Probing the Roles of Active Site Residues in D-Xylose Isomerase*

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Richard D. Whitaker‡§, Yunje Cho†, J. aeoh Cho‡‡, H. L. Carrell**††, Jenny P. Glusker***‡‡, P. Andrew Karplus§§, and Carl A. Batt¶¶

From the Department of Food Science and §§Section of Biochemistry, Cell and Molecular Biology, Cornell University, Ithaca, New York 14853 and the **Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

The roles of active site residues His\(^54\), Phe\(^94\), Lys\(^183\), and His\(^220\) in the Streptomyces rubiginosus D-xylose isomerase were probed by site-directed mutagenesis. The kinetic properties and crystal structures of the mutant enzymes were characterized. The pH dependence of diethylpyrocarbonate modification of His\(^54\) suggests that His\(^54\) does not catalyze ring-opening as a general acid. His\(^54\) appears to be involved in anomic selection and stabilization of the acyclic transition state by hydrogen bonding. Phe\(^94\) stabilizes the acyclic-exo transition state directly by hydrophobic interactions and/or indirectly by interactions with Trp\(^137\) and Phe\(^26\). Lys\(^183\) and His\(^220\) mutants have little or no activity and the structures of these mutants with D-xylose reveal cyclic \(\alpha\)-D-xylopyranose. Lys\(^183\) functions structurally by maintaining the position of Pro\(^187\) and Glu\(^186\) and catalytically by interacting with acyclic-extended sugars. His\(^220\) provides structure for the M2-metal binding site with properties which are necessary for extension and isomerization of the substrate. A second M2 metal binding site (M2\(^-\)) is observed at a relatively lower occupancy when substrate is added consistent with the hypothesis that the metal moves as the hydride is shifted on the extended substrate.

D-Xylose isomerase (EC 5.3.1.5) catalyzes the reversible interconversions of D-xylose to D-xylulose and D-glucose to D-fructose. The D-xylose isomerase gene (xyLA) has been cloned from a variety of bacterial sources (1–12) and structures of the Streptomyces rubiginosus (13–15), Arthrobacter (16–18), Actinoplanes missouriensis (19), and Streptomyces olivochromogenes (20, 21) enzymes have been determined by x-ray crystallography. The catalytic domains fold as eight-stranded \(\beta\) barrel motifs and contain a well-conserved active site with two divalent metal ions (17). All D-xylose isomerases require Mg\(^2+\), Co\(^2+\), or Mn\(^2+\) ions for activity, suggesting that they have similar enzymatic mechanisms.

Initially, a cis-enediol mechanism was proposed for D-xylose isomerase (14, 22), similar to the mechanism of triose-phosphate isomerase. However, isotope exchange experiments (23) and crystallographic analyses (15, 16) with various substrates and inhibitors suggests that the reaction proceeds via a metal-mediated hydride shift (Fig. 1a). The currently accepted pathway for the reaction involves the preferential binding of \(\alpha\)-D-xylopyranose (24, 25) followed by ring opening (25), extension of the substrate, and then the hydride shift (15, 16, 26). Recently, Meng et al. (27) have proposed that the hydride shift occurs on the cyclic form of sugar (Fig. 1b).

Site-directed mutagenesis has been used to probe the functions of specific active site residues in D-xylose isomerase (27–32), however, only a few structures of mutant enzymes have been reported (19, 33–35). Kinetic data can be misleading if the substitutions affect the properties of catalytically important residues other than those changed by mutagenesis. For this reason, we have shifted our mutagenic studies from the Escherichia coli D-xylose isomerase (28, 29), which has not been successfully crystallized, to the S. rubiginosus enzyme which readily forms crystals diffracting x-rays beyond 2.0 Å (15, 34, 36). Here, we report the cloning and expression of S. rubiginous xyLA in E. coli, and investigate the roles of His\(^54\) and Phe\(^94\) in anomic recognition, the putative role of His\(^54\) in ring opening, and the functions of His\(^54\), Phe\(^94\), Lys\(^183\), and His\(^220\) in isomerization.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—S. rubiginosus (ATCC 21175) was obtained from the American TypeCulture Collection. E. coli BL21(DE3) (F\(^-\)ompT recF mcrB (λ cts857 ind1 Sam7 nin5 lacUV5-T7 gene I)) was from Novagen. E. coli TG1 (supE hsdS d5 thi Δ(lac-proAB) F\(^{−}\)traD36 proAB lacIq [lacZ M15]) was used to propagate plasmid and bacteriophage M13. M13 mp18 and M13 mp19 DNA were purchased from U. S. Biochemical Corp. and pET11d plasmid DNA was from Novagen. S. rubiginous xyLA cloned into pET11d is called pRDW100 (its construction is described below) and is regulated by T7 RNA polymerase and LacZ, using the strain BL21(DE3) as a host.

Biocultural Reagents—All compounds were reagent grade and purchased from Sigma, except acetaldehyde and 2-thio-\(\alpha\)-D-glucopyranose (THG),\(^1\) which were from Aldrich. The sugar 5-deoxy-\(\alpha\)-xylose was synthesized by aldol condensation of dihydroxyacetone and acetaldehyde, using rabbit muscle aldolase with the cofactor sodium arsenate as a catalyst, and was purified by ion-exchange chromatography,\(^2\) using a scheme similar to that described by Durrwachter et al. (37) for the synthesis of 5-deoxy-\(\alpha\)-fructose.

DNA Isolation, Transformation, and Manipulations—S. rubiginosus was grown in yeast and maltose extract medium supplemented with 34% (w/v) sucrose and chromosomal DNA was isolated as Hopwood et al. (38). Both plasmid and bacteriophage DNA were isolated from cul-

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\(^1\) The abbreviations used are: THG, 2-thio-\(\alpha\)-D-glucopyranose; PCR, polymerase chain reaction; DEPC, diethylpyrocarbonate; M1, metal 1; M2, metal 2; Wat, water; Pipes, 1,4-piperazinediethanesulfonic acid.

\(^2\) S. Seeholzer, unpublished data.
Active Site of d-Xylose Isomerase

FIG. 1.
Fig. 1. Cartoons of the metal-mediated hydride shift (a) (15, 16, 26) and cyclic-hydride transfer mechanisms (b) (27). Residues relevant to this study (His\(^{54}\), Phe\(^{94}\), Lys\(^{183}\), and His\(^{220}\)) are shown, as well as Asp\(^{287}\), which is important in the cyclic-hydride transfer mechanism (b). Metal ions are labeled M1 and M2. In both mechanisms the \(\alpha\)-pyranose (\(\alpha\)-xylene is shown here) binds to the active site and after isomerization, the \(\alpha\)-furanose form (\(\alpha\)-xylene is shown here) is released. In the metal-mediated hydride shift, ring opening is required prior to isomerization, while in the cyclic-hydride transfer ring opening and isomerization occur at the same step.

