å less than in the native xylose isomerase structure (Lavie et al., 1994). As in the native structure, Asp216, His219, and H_2O1700 are ligands to Mg-2. The ligand H_2O1700 (1800 in monomer 2) was also observed in the structures of enzyme plus

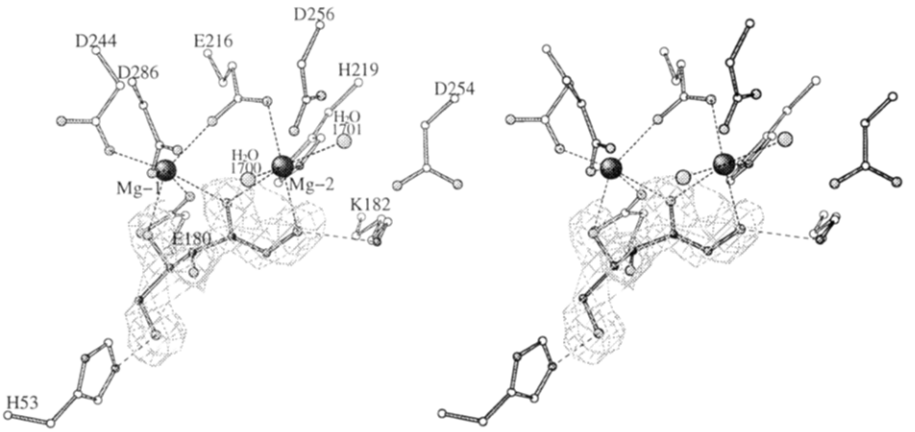


FIGURE 1: Stereoview of the fit of THA into the 1.6-Å resolution electron density map calculated with coefficients ($F_o - F_c$) at the 3.0σ contour level. Also depicted are the molecular models for the two magnesium ions and important active-site amino acid side chains and waters. Metal ligands are represented by lines with short dashes, and hydrogen bonds are represented by lines with long dashes. Residues are labeled using the numbering from monomer 1 of the enzyme. This figure was rendered using the program Molscript (Kraulis, 1991).

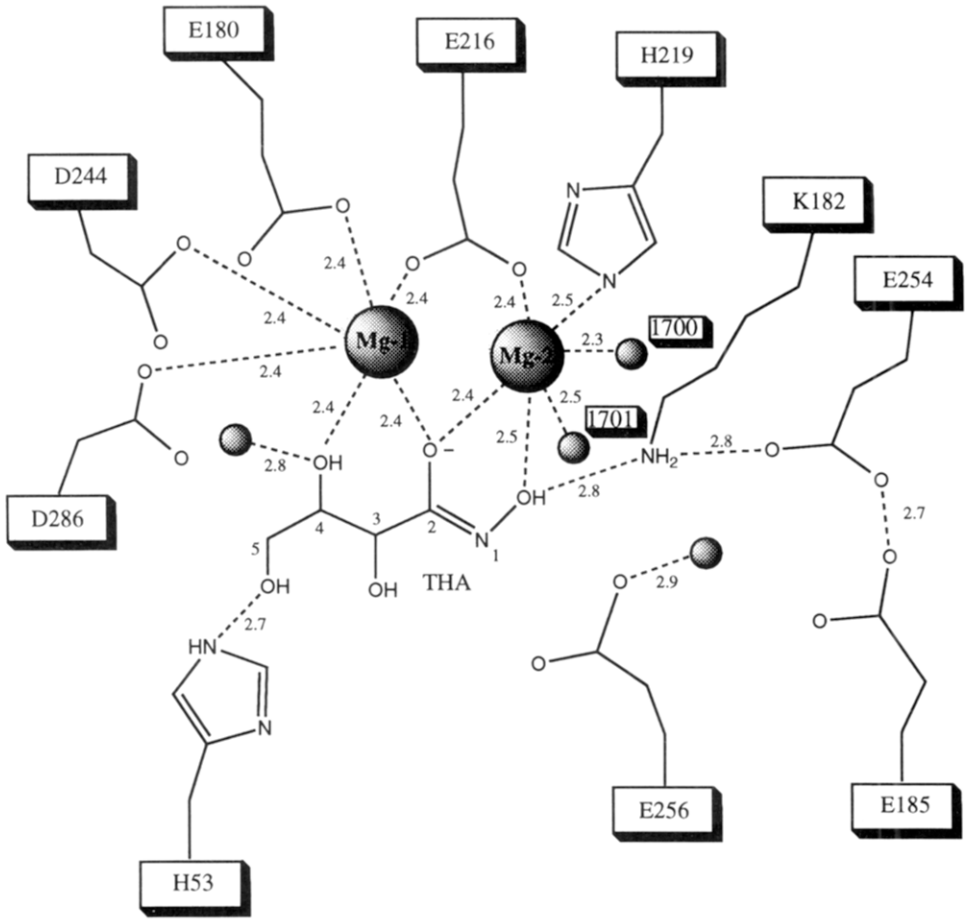


FIGURE 2: Schematic representation of the active site of D-xylose isomerase with the inhibitor THA bound. All ligands and hydrogen bonds formed with THA are shown as well as all metal ligands. Magnesium ions are depicted as large gray spheres and water molecules as small gray spheres. Dashed lines represent ligands which are ≤2.5 Å in length to Mg-1 and Mg-2 or hydrogen bonds ≤3.0 Å in length. Each ligand and hydrogen bond is labeled with the length in angstroms (±0.2 Å). The numbering scheme for the backbone of THA is also shown.

substrate (Collyer et al., 1990; Whitlow et al., 1991; Lambeir et al., 1992; Lavie et al., 1994). However, due to the short ligand−Mg-2 distance of 1.95 Å, it has been modeled by us as a hydroxide in the structures of the native xylose isomerase and the structures of the mutant E180K (which does not carry out the hydride-shift step). Thus, the protonation of this hydroxide also occurs in the interaction of THA with xylose isomerase. Asp254 and Asp256, which form ligands to Mg-2 in the native structure, are too distant from Mg-2 to form metal−carboxylate ligands in the XyI−THA complex. Asp254 is turned away relative to its position in the native structure, forming a new hydrogen bond to Glu185. For Asp256, no significant conformational change in the side chain occurs when the interaction with Mg-2 is lost. These two carboxylates form three ligands to Mg-2, and only two are replaced by THA. For Mg-2 to remain octahedrally coordinated, a second water molecule forms a ligand to this metal (H_2O1701, see Figure 2). The only interactions with

active-site residues made by THA are hydrogen bonds between the C5 hydroxyl and the N1 hydroxyl to His53 and Lys182.

DISCUSSION

The Hydroxamate as a Transition-State Analog. Since the inhibition constant (K_i) for THA was not corrected for the binding of THA to free metal in solution, the observed K_i of 100 nM represents an upper limit. The Michaelis constant for the substrate glucose is approximately 10^6-fold higher than this K_i. The binding constant for the linear inhibitor xylitol (K_i = 0.3 mM) is 3000-fold higher than that of THA (Collyer et al., 1990). Such strong binding suggests, but is not proof, that THA may act as a transition-state analog (Haldane, 1930; Jencks, 1969; Wolfenden, 1969) or as an intermediate analog (Schloss, 1988). The potent binding cannot be attributed solely to the binding of THA to the divalent metals at the active site since the smallest analogous hydroxamate, acetohydroxamic acid, is not a good inhibitor of xylose isomerase, with a K_i of 0.1 mM (Gregory K. Farber, unpublished results). This demonstrates that the C4 hydroxyl plus the hydrogen bond between the C5 hydroxyl and His53 are necessary for strong binding. Electron density interpreted as two magnesium ions is clearly present in the structure with THA bound to the active site. The metal ions refine with an occupancy of 1.0 and low *B* factors, as is the case for the structure of xylose isomerase determined in the absence of THA. Therefore, inhibition is not achieved by pulling the metal out of the enzyme active site. On the basis of the strong binding and the structural similarities with glucose binding (see next section), we postulate that THA resembles the proposed transition state for the enzyme-catalyzed hydride transfer reaction.