### Table I

Crystallographic refinement, and kinetic data

| Model | [Sugar] | Space group | Cell dimensions (Å) | Total observations | Uniq. reflections | Resolution (Å) | Completeness % | R\(_{sym}\) % | R\(_{ave}\) % | r.m.s. bond differences (Å) | r.m.s. angle differences (°) | Activity on xylene | K\(_{cat}\) (10\(^{-3}\) s\(^{-1}\)) | k\(_{m}\) (mM) |
|-------|---------|-------------|---------------------|--------------------|------------------|----------------|----------------|-------------|-----------|-----------------------------|-----------------------------|-------------------|----------------|----------------|
| Wild-type | None | 1222 | 93.89, 99.81, 102.92 | 83,657 | 44,249 | 10 to 1.70 | 83 | 0.06 | 0.165 | 0.006 | 1.27 |
| Wild-type/THG | 0.50 | 1222 | 93.86, 99.76, 102.92 | 121,340 | 33,496 | 10 to 1.80 | 85 | 0.07 | 0.155 | 0.013 | 2.50 |
| His\(^{54}\)-Ser | 1.00 | 1222 | 93.88, 99.70, 102.92 | 140,135 | 43,439 | 10 to 1.70 | 93 | 0.07 | 0.167 | 0.016 | 2.60 |
| His\(^{54}\)-Asn | 1.00 | 1222 | 93.96, 99.76, 103.06 | 169,236 | 36,903 | 10 to 1.80 | 91 | 0.09 | 0.158 | 0.021 | 2.80 |
| Phe\(^{94}\)-Ser | 1.25 | 1222 | 93.88, 99.43, 102.76 | 36,346 | 36,346 | 10 to 1.88 | 84 | 0.06 | 0.157 | 0.006 | 1.29 |
| Lys\(^{183}\)-Met | 1.25 | 1222 | 94.08, 100.18, 102.91 | 81,703 | 48,753 | 10 to 1.70 | 89 | 0.06 | 0.166 | 0.006 | 1.30 |
| His\(^{220}\)-Glu | 1.25 | 1222 | 93.89, 99.60, 102.90 | 64,809 | 27,370 | 10 to 2.09 | 85 | 0.05 | 0.153 | 0.007 | 1.35 |
| His\(^{54}\)-Ser | 1.50 | 1222 | 93.64, 99.79, 102.64 | 84,653 | 46,955 | 10 to 1.70 | 70 | 0.08 | 0.179 | 0.006 | 1.34 |

*Structure of the wild-type \(\alpha\)-xylene isomerase (Data set 1) is from Carrell et al. (36).

**Kinetic data with Mg\(^{2+}\) at pH 7.7 and 30°C, using the coupled sorbitol dehydrogenase assay (42).

**Kinetic data with Mn\(^{2+}\) at pH 7.3 and 37°C, from Cha et al. (34).

*Refined without sugar.

**Kinetic data with Mn\(^{2+}\) at pH 7.3 and 37°C, using the method of Dische and Borenfreund (41).
FIG. 2. Electron densities for α-xylose isomerase complexes with ligands. For all of the panels, residues are labeled at CA, metal ions (asterisks) are labeled M1 and M2, and water molecules (asterisks) are labeled Wat. The dashed lines depict metal ligation and hydrogen bonds which have distances \( \leq 3.2 \text{ Å} \) and reasonable geometry. Cross-hatched lines represent electron density of the \( F_o - F_c \) "omit" map. The contour level is 3\( \sigma \) in panels a and b, and 4\( \sigma \) in panels c-f. a, wild-type THG. THG was modeled in two conformations (ALT1 and ALT2) and each was refined at 0.5 occupancy. The main difference in ALT1 and ALT2 of THG is the position of O6. In ALT1, O6 is 2.8 Å from Thr90 OG, and in ALT2, O6 is 2.5 Å from Wat 408. The B factors for both models of THG, M1, and M2 are 17, 10, and 7 Å\(^2\), respectively. Although not indicated in this figure, the positions of both metals shift 0.6 Å compared to the wild-type structure without THG. The protein side chains liganded to M1 shift 0.3 to 0.8 Å as a result of the new position of the metal and also because of the proximity of the sugar hydroxyls O3 and O4. Shifts in the positions of side chains liganded to M2 are also observed, most likely because the ligands to M1 are repositioned. The indole group of Trp137 rotated 9° about \( x_2 \) and the phenyl ring of Phe26* (*, from a neighboring subunit) rotated 5° about \( x_1 \) when THG was added. The amino side chain of Lys183 moved 0.5 Å in order to accommodate the new position of Wat 565 which is hydrogen bonded to O2 of THG. His54 NE2 is 2.9 Å from the ring sulfur and 3.5 Å from the anomeric hydroxyl, and both O3 and O4 are liganded to M1 at 2.3 Å. b, His54-Ser/xylose. D-Xylose was placed in the electron density, but not added during refinement because the electron density of the sugar is weak and in the absence of sugar, water molecules are located close to the positions of the sugar hydroxyls and would complicate an accurate determination of sugar occupancy (not shown). Sugar binding would be comparable to a wild type-xyulose complex (36), except there is no hydrogen bond between Ser54 and O5. Instead, O5 of sugar could hydrogen bond to Wat585 and Wat708, a water not observed in a wild-type/xylose structure (36). No water or metal were added to the electron density near M2, although we suspect that this electron density represents an alternate position for M2 (see Fig. 5). c, Lys183-Met/xylose. α-Xylose was modeled and refined in Lys183-Met. The B factors for the sugar, M1, and M2 were 16, 12, and 13 Å\(^2\), respectively. The binding of α-α-xylopyranose is comparable to wild-type THG, except α-α-xylopyranose lacks C6 and O6. The sugar hydroxyls O3 and O4 are liganded to M1 at 2.4 and 2.3 Å, respectively.
and 100-μl aliquots were removed at various times. The activity of wild-type, His54-Asn, or Phe94-Ser enzymes on the mutarotating D-xylose (200 mM final D-xylose concentration) was determined in the sorbitol dehydrogenase assay, described above.