The tautomer of THA that is bound can be deduced from the crystal structure. From the interactions made, we suggest that bound THA possesses a negatively charged C2 hydroxyl and a double bond between N1 and C2. Often the resemblance of an inhibitor to a proposed transition state is used to support one mechanism over another mechanism possessing a different transition state. In the case of xylose isomerase, the mechanisms in question, proton transfer versus hydride shift, have transition states which are sterically and electronically similar. As a result, the identification of THA as a transition-state analog cannot be used to distinguish between the two mechanisms.

The Xylose Isomerase−THA Complex. The X-ray structure of xylose isomerase with THA bound was solved at 1.6-Å resolution and refined to a final *R* factor of 21.6%. Although XyI−THA has unique active-site features as compared to the native enzyme, there is no significant difference in the structure of the enzyme main chain (Cα rms deviation 0.27 Å). There is also no significant deviation in overall structure between the native enzyme and the enzyme−substrate complexes (Lavie et al., 1994). These results show that the strong binding of the inhibitor does not induce any gross conformational changes.

THA binds to xylose isomerase in an analogous fashion to glucose by forming ligands with both metals and hydrogen bonds with His53 and Lys182. This is consistent with the kinetic data which demonstrate that the hydroxamate is a competitive inhibitor with the substrate glucose. The inhibitor is bound strongly to the active site as demonstrated by the exceedingly low *B* factors of the inhibitor (average *B* ~12 Å^2) as compared to the enzyme backbone (average *B* ~16 Å^2) and in sharp contrast to the *B* factors of the substrate glucose when bound to xylose isomerase (average *B* ~60 Å^2). There is only a single mode of binding in which the inhibitor may be fitted into the electron density (see Figure 1). The fact that the inhibitor is strongly bound in a fashion so similar to that of the substrate is consistent with the identification of THA as a transition-state analog.

Comparison of the Enzyme−Glucose and Enzyme−THA Complexes. In Figure 3 an overlay of the structures of glucose bound to the enzyme and THA bound to the enzyme shows the many common interactions made with the enzyme and metal cofactors (see Figure 2 and Scheme 1 for numbering of THA and glucose, respectively). It should be noted that THA was designed to mimic the transition state of the reaction with the substrate xylose, so there is no carbon corresponding to C6 of glucose. The comparison is made with glucose since there is no structure available for the *S. olivochromogenes* enzyme with xylose bound. THA and glucose bind in the same location and adopt an extended linear conformation. In both cases two hydrogen bonds are formed between the enzyme and bound compound: one to His53, the other to Lys182. The interaction of substrate with His53, though important, is not essential as shown by site-directed mutants of this residue which retain ~10% of the wild-type catalytic activity (Lee et al., 1990; Gerhardt-Müller, 1993). THA mimics this important interaction of the substrate with the enzyme. It seems that Lys182 may play a role in catalysis, since mutations of this conserved residue abolish activity (Lambeir et al., 1992). Again, THA makes the same interactions as glucose with this moiety. Thus, all hydrogen-bonding interactions between glucose and enzymic amino acid side chains are duplicated by THA.

THA, like glucose, forms further interactions with the two metal cofactors, Mg-1 and Mg-2. In the enzyme−glucose complex structure, the electron density for Mg-2 was interpreted as representing two alternate positions: position-1, which was the sole position occupied by Mg-2 in the native structure, and position-2, which is 0.9 ± 0.2 Å closer to Mg-1 (Lavie et al., 1994). Figure 4 is an overlay of the positions of the two magnesium ions in the free enzyme, enzyme−glucose, and enzyme−THA complexes. In the XyI−THA structure, Mg-2 is observed in a single position (4.2 Å from Mg-1, compared to the 5.1 Å intermetal distance observed in the native structure). This position, which is close to Mg-2 position-2 in the glucose structure, allows the C2 hydroxyl of THA to become a bridging ligand between the two metals. Thus, the XyI−THA has captured a single stable position for Mg-2. The ability of the enzyme in XyI−THA to stabilize this metal position taken together with the evidence for a similar metal position in the glucose structure is strong evidence for metal movement during catalysis.

In XyI−THA, when Mg-2 moves from position 1 to position 2, closer to Mg-1, the bidentate ligands to Asp254 and the monodentate ligand to Asp256 seen in the native structure are no longer present. Asp254 changes conformation to form a new hydrogen bond with Glu185 and Lys182. Mg-2 remains octahedrally coordinated as a result of the formation of two ligands to the N1 and C2 hydroxyls of THA and one ligand to a new water molecule (H_2O1701). Detection of this water molecule in the enzyme−glucose complex structure was not possible because its electron

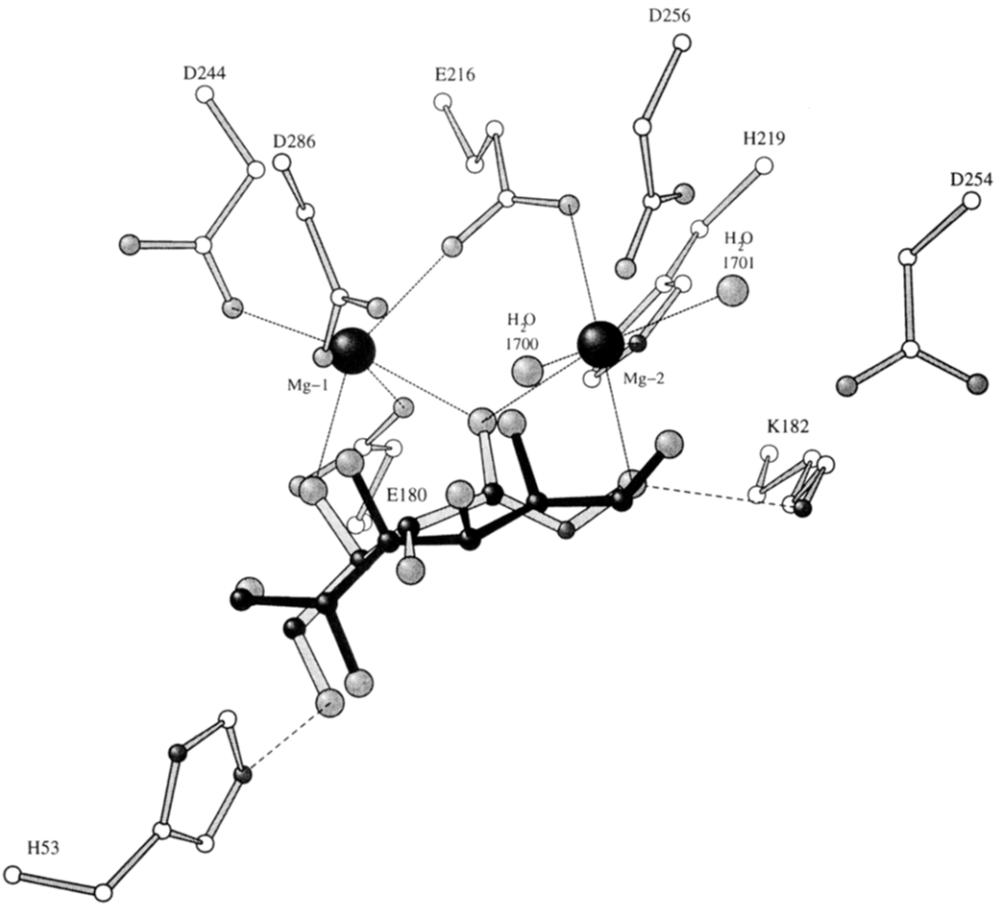


FIGURE 3: Structure of the active site of D-xylose isomerase as determined to 1.6-Å resolution in the presence of the inhibitor THA. The model of α-D-glucose (dark gray bonds) from a 1.96-Å resolution structure, refined to an *R* factor of 16.6% (Lavie et al., 1994), is overlaid with the model of THA (light gray bonds). Metal ligands are represented by lines with short dashes, and hydrogen bonds are represented by lines with long dashes. This figure was rendered using the program Molscript (Kraulis, 1991).