Crystallography—Crystallization of D-xylose isomerase was as described previously (34). Crystals of D-xylose isomerase were stored in 1 mM MnCl₂, 2 mM Pipes (pH 7.2), and 2 M ammonium sulfate. The concentrations of D-xylose, THG, and xylitol used in crystal soaks containing 1 mM MnCl₂, 2 mM Pipes (pH 7.2), and 2 M ammonium sulfate are shown in Table I, along with crystallographic and refinement data. X-ray diffraction data from crystals of His54 mutants with and without D-xylose were measured on a Nicolet X100A area detector, the data

His²²₀-Glu/xylose, α-β-Xylopyranose was modeled and refined in His²²₀-Glu. The B factor for the sugar is 13 Å². Both metals are observed and metal ligation is similar in the absence of sugar (34), except M₁ is liganded by O3 and O4 of sugar at 2.4 and 2.2 Å, respectively. Glu²²₀ does not ligand M₂, but can interact with O3 of sugar at 3.0 Å. e, His²²₀-Ser/xylose. α-β-Xylopyranose was modeled and refined in His²²₀-Glu. The B factor for the sugar is 14 Å². Both metals are observed and metal ligation is similar to that observed in the absence of sugar (34), except M₁ is liganded by O3 and O4 of sugar at 2.3 and 2.2 Å, respectively. Two water molecules (Wat⁷⁰⁰ and Wat⁷⁰¹) are located near Ser²²₀ and one water (Wat⁷⁰⁰) replaces His²²₀ NE2 as a ligand to M₂. f, His²²₀-Ser/xylitol. The orientation of xylitol is such that O₁ interacts with Lys₁₈₃ NZ and O₅ hydrogen bonds to His⁹⁴ NE2. M₁ is liganded by O₂ and O₄ at 2.2 and 2.3 Å, respectively. The B factor for xylitol is 6 Å². M₂ is absent, alternate conformations (ALT₁ and ALT₂) for the side chain of Asp²⁵⁵ and two new water molecules (Wat⁷⁰⁶ and Wat⁷⁰⁷) are shown. Additional differences are shown and described in Fig. 7.

FIG. 2—continued
Cloning and Expression of *S. rubiginosus* xylA—Two oligonucleotide primers, one of which was complementary to the 5' end and the other complementary to the 3' end of *S. rubiginosus* xylA (12), were used in a PCR of *S. rubiginosus* chromosomal DNA. The annealing temperature for the PCR was varied from 37 to 70°C, and MgCl₂ was added at 1.5–4 mM, but no products were amplified. Some DNA sequences with a high G + C content cannot be amplified unless 7-deaza-dGTP is included in the reaction mixture (43). When 50% of the dGTP in the reaction mixture was substituted with 7-deaza-dGTP, a PCR product with the expected size (1.2 kilobases) was amplified. The DNA sequence of the cloned PCR product had differences at nucleotides 1735, 1857, and 2562 from the reported sequence of Wong et al. (12). The differences at 1857 and 2562 were silent mutations (both TTT instead of TTC), but the difference at nucleotide 1735 corresponded to a Phe→Leu mutation (TTC to CTC) at residue 13 of the protein. The PCR product was cloned into pET11d, under the control of the T7 promoter and LacZ, and several transformants were obtained that expressed a 43-kDa protein only upon addition of isopropyl-1-thio-β-β-galactopyranoside. This protein migrated the same in SDS-polyacrylamide gel electrophoresis as non-recombinant D-xylose isomerase, but this protein from the cell lysate was not soluble and had no enzymatic activity. To see if Leu₁₃ may have caused the insolubility of recombinant d-xylose isomerase, it was mutated to Phe. Also, to potentially improve protein expression, the AGG codon for Arg₅⁰, rarely observed in *S. rubiginosus*, was replaced with the more common CTT codon. The Phe₅⁰ recombinant d-xylose isomerase (wild-type) was soluble and had kinetic properties identical to non-recombinant d-xylose isomerase (34). This expression system was used to overexpress d-xylose isomerases containing active site mutations. As summarized in Table I, six of these mutant enzymes have been kinetically characterized and crystallographically studied alone and in complex with d-xylose, and in one case, with xyitol.

Wild-type d-Xylose Isomerase Complexed with THG—The structure of the Arthrobacter d-xylose isomerase complexed with the cyclic inhibitor THG (Kᵣ₃ = 33 mM) showed that His₅⁴ is approximately 3 Å from both the ring sulfur and atom O₁, the anomeric oxygen (16). The structure of the *S. rubiginosus* d-xylose isomerase with THG was determined to assess the His₅⁴ interactions in this enzyme with a cyclic α-pyranose sugar. THG can clearly be seen in the electron density map (Fig. 2a). The binding of THG in *S. rubiginosus* is comparable to that observed in the Arthrobacter enzyme, except His₅⁴ NE₂ is 2.9 Å from the ring sulfur and 3.5 Å from the anomeric hydroxyl and two alternate positions for O₆ are seen in the electron density map (Fig. 2a). One position of O₆ (ALT1) is nearly the same as that reported by Collyer et al. (45) and forms a hydrogen bond to Thr⁹⁰ OG. In the other position (ALT2), O₆ interacts with Wat⁴⁰⁹. The different positions of O₆ did not affect the hydrogen bonding or metal ligation of the other sugar hydroxyls. The B factors for M1 and M2 were 10 and 7 Å², respectively, indicating that the occupancy of both metals is high and their mobility is low.

pₖₐ of His₅⁴—The ring-opening mechanism proposed by Collyer et al. (16) and Whitlow et al. (15) involves His₅⁴ acting as base to remove a proton from the anomeric oxygen of the substrate. However, in the wild-type/THG structure described above, His₅⁴ is closer (2.9 Å) to the ring sulfur than the anomeric hydroxyl (3.5 Å), suggesting that it might acid catalyze ring opening of the analogous sugar, D-xylose, via the protonation of the ring oxygen. Also, the His₅⁴ imidazole is held in place by a hydrogen bond to the carboxylate of Asp³⁷ (36) which may help to raise the pKₐ of His₅⁴. The protonation state of His₅⁴ was determined by chemical modification with DEPC, a reagent that reacts with histidines only in their deprotonated form (46). Previous studies had shown that His₅⁴ is responsible for DEPC sensitivity (31, 47–49), and this was reconfirmed in this study. The wild-type enzyme retained only 9.3% activity when incubated with DEPC (0.157 mM) at 25°C for 10 min at pH 7.0, while His₅⁴-Ser and His₅⁴-Asn mutants (described below) retained 100% activity after incubation with DEPC. To determine the pKₐ of His₅⁴, the rate of DEPC inactivation was measured in the pH range of 5.5–7.5 (Fig. 3). The inflection point (pKₐ) from two separate DEPC inactivation experiments was 6.40 ± 0.01. Parallel studies, using the same enzyme preparation, yielded a pH optimum for catalysis of D-xylose between pH 8 and 9 (Fig. 3).