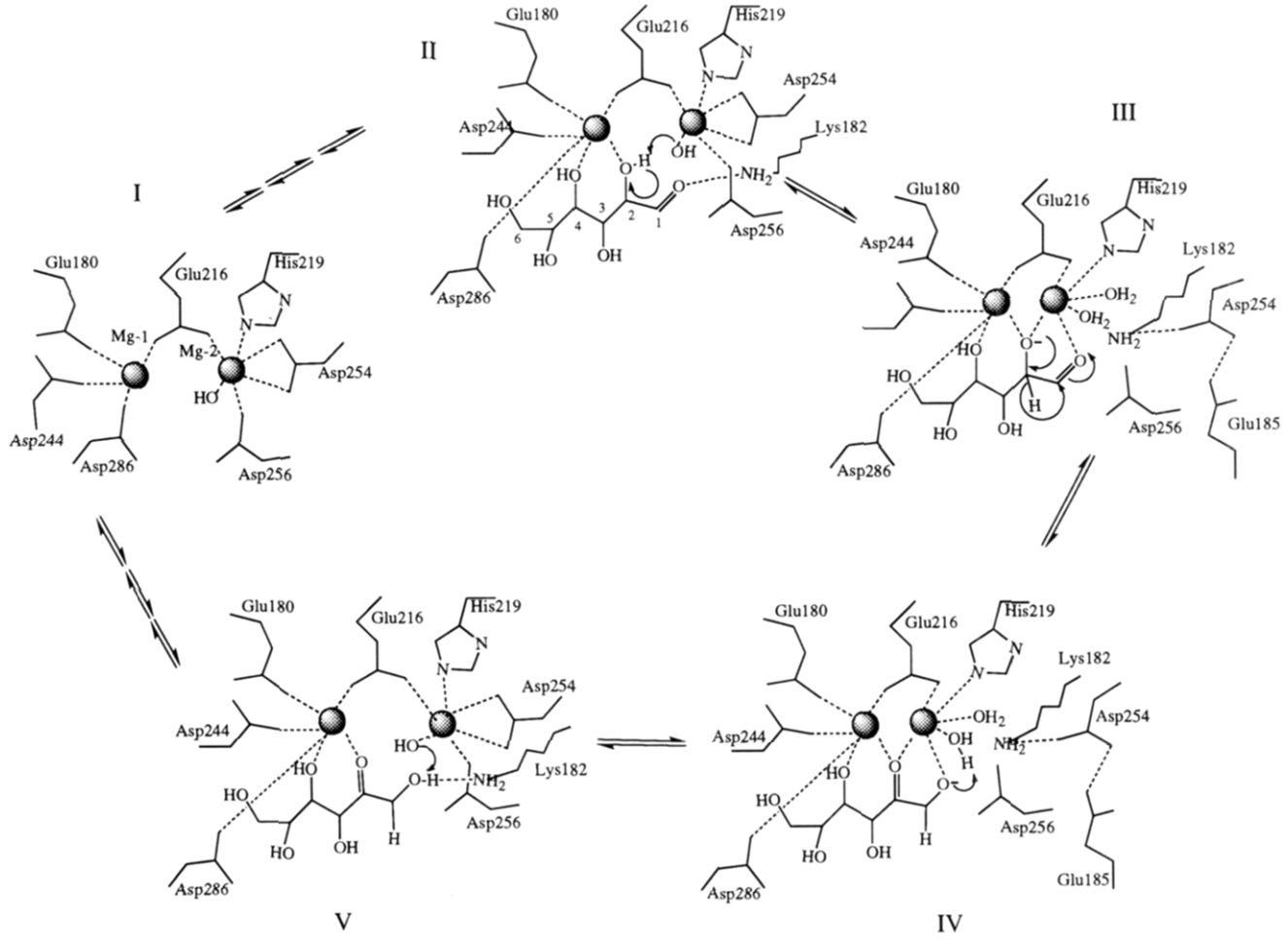
density would have been obscured by that of Mg-2 in position-1. Other evidence of the changes in coordination of Mg-2 such as the alternate conformation of Asp254 is not seen in the enzyme−substrate structures. However, the observation of that alternate conformation would be dependent on the percentage of time the residue occupies this position (subject to kinetic and thermodynamic barriers). We conclude that the structure of XyI−THA is consistent with changes in coordination of Mg-2 upon metal movement during catalysis with no net change in the number of metal ligands.

In the native structure, a hydroxide ion (OH1700) is observed making a ligand to Mg-2 while in the substrate soaked structures this ligand is assigned as a water molecule (based on a Mg-2-oxygen distance of 1.9 Å for hydroxide, as opposed to >2.3 Å for water). In XyI−THA this ligand is at a distance of 2.4 Å, indicating that it is a water molecule. Since THA is a linear molecule (it cannot cyclize like glucose), the protonation of OH1700 to form water must occur in a step following ring opening. The proton donor to the hydroxide may be the bridging C2 hydroxyl of THA which must be deprotonated in order to be a ligand to both Mg-1 and Mg-2. The pK_a of 7.2 obtained from plots of pH versus log V_{max}/K_M for the wild-type enzyme with the substrate glucose has been attributed to the metal-bound OH1700 (Allen et al., 1994b). However, the C2 hydroxyl of glucose in close proximity to the two divalent cations might also posses such a pK_a. One of the roles of the magnesium ions may be to act as Lewis acids in the depression of the pK_a of the C2 hydroxyl. By analogy we propose that in the catalytic reaction the C2 hydroxyl of glucose must become deprotonated and that this proton can be moved to the C1 hydroxyl via the hydroxide ligand that acts as a general acid/base (Scheme 1).

Mechanistic Implications of the Xylose Isomerase−THA Complex. The mechanism of isomerization carried out by xylose isomerase is depicted in Scheme 1. The new position observed for Mg-2 in XyI−THA plus the elliptical density observed for Mg-2 in the enzyme−substrate complexes suggest two alternate positions for that metal. This is consistent with movement of this metal during the isomerization step. The driving force for this movement can be attributed to the negative charge formed by the deprotonation of the C2 hydroxyl by the hydroxide-Mg-2 ligand. Since tautomers of THA have a negative charge on this oxygen, THA may mimic the deprotonated state of linear glucose which is stabilized by the two metals in close proximity. The fact that the ligand to Mg-2 is a water and not a hydroxide ion (as seen in the native structure and in the structure of the mutant E180K) is consistent with the interpretation that the hydroxide form can act as a base to deprotonate the C2 hydroxyl of THA. This step would be followed by the hydride-transfer step which may be concomitant with the transfer of a proton from the protonated hydroxide to the C1 oxygen of the substrate.

Conclusions. The reaction catalyzed by the enzyme xylose isomerase can be considered in two parts: enzyme-catalyzed sugar ring opening and isomerization. The isomerization

Scheme 1: Proposed Hydride-Transfer Mechanism for D-Xylose Isomerase with the Substrate α-D-Glucose (see Discussion)