Kinetic Properties of His₅⁴ Mutants—To examine the role of His₅⁴ in anameric recognition, ring opening, and isomerization, His₅⁴ was changed to Ser, Asn, and Asp. The His₅⁴-Ser and His₅⁴-Asn mutants had activity (Table I), while His₅⁴-Asp was insoluble and crude cell extracts containing this mutant enzyme had no activity. The pKₐ values for the His₅⁴-Ser and His₅⁴-Asn enzymes on D-xylose were 12- and 5-fold lower and the Kₘ values were 2- and 7-fold higher than that observed for wild-type. It has been suggested that the lower kₗₑ₅₅ of His₅⁴ mutants is due to the loss of a hydrogen bond to O₅ of the acyclic-extended substrate (30, 31). The importance of this interaction was tested by examining the kinetics of D-xylose isomerase on the acyclic sugar 5-deoxy-D-xylulose. The His₅⁴ mutants had kₐ values on the 5-deoxy-sugar similar to D-xylose, while the wild-type had a 20-fold lower kₗₑ₅₅ on the deoxy-sugar than on D-xylose (Table II). The Kₘ values on the 5-deoxy-sugar for the mutants (22.5 and 35.0 mM) were similar to the value for the native enzyme (22.0 mM).

Anomeric Specificity of D-Xylose Isomerase—D-Xylose isomerase preferentially binds the α-pyranose form of aldose (24, 25). It has been reported that His₅⁴ is important for recognizing the α-pyranose (30) and that the Phe⁴⁰ side chain prevents the β-pyranose form from binding by steric exclusion of the O₁ hydroxyl (15, 50). To possibly allow β-pyranose binding, the Phe⁴⁰ side chain was replaced with the smaller hydrophilic Ser residue. The kinetics of this mutant show a 5-fold decrease in the kₛₑ₅₅ and a 7-fold increase in Kₘ with D-xylose as a substrate.
Table I. The roles of His$^{54}$ and Phe$^{94}$ in anomeric recognition were directly explored by dissolving crystalline $\alpha$-D-xylo-lose in water and assaying relative activity, using experimental conditions similar to Lambier et al. (30). Both wild-type and Phe$^{94}$ Ser had the highest activity immediately after $\alpha$-D-xylo-lose was dissolved and showed diminished activity as the $\beta$-anomer was forming, until an activity equilibrium was reached (Fig. 4). His$^{54}$-Asn retained 100% activity over time (Fig. 4), indicating that this mutant has no preference for the $\alpha$-pyranose over the $\beta$-pyranose and is consistent with the results of Lambier et al. (30).

Structures of His$^{54}$-Asn and His$^{54}$-Ser—In wild-type $\alpha$-D-xylo-lose isomerase, His$^{54}$ NE2 hydrogen bonds to one water (Wat$^{133}$) and His$^{54}$ ND1 interacts with Asp$^{57}$ OD1 (36). The structures of His$^{54}$-Ser and His$^{54}$-Asn show only slight structural differences, and these differences are localized to the region around the substituted histidine. The electron densities of the His$^{54}$, Ser and His$^{54}$-Asn side chains appear well ordered and the average temperature factors for the Ser$^{54}$ and Asn$^{54}$ side chains are 17 and 13 Å$^2$, respectively. In the His$^{54}$-Ser structure, the side chain hydroxyl of Ser$^{54}$ rotated approximately 150° from the position observed for His$^{54}$ CG. In this position, Ser$^{54}$ OG hydrogen bonds to the amide nitrogen and the OD carboxylate of Asp$^{57}$ at 3.1 and 2.8 Å, respectively. One new water molecule (Wat$^{708}$) is observed 1.4 Å from the position normally occupied by His$^{54}$ NE2 and it forms a hydrogen bond to Wat$^{133}$ and Wat$^{585}$. The latter solvent molecule is 2 Å from its position in the native enzyme. In His$^{54}$-Asn, the Asn$^{54}$ ND hydrogen bonds to Asp$^{57}$ OD1 at 2.7 Å, replacing the His$^{54}$ ND1-Asp$^{57}$ OD1 interaction, and Asn$^{54}$ OD hydrogen bonds to Wat$^{585}$ at 2.9 Å. No other differences greater than 0.3 Å for either structure were observed.

Structure of His$^{54}$-Ser Complexed with $\alpha$-D-xylo-lose—In many structures of wild-type $\alpha$-D-xylo-lose isomerase complexed with $\alpha$-D-xylo-lose, His$^{54}$ NE2 hydrogen bonds to O5, Lys$^{183}$ NZ interacts with O1, and both O2 and O4 are liganded to M1 of the extended sugar (15, 36). Although 1.5 M $\alpha$-D-xylo-lose was used to soak a His$^{54}$-Ser crystal, the electron density attributed to the sugar is disordered. Since several different conformations of cyclic, pseudo-cyclic (i.e. the sugar ring is cleaved and the sugar is somewhat cyclic) and acyclic-extended forms of $\alpha$-xylo-lose could fit into the electron density, sugar was omitted from the model. The position of M1 and M2 shift 0.2 and 0.4 Å, respectively, when $\alpha$-D-xylo-lose is added, but the B factors of M1 and M2 remain low (10 Å$^2$). The other differences in the $\alpha$-xylo-lose complex are 0.4 Å shifts in the side chains of the metal ligands Glu$^{181}$, Glu$^{217}$, Asp$^{255}$, and Asp$^{287}$, equivalent to those observed in the wild-type/THG structure.

Structure of Lys$^{183}$-Met—In the metal-mediated hydride shift mechanism, Lys$^{183}$ would assist in holding the substrate in the proper orientation and polarizing O1 by hydrogen bonding (15, 16, 30). In the cyclic-hydride transfer, Lys$^{183}$ has no direct catalytic role (27). Lys$^{183}$ was substituted with Met and

![Fig. 4. Anomeric preference of wild-type (C), Phe$^{94}$-Ser (A), and His$^{54}$-Asn (B) $\alpha$-xylo-lose isomerase. Crystalline $\alpha$-D-xylopyranose (96%) was dissolved, aliquots were removed, and the relative rate on the mutarotating sugar was assayed at different time intervals by the coupled-orbital dehydrogenase assay as described under "Materials and Methods."](http://www.jbc.org/)

An equivalent, low occupancy site for M2 (M2') has been described for the native S. rubiginosus $\alpha$-D-xylo-lose isomerase complexed with $\alpha$-xylo-lose (15) and is observed in our wild-type $\alpha$-xylo-lose complex. The M2' position has been proposed to stabilize the transition state of the acyclic-extended sugar in the metal-mediated hydride shift mechanism through interactions with O1 and O2 of sugar (15).

In our wild-type structure, the relative difference peak heights calculated from a $F_{o}-F_{c}$ map with M2 and M2' omitted is 0.12 eÅ$^2$ and 0.0092 eÅ$^2$ for M2'. This yields an estimated M2:M2' ratio of 13:1. Since the occupancy of M2' is 13-fold lower and side chain atoms and water molecules have fewer electrons than Mn$^{2+}$, low occupancy positions for the ligands of M2' would not be seen above the noise in the electron density, as noted by others (15). The potential liganding of the metal at M2' by Wat$^{409}$, His$^{220}$ NE2, both carboxylate oxygens of Glu$^{217}$, and O1 and O2 of sugar is shown in Fig. 5.

Structure of Phe$^{94}$-Ser—In the wild-type enzyme, Phe$^{94}$ is in the active site near the side chains of Trp$^{137}$ and Phe$^{26}$ (*, from a neighboring subunit). As a result of replacing the larger Phe with the smaller Ser, two alternative positions for Ser$^{94}$ OG hydrogen bonding to the protein and metal liganding are compared to the wild-type enzyme. When Phe$^{94}$-Ser is added to THG, the electron density attributed to the sugar is disordered. Since several different forms of $\alpha$-xylo-lose could fit into the electron density, sugar was omitted from the model. The position of M1 and M2 shift 0.2 and 0.4 Å, respectively, when $\alpha$-xylo-lose is added, but the B factors of M1 and M2 remain low (10 Å$^2$). The other differences in the $\alpha$-xylo-lose complex are 0.4 Å shifts in the side chains of the metal ligands Glu$^{181}$, Glu$^{217}$, Asp$^{255}$, and Asp$^{287}$, equivalent to those observed in the wild-type/THG structure.

Structure of Phe$^{94}$-Ser Complexed with $\alpha$-D-xylo-lose—In the wild-type THG structure, CZ of Phe$^{94}$ is 3.8 Å from C1 of THG. In structures of S. rubiginosus $\alpha$-D-xylo-lose isomerase with an acyclic-extended pentose sugar bound in the active site (15, 36), Phe$^{94}$ is far from C1 (6.6 Å) and is closest to C3 (5.4 Å) of the sugar. When $\alpha$-xylo-lose is added to Phe$^{94}$-Ser, the electron density attributed to the sugar is disordered. Since several different conformations of cyclic, pseudo-cyclic (i.e. the sugar ring is cleaved and the sugar is somewhat cyclic) and acyclic-extended forms of $\alpha$-xylo-lose could fit into the electron density, sugar was omitted from the model. The position of M1 and M2 shift 0.2 and 0.4 Å, respectively, when $\alpha$-xylo-lose is added, but the B factors of M1 and M2 remain low (10 Å$^2$). The other differences in the $\alpha$-xylo-lose complex are 0.4 Å shifts in the side chains of the metal ligands Glu$^{181}$, Glu$^{217}$, Asp$^{255}$, and Asp$^{287}$, equivalent to those observed in the wild-type/THG structure.
this mutant enzyme had no detectable activity (Table I). In wild-type D-xylose isomerase, Lys183 NZ hydrogen bonds to Asp255 OD1, Glu186 O, and Wat565 (Fig. 6, thin lines). Since Asp255 is a M2 ligand, it was possible that the Met183 substitution indirectly abolished activity by perturbing the structure around M2. The Lys183-Met structure was determined and no large differences (>0.4 Å) in the conformations of residues 183 or 255 were observed, however, major structural changes occurred outside the active site at Glu186 and Pro257 (Fig. 6) and to a lesser extent in the active site at Phe269 and Trp317.

The loss of the Lys183, Glu186 hydrogen bond and the increased van der Waals radii associated with the Met side chain leads to the most dramatic structural change, with the Glu186, cis-Pro257 peptide bond flipped from the cis to the trans conformation (Fig. 6). The Glu186 cis angles change from 76/107° to 116/−65, both of which are angles normally not observed for non-glycine residues (51). The energy of a Xaa-trans-Pro peptide bond is roughly 5-fold more favorable than a Xaa-cis-Pro peptide bond (51).

Structure of Lys183-Met Complexed with D-xylose—When D-xylose is soaked into a Lys183-Met crystal, electron density indicative of the cyclic α-D-xylopyranose is observed (Fig. 2c). The binding of α-D-xylopyranose is comparable to that of THG in wild-type, except α-D-xylopyranose lacks C6 and O6. There is no significant movement (>0.2 Å) of either metal in the Lys183-Met structure compared to the Lys183-Glu or the wild-type/THG structures. His220 NE2 is 2.7 Å from the ring-oxygen and is 3.4 Å from the anionic oxygen. The positions of Glu186, Pro257, Phe269, and Trp317 remain as in the unliganded Lys183-Met structure.

Structures of His220-Glu and His220-Ser Complexed with D-xylose—In wild-type D-xylose isomerase, His220 is the terminal residue on a short α-helix that includes residues 216–220. At neutral pH, the imidazole ND1 is protonated and hydrogen bonded to Pro182 O, and NE2 of the imidazole is ligated to M2. It was previously reported that substitutions to His220 result in almost a complete inactivation of D-xylose isomerase (Table I) (34) and a decrease in thermostability. Structures of His220-Glu, Ser, His220-Asn, and His220-Glu show that both M1 and M2 are still bound, but there are some perturbations (0.3 to 0.4 Å differences from the wild-type) in the protein around M2 (34). Crystal structures of His220-Glu and His220-Ser with D-xylose were determined to discover why these mutants have almost no activity. Both His220 mutants have clear ligand electron density into which α-D-xylopyranose (Fig. 2, d and e) was fitted and refined at full occupancy. The positions of M1 and M2 in the His220-Glu and His220-Ser do not differ greater than 0.3 Å from the sites they occupy in the wild-type/THG structure. The temperature factors for M1 are very similar in the wild-type/THG, His220-Ser/xylose, and His220-Glu/xylose structures (all 8 to 10 Å²); the temperature factor for M2 is slightly higher in His220-Ser/xylose (14 Å²) than wild-type/THG (7 Å²) and is significantly higher in His220-Glu/xylose (26 Å²), indicating that M2 is either more mobile or has a lower occupancy in the His220 mutants.