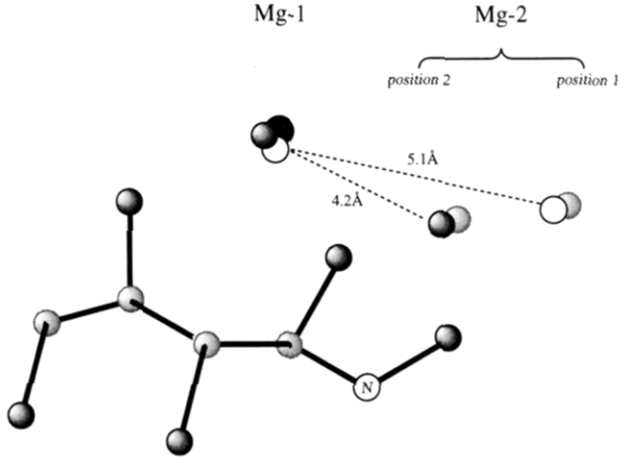


FIGURE 4: Overlay of the positions of Mg-1 and Mg-2 in the native enzyme (white spheres), enzyme−glucose complex (black sphere for Mg-1, light gray spheres for Mg-2), and XyI−THA (dark gray spheres). The two alternate positions for Mg-2 in the enzyme−glucose complex structure are labeled. The relative position of THA in XyI−THA is shown. The intermetal distances for the native and XyI−THA structures are also shown.

reaction is proposed to proceed via a hydride-shift mechanism on the basis of kinetic results (Rose et al., 1969; Rose, 1981; Allen et al., 1994a,b) and crystallographic observations (Collyer et al., 1990; Whitlow et al., 1991; Lambeir et al., 1992; Lavie et al., 1994). THA was designed as a transition-state analog of the isomerization reaction with the substrate xylose. THA is the most potent inhibitor yet reported for xylose isomerase with $K_i \leq 100$ nM. Previous crystallographic studies of xylose isomerase bound to cyclic inhibitors [to examine the ring-opening step (Collyer & Blow, 1990; Blow et al., 1992; Collyer et al., 1992)] and linear inhibitors [to examine the isomerization step (Collyer & Blow, 1990; Collyer et al., 1990; Whitlow et al., 1991; Blow et al., 1992; Jenkins et al., 1992)] were all performed with inhibitors which were not strongly bound.

If THA acts as a transition-state or intermediate analog, then the structures of the native enzyme, enzyme−glucose complex, and enzyme−THA complex represent "snap shots" along the catalytic pathway of xylose isomerase. The structure of the enzyme−glucose complex shows the linear mode of binding of glucose and suggests two alternate positions for one of the metal cations. The enzyme−THA complex demonstrates the ability of enzyme to stabilize a new position for the metal ion such that a deprotonated C2 hydroxyl of glucose is stable, forming a bridging ligand between the two metal ions. If the metal did not move, simultaneous coordination of both metals to this critical oxygen would be impossible. The THA complex also shows the necessary reorganization of ligands to the mobile metal. Comparison of the structure of the free enzyme to the structure of the enzyme bound to substrate (glucose) and inhibitor (THA), supports a change in the protonation state of the metal-bound hydroxide during the isomerization reaction.

The dimetallic center of xylose isomerase provides an electron sink to stabilize the transition state of the hydride transfer step. As demonstrated by the binding of THA, it

may do so through the ligation of both the substrate C1 and C2 hydroxyls. Due to their liganding ability, hydroxamic acids may be general inhibitors any of dimetallic enzymes. Leucine aminopeptidase (2 mol of Zn^{2+}/mol of enzyme) is inhibited by L-leucine hydroxamate (Chan et al., 1982) and enolase (2 mol of Mg^{2+}/mol of enzyme) is inhibited by phosphenoacetohydroxamic acid (Russell & Reed, 1992). It would be of interest to test hydroxamates as inhibitors of other dimetallic enzymes.

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REFERENCES

Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5627−5631.

Allen, K. N., Lavie, A., Petsko, G. A., & Ringe, D. (1994a) *Biochemistry 33*, 1481−1487.

Allen, K. N., Lavie, A., Glasfeld, A., Tanada, T. N., Gerrity, D. P., Carlson, S. C., Farber, G. K., Petsko, G. A., & Ringe, D. (1994b) *Biochemistry 33*, 1488−1494.

Blow, M. D., Collyer, C. A., Goldberg, J. D., & Smart, O. S. (1992) *Faraday Discuss. Chem. Soc. 93*, 67−73.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Brünger, A. T. (1992) in *X-PLOR (Version 3.0) Manual,* Yale University, New Haven, CT.

Carrell, H. L., Glusker, J. P., Burger, V., Manfre, F., Tritsch, D., & Biellmann, J.-F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4440−4444.