The largest change observed when D-xylose is added to His220-Glu, is a 0.4 Å shift in the position of Asp287 OD. Glu220 does not replace the function of His220 NE2 in serving as a ligand to M2 (Fig. 2d), as previously observed in His220-Glu without sugar (34), but can interact with O3 at 3.0 Å. The position, coordination, and temperature factors for both metals is similar in His220-Glu with or without D-xylose (34).

The structure of His220-Ser/xylose shows only slight rotation of Trp137 and Phe269, but these positions are observed in the wild-type/THG structure. There were no other significant changes (>0.3 Å) in the positions of the metals or their ligands compared to His220-Ser without sugar (34). The two new water molecules seen near the Ser220 side chain, Wat700 and Wat701 as well as the ligation of M2 by Wat700 in His220-Ser/xylose (Fig. 2e), are also observed in His220-Ser without D-xylose (34).

Structures of His220-Glu and His220-Ser Complexed with Xylitol—Xylitol is an acyclic polyol inhibitor of D-xylose isomerase. The Kᵅ value of xylitol for the Streptomyces violaceoruber D-xylose isomerase is 0.45 mM (52). The structure of wild-type S. rubiginosus D-xylose isomerase complexed with xylitol has been reported and xylitol is observed bound in an extended conformation and oriented such that O1 hydrogen bonds to Lys183 NZ and O5 with His54 NE2 (14, 15). Both metals are observed and M1 is liganded by the sugar hydroxyl O2 and O4.

Xylitol was added to His220-Ser to mimic the acyclic sugar binding typically seen in native D-xylose isomerase-xylitol complexes. The electron density of xylitol is well defined and xylitol binds in an extended conformation (Fig. 2f). However, significant structural changes are observed in and near the active site.
of His220, Ser/xylitol (Fig. 2f and Fig. 7). These changes include the disappearance of M2, the addition of two new waters (Wat706 and Wat707), alternative conformations for the side chains Asp255 and Glu186, rotation of Phe26*, and changes in the position of both M1 and its carboxylate ligands. The electron density attributed to metal (Mn$^{2+}$) at both M1 and M2 in all of the structures described in this work can easily be seen at 12 to 15Å. However, in His220-Ser/xylitol only density around M1 is seen at 12 to 15Å, and no peaks greater than 5Å are observed around the M2 site, strongly suggesting that M2 is not bound. In order to accommodate O1 of xylitol, Wat705 has moved 1.8Å, and is only 1.0Å from the site once occupied by M2. Glu217 OE2 shifted 1.2Å and now hydrogen bonds to Wat705 at 2.9Å and to a new water molecule (Wat707) at 2.7Å.

**DISCUSSION**

The *S. rubiginosus* xylA was cloned via PCR and expressed in E. coli. The cloned PCR fragment contained several nucleotide sequence differences, one of which gives rise to a Phe13→Leu mutation. In *S. rubiginosus* d-xylose isomerase, Phe13 and the side chains of Val18, Leu43, Phe286, and Phe288 are buried in a hydrophobic pocket, approximately 8.0Å from the active site. Although we would not predict the Leu13 substitution to affect activity, it resulted in the formation of insoluble inclusion bodies. After Leu13 was changed back to Phe, soluble, active xylitol was obtained, suggesting that the Phe13-Leu mutation caused problems with protein folding.

Isomerization—A cyclic-hydride transfer isomerization mechanism has been proposed (Fig. 1b; Ref. 27) to unify the observations: (i) that rate-limiting step in the reaction is the C2-C1 hydride transfer and (ii) both the wild-type and mutant α-xylose isomerases from Clostridium thermosulfurogenes display a 2.5-fold difference in $k_{cat}$ for α-o-glucose as compared to β-o-glucose (45). Because ring opening of both α and β-o-glucose produces chemically identical molecules, Meng et al. (27) argued that the different $k_{cat}$ values necessitate that the hydride shift must be concerted with ring opening.

While we do not have an alternative explanation for the results of Meng et al. (27) the data from our study and others are not readily consistent with the cyclic-hydride transfer mechanism. First, Lys183-Met, His220-Ser, and His220-Glu complexed with d-xylose and wild-type/THG show that O2 is 4.5Å from the closest of the carboxylate oxygens (OD 2) of Asp287, and that this carbohydrate oxygen is liganded to M1. It is highly unlikely that Asp287 could be a base catalyst given the orientation of the α-pyranose. Second, in the cyclic-hydride transfer mechanism, M2 and Lys183 would have no direct function in catalysis, even though our biochemical and crystallographic data indicate that both are important catalytically. The structure of His220-Ser and His220-Asn do not show any major structural perturbations at M1 or Asp287, yet these mutants are almost totally inactive (34). Structures of His220-Ser, His220-Glu, and Lys183-Met with d-xylose have α-d-xyl-C186-xylose isomerase and Lys183-Met with d-xylitol in the active site and display binding similar to wild-type/THG. Additional evidence for a role of M2 in catalysis is shown by substitutions at residues located near M2 ligands. Substitutions to Lys294 (Lys289 in S. rubiginosus) and Glu186 change the metal specificity and pH profile of the M. auxorossis d-xylose isomerase (30, 35) and structures of the Glu186-Gln mutant with different metals show differences at the M2 site but not at M1 (35). Lys183 is essential for activity. Replacement of Lys183 with Met, Ser, Gln, or Arg renders the enzyme inactive (30). The structures of Lys183-Met reveals no dramatic changes to M2, or their ligands and is further evidence against the cyclic-hydride transfer. However, the Lys183-Met structures should be interpreted with caution since other structural changes occur in this enzyme.

With the exception of the results of Meng et al. (27), the aforementioned data and observations are consistent with the proposed metal-mediated hydride shift mechanism (Fig. 1a) (15, 16). When d-xylose, d-glucose, xylitol, or sorbitol are added to d-xylose isomerase, an acyclic-extended form of sugar is observed which may represent substrate, product, or intermediate(s) (15, 16, 18, 19, 21, 26, 35, 36). The orientation of substrate is such that O1 is hydrogen bonded to Lys183 NZ, O5 is hydrogen bonded to His154 NE2, and O2 and O4 of the extended sugar are liganded to M1 (Fig. 1a). In the metal-mediated hydride shift mechanism proposed by Collyer et al. (16), Whitlow et al. (15), and Lavie et al. (26), a M2 bound hydroxide initiates isomerization by removal of the O2 hydrogen. M2 would shift approximately 1.9Å to a site where it could be liganded by both carboxylate oxygens of Glu217, the imidazole NE2 of His220, Wat409, and both O1 and O2 of substrate. The
resulting negative charge on O2 would be stabilized by both M1 and M2 interactions. The C2 hydrogen would transfer directly to the partially positively-charged C1 and either a water molecule or Lys183 NZ might protonate O1. Lys183 NZ would help stabilize the sugar by interacting with O1 of the acyclic-extended substrate, intermediate(s), and product.

The results from this study and the possible functions of His54, Phe94, Lys183, and His220 in the reaction are discussed below in terms of the metal-mediated hydride shift mechanism. His54 and Phe94—Prior to isomerization, sugar binding and ring-opening must occur. In solution, d-xylose and d-glucose exist predominately as hemiacetals, forming six-member pyranose rings with two anomeric forms (α and β) that differ in their stereochemistry at C1. NMR and kinetic experiments have shown that d-xylose isomerase prefers the α-pyranose form of hemiacetal (24, 25). His54 appears to be one determinant of the preference for the α-pyranose, by interacting with the anomeric hydroxyl (30). The results with His54-Asn suggest that this mutant enzyme has no preference for the α-pyranose over the β-pyranose and is consistent with the proposal of Lambir et al. (30). It was proposed that Phe94 could be involved in anomeric selection by preventing β-pyranose binding due to unfavorable interactions between the hydrophilic anomeric hydroxyl and the hydrophobic phenyl ring (15, 50). Although changing the phenyl side chain to the smaller Ser decreased activity (Table I), this mutation did not affect anomeric specificity, indicating that Phe94 is not a major determinant in anomeric recognition.

Phe94 does have a role in maintaining the structure of the active site, sugar binding, and stabilization of the transition state. The structure of Phe94-Ser shows two new waters and changes in the positions of both the nearby hydrophobic side chains of Phe260 and Trp137. Upon addition of d-xylose, disordered density is observed in the active site, to which different forms of sugar could be fit. As mentioned previously, the Phe94-Ser mutation has a reduced k_{cat} and an increased K_m (Table I). These results suggest that Phe94 is involved in stabilization of the acyclic extended transition state, through hydrophobic interactions directly with the sugar or indirectly by interacting with the nearby Trp137 and Phe260 side chains which in turn contact the sugar.

After binding of the α-pyranose, d-xylose isomerase catalyzes ring opening (25). It was suggested that His54 NE2 could act as a catalytic base, abstracting a proton from the anomeric oxygen (O1) of the α-pyranose, and facilitating sugar ring cleavage (15, 16). However, in the wild-type/THG structure, His54 NE2 is closer to the ring-sulfur of THG (2.9 Å) than the anomeric hydroxyl (3.5 Å), suggesting that the His54 imidazole could act as an acid catalyst and thus protonate the ring oxygen. The pH dependence of DEPC modification indicates that the pK_a of His54 is 6.40 ± 0.01. From kinetic studies of the Mg^{2+}-activated Arthrobacter d-xylose isomerase with fructose as a substrate, it was reported that the pK_a for a group controlling K_m was 6.2 ± 0.1 and it was suggested that this group was His54 (His54 in S. rubiginosus) (53). The pK_a of His54 determined by DEPC inactivation is consistent with the kinetic study of the Arthrobacter enzyme and indicates that at the pH optimum of the enzyme (near 8.0–9.0), His54 NE2 is deprotonated and probably could not be an acid catalyst in ring opening. Changing His54 to non-basic residues (Ser, Ala, Asn, and Gln) reduces activity, but the rate-limiting step in the overall reaction has been reported to be isomerization, not ring opening (30, 31). The acidic sugar 5-deoxy-d-xyulose can be used as a substrate, indicating that enzyme-catalyzed ring opening is not a step absolutely required prior to isomerization. Other than crystallographic observations noting the proximity of His54 to cyclic sugars (15, 16), there is no biochemical evidence suggesting that His54 catalyzes ring opening.

Initial site-directed mutagenesis experiments of the E. coli d-xylose isomerase suggested that His54 (His54 in S. rubiginosus) might be catalytically important in a cis-enediol mechanism, as replacement with Arg or Tyr rendered the enzyme inactive (28). However, when His54 was replaced with smaller residues (Ala, Ser, Asn, Asp, Glu, and Gln), these mutant enzymes retained activity (29–31), proving that His54 is not crucial for catalysis and providing further evidence against the cis-enediol mechanism. His54 does, however, interact with the transition state. In most crystal structures of d-xylose isomerase complexed with d-xylose, His54 interacts with O5 of an acyclic-extended form of sugar. Biochemical evidence for a His54-sugar interaction is shown by comparing the kinetics of the wild-type enzyme and His54 mutants on d-xylose, d-xyulose, and 5-deoxy-d-xyulose (Table II). Both mutants have lower k_{cat} and higher K_m values.
on D-xylose and D-xylulose when compared to the native enzyme. This is likely due to the loss of a hydrogen bond from O5 of the extended sugar to the substituted side chain. The interaction with the C5 hydrogen of 5-deoxy-D-xylulose and His54-NE2 of the native enzyme is clearly unfavorable, as reflected in both the diminished \( k_{cat} \) and elevated \( K_m \). A possible reason for the His54 mutations having a similar \( k_{cat} \) on D-xylose and 5-deoxy-D-xylulose is that their smaller side chains cannot hydrogen bond to O5 of D-xylose, and the replaced side chains are not close enough to interact unfavorably with the C5 methyl group of 5-deoxy-D-xylulose.

Additional evidence for the importance of the hydrogen bond from His54 to O5 of the extended substrate can be inferred from the His54-Ser/D-xylose structure. The Ser54 side chain is 6.9 Å away from O5 of the extended sugar and thus cannot form a hydrogen bond to substrate. The electron density contributed by the sugar in the active site is weak and the temperature factors of the metals are high, possibly because the hydrogen bond between substrate is lost and/or the His54-Ser mutation makes the active site larger.

Lys183 and His220—As mentioned previously, virtually all reports of wild-type D-xylose isomerase complexed with D-xylose have shown an acyclic conformation of sugar bound to the enzyme. The exception where \( \alpha-\beta-D-xlyopyranose \) was observed was attributed to the low occupancy of both metals (15). The \( \alpha-\beta-D-xlyopyranose \) form is observed in Lys183-Met, His220-Ser, and His220-Glu mutants none of which have appreciable activity (Table I). The Lys183-Met mutant is likely blocked at isomerization, since D-xylose exists in small amounts in solution as an acyclic form (0.3% in unbuffered solutions (54)).