Chan, W. W.-C., Dennis, P., Demmer, W., & Brand, K. (1982) *J. Biol. Chem. 257*, 7955−7957.

Collins, K. D. (1974) *J. Biol. Chem. 249*, 136−142.

Collyer, C. A., & Blow, D. M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1362−1366.

Collyer, C. A., Henrick, K., & Blow, D. M. (1990) *J. Mol. Biol. 212*, 211−235.

Collyer, C. A., Goldberg, J. D., Viehmann, H., Blow, D. M., Ramsden, N. G., Fleet, F. J., Montgomery, F. J., & Grice, P. (1992) *Biochemistry 31*, 12211−12218.

Dauter, Z., Terry, H., Witzel, H., & Wilson, K. S. (1990) *Acta Crystallogr. B46*, 833−841.

Dische, Z., & Borenfreund, E. (1951) *J. Biol. Chem. 192*, 583−587.

Farber, G. K., Petsko, G. A., & Ringe, D. (1987) *Protein Eng. 1*, 459−466.

Farber, G. K., Glasfeld, A., Tiraby, G., Ringe, D., & Petsko, G. A. (1989) *Biochemistry 28*, 7289−7297.

Gerhardt-Müller, E. (1993) Ph.D. Thesis, Heidelberg University, Heidelberg, FRG.

Haldane, J. B. S. (1930) *Enzymes,* pp 180−185, Longmans Green, New York.

Hodel, A., Kim, S.-H., & Brünger, A. T. (1992) *Acta Crystallogr. A48*, 851−858.

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp 282−320 and 490, McGraw Hill, New York.

Jenkins, J., Janin, J., Rey, F., Chiadmi, M., van Tilbeurgh, H., Lasters, I., De Maeyer, M., Van Belle, D., Wodak, S. J., Lauwereys, M., Stanssens, P., Mrabet, N. T., Snauwaert, J., Matthyssens, G., & Lambeir, A.-M. (1992) *Biochemistry 31*, 5449−5458.

Jones, T. A. (1978) *J. Appl. Crystallogr. 11*, 268−272.

Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.

Lambeir, A.-M., Lauwereys, M., Stanssens, P., Mrabet, N. T., Snauwaert, J., van Tilbeurgh, H., Matthyssens, G., Lasters, I., De Maeyer, M., Wodak, S. J., Jenkins, J., Chiadmi, M., & Janin, J. (1992) *Biochemistry 31*, 5459−5466.

Lavie, A., Allen, K. N., Petsko, G. A., & Ringe, D. (1994) *Biochemistry 33*, 5469−5480.

Lee, C., Bagdasarian, M., Meng, M., & Zeikus, G. (1990) *J. Biol. Chem. 265*, 19082−19090.

Meng, M., Lee, C., Bagdasarian, M., & Zeikus, G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4015−4019.

Moore, S., & Link, K. P. (1940) *J. Biol. Chem. 133*, 293−311.

Reider, S. V., & Rose, I. A. (1959) *J. Biol. Chem. 234*, 1007−1010.

Rose, I. A. (1981) *Phil. Trans. R. Soc. London B293*, 131−143.

Rose, I. A., O'Connell, E. L., & Mortlock, R. P. (1969) *Biochim. Biophys. Acta 178*, 376−379.

Russell, R., & Reed, G. H. (1992) *Biochemistry 31*, 7166−7173.

Schloss, J. V. (1988) *Acc. Chem. Res. 21*, 348.

Schray, K. J., & Rose, I. A. (1971) *Biochemistry 10*, 1058−1062.

van Tilbeurgh, H., Jenkins, J., Chiadmi, M., Janin, J., Wodak, S. J., Mrabet, N. T., & Lambeir, A.-M. (1992) *Biochemistry 31*, 5467−5471.

Whitlow, M., Howard, A. J., Finzel, B. C., Poulos, T. L., Winborne, E., & Gilliland, G. L. (1991) *Proteins: Struct., Funct., Genet. 9*, 153−173.

Wolfenden, R. (1969) *Nature 223*, 704−705.

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