There are several possible reasons for observing cyclic sugar in these mutants: (i) the extended form of sugar represents product, (ii) the mutated side chains directly or indirectly block the ring opening step, or (iii) the binding energy of cyclic (or pseudo-cyclic) sugar is improved to the binding energy of the extended substrate. Computer simulations of D-xylose isomerase with D-xylose estimated that there is an increase of 8 kcal/mol when D-xylose goes from pseudo-cyclic (O3 and O4 ligated to M1) to acyclic (O2 and O4 ligated to M1) conformations (55). Entropic contributions were not included in their calculation and might lower this estimate. One possibility is that the acyclic form of sugar observed bound to the wild-type D-xylose complexes is actually D-xylose (15). Since Lys183-Met has no activity, and His220-Ser and His220-Glu have only 0.3 and 0.5% activity (Table I), respectively, it might be expected that \( \alpha-\beta-D-xlyopyranose \) would be observed. However, one S. olivochromogenes D-xylose isomerase mutant, Glu180Lys (Glu180Lys in S. rubiginosus), has no activity but shows an acyclic-extended conformation of D-glucose bound in the active site (33). A direct role for either Lys183 or His220 in ring opening is unlikely since both are far (>7 Å) from the anomeric and ring oxygens of substrate. We postulate that \( \alpha-\beta-D-xlyopyranose \) is observed in the His220 mutant structures because this form is energetically more stable in the mutants than the acyclic-extended conformation as M2 has dissociated from His220-Ser/xylitol and the altered binding site cannot provide the proper metal geometry and/or enough ligands. In the case of Lys183-Met, we believe \( \alpha-\beta-D-xlyopyranose \) is observed in the active site because the extended sugar is not stabilized by the absent Lys183 N2-O1 sugar interaction and thus the \( \alpha-\beta-D-xlyopyranose \) conformation becomes more stable.

Lys183 could function catalytically in the metal-mediated hydride shift by assisting in the polarization of O1 of the transition state (15, 16, 30). Substituting the Lys side chain with Met rendered D-xylose isomerase inactive. In studies of the A. missouriensis D-xylose isomerase, substitution with Ser, Gln, and Arg also inactivated the enzyme (30). The absence of activity may, however, result from a structural defect that indirectly affects the function of another catalytically important residue. The structures of both Lys183-Met and Lys183-Met/xylitol have large perturbations at and around Glu180 and Pro187. Other changes are observed at Phe260 and Trp137 in the active site. Comparison of the wild-type enzyme complexed with D-xylose (i.e., acyclic-extended sugar) to the Lys183-Met/xylitol complex suggests that the extended sugar could be accommodated in the active site of Lys183-Met. These results clearly indicate that Lys183 is structurally important but they also suggest that Lys183 has a role in extending the pseudo-cyclic sugar, stabilization of the acyclic-extended sugar, and isomerization, by interacting with O1 of the sugar.

His220 NE2 serves as a ligand to M2 when M2 is in either the high occupancy site (M2) or the low occupancy site (M2'). Movement to the M2' may be concurrent with deprotonation of the C2 hydroxy (56). Perturbing the structure at M2 or M2' could affect sugar extension by altering the properties of the metal(s) or the metal-bound Wat409, both of which interact with O2 and/or O1 of the extended sugar (15, 16, 19, 26, 36). The reason why the His220 mutants have little activity could be that the introduced side chains cannot stabilize the M2' site. Indirect evidence for M2' destabilization in His220 mutants is shown by the weaker binding at M2, measured kinetically (34) and observed crystallographically. Further evidence is provided by the ability of xylitol to eject the M2 when it is soaked into the mutant enzyme.

In contrast to the A. missouriensis His220-Asn/xylitol structure in which M2 is absent and acyclic-extended D-xylose is observed (19), M2 and \( \alpha-\beta-D-xlyopyranose \) are observed in the structures of His220-Ser (this study) and His220-Glu (this study). The difference in M2 occupancy might be due to different metals used in the two studies; they employed Mg\(^{2+}\), while in this study Mn\(^{2+}\) was used. Depending upon the substitution, there is a 48–200-fold decrease in metal affinity with Mg\(^{2+}\) compared to Mn\(^{2+}\) in S. rubiginosus His220 mutants (34). The reason(s) that different sugar conformations are observed might be because different metals were used (as above) and/or that different substitutions might affect substrate binding or catalysis differently. It is unlikely these mutants operate using a different reaction mechanism. Attempts to recover second-site mutations that restore partial activity in either the E. coli or S. rubiginosus enzymes have yielded only reversions back to the original amino acid residue (i.e. His54-Ser back to His54Lys, data not shown). Not all mutations at His220 result in the same activity. His54-Gln has 3.4% activity, His220-Ser, His220-Asn, and His220-Glu have 0.5–0.8% activity, and His220-Lys has no activity (30, 34).

His220 appears to be important for maintaining the structure around M2 when the substrate is extended. Both the structure of His220-Ser/xylitol determined in this study and the structure of His220-Asn/xylitol from A. missouriensis (19) display similar sugar binding, show that M2 is absent, and reveal new positions for metal ligands and other residues which are located in and near the active site. These differences are presumably caused directly and indirectly by bringing O1 and O2 of the extended sugar near a M2 site with lower affinity for metal.

CONCLUSIONS

The functions of active site residues in D-xylose isomerase were investigated kinetically and structurally. Some of the main conclusions and supporting observations are summarized here.

1) His54 is not essential for catalysis, but appears to be responsible for anomeric recognition and to contribute to the stabilization of the transition state via hydrogen bonding to O5. His54 may act as a base-catalyst but not likely as an acid catalyst in...
ring-opening where enzyme activity is maximal. 2) Phe<sup>94</sup> is not important for anomer recognition or essential for enzyme activity, but it clearly contributes to the optimal binding of the transition state. 3) Lys<sup>183</sup> is important structurally and probably catalytically. Lys<sup>183</sup> has structural perturbations at the catalytic center only experience minor ably catalytically. Lys<sup>183</sup>-Met has structural perturbations at the catalytic center only experience minor ably catalytically. Lys<sup>183</sup>-Met as we suggest it is necessary for catalysis. His<sup>220</sup>-Ser important because it coordinates metal at both the M2 and M2 positions, as we suggest is necessary for catalysis. His<sup>220</sup>-Ser and His<sup>220</sup>-Ser mutants have little activity and their structures have M1, M2, and α-D-xylopyranose bound to the active site. Xyitol binds in an extended form to His<sup>220</sup>-Ser, but the metal at the M2 position is lost from the enzyme rather than simply being shifted.

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