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Protein Engineering of Xylose (Glucose) Isomerase from *Actinoplanes missouriensis*. 2. Site-Directed Mutagenesis of the Xylose Binding Site[†,‡]

Anne-Marie Lambeir,[*,§,‖] Marc Lauwereys,[§,‖] Patrick Stanssens,[§,‖] Nadir T. Mrabet,[§,⊥] Johan Snauwaert,[§,#] Herman van Tilbeurgh,[§,○] Gaston Matthyssens,[§,‖] Ignace Lasters,[Δ,‖] Marc De Maeyer,[Δ,‖] Shoshana J. Wodak,[×] John Jenkins,[∇,+] Mohammed Chiadmi,[∇] and Joël Janin[∇]

Plant Genetic Systems N.V., Jozef Plateaustraat 22, B-9000 Gent, Belgium, PGS/UCMB and UCMB, Université Libre de Bruxelles, Avenue Paul Héger P2, B-1050 Brussels, Belgium, and Laboratoire de Biologie Physicochimique, CNRS UA1131, Bâtiment 433, Université Paris-Sud, F-91405 Orsay, France

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ABSTRACT: Site-directed mutagenesis in the active site of xylose isomerase derived from *Actinoplanes missouriensis* is used to investigate the structural and functional role of specific residues. The mutagenesis work together with the crystallographic studies presented in detail in two accompanying papers adds significantly to the understanding of the catalytic mechanism of this enzyme. Changes caused by introduced mutations emphasize the correlation between substrate specificity and cation preference. Mutations in both His 220 and His 54 mainly affect the catalytic rate constant, with catalysis being severely reduced but not abolished, suggesting that both histidines are important, but not essential, for catalysis. Our results thus challenge the hypothesis that His 54 acts as an obligatory catalytic base for ring opening; this residue appears instead to be implicated in governing the anomeric specificity. With none of the active site histidines acting as a catalytic base, the role of the cations in catalyzing proton transfer is confirmed. In addition, Lys 183 appears to play a crucial part in the isomerization step, by assisting the proton shuttle. Other residues also are important but to a lesser extent. The conserved Lys 294 is indirectly involved in binding the activating cations. Among the active site aromatic residues, the tryptophans (16 and 137) play a role in maintaining the general architecture of the substrate binding site while the role of Phe 26 seems to be purely structural.

Xylose isomerase requires the binding of two divalent metal ions for optimal activity (Callens et al., 1988a,b). The type of activating cation partly determines the substrate specificity. For xylose isomerases derived from *Streptomyces* (including *Actinoplanes missouriensis*), magnesium ions are the best activators in the presence of xylose, followed by cobalt and manganese. In the presence of glucose, magnesium and cobalt are good activators, but the activity in the presence of manganese is significantly lower. The role of the cations and the effects of mutations in the cation binding sites were discussed in an accompanying paper (Jenkins et al., 1992).

The stereochemistry and substrate specificity of xylose isomerase can be summarized as follows [see also Whitlow et al. (1991)]: the enzyme is specific for the α-anomers of its substrates, prefers pentoses over hexoses, and only uses substrates with conserved stereochemistry at the C2 and C4 positions (xylose, glucose, ribose, etc.). The hydrogen transfer is stereospecific, consistent with a planar intermediate in which the O1 and O2 oxygens are in a cis position, and the hydrogen transfer occurs at one side of the plane. The transferred proton does not exchange with solvent protons (Feather et al., 1970; Schray & Rose, 1971; Young et al., 1975; Kersters-Hilderson et al., 1976; McKay & Tavlarides, 1979; Bock et al., 1983; Makkee et al., 1984). Because of the similarity in stereochemistry between the two reactions, it has been accepted for a long time that xylose isomerase and triose-phosphate isomerase share the same enzymatic mechanism: base-catalyzed proton transfer involving a stable enediol intermediate (Rose et al., 1969). Recently, this mechanism has been questioned by a number of investigators who found it difficult to reconcile with the structure of the active site (Farber et al., 1989; Collyer et al., 1990; Collyer & Blow, 1990). It is now generally accepted that the isomerization occurs with a metal-assisted hydride shift mechanism (Whitlow et al., 1991).

On the basis of crystallographic studies of various xylose isomerase complexes, the following detailed mechanism was proposed (Collyer et al., 1990). The cyclic α-anomer of the sugar binds to the dimetal–enzyme complex, coordinating with its O2 and O3 oxygens to the cation bound at site 1 (M1). Opening of the bound sugar is followed by a rearrangement during which the sugar adopts an extended form, now bound to the M1 cation by O2 and O4. The isomerization occurs with a hydride shift, as there is no evidence for a catalytic base in the crystal structure. The "activated" complex may involve a change in relative position of the second cation (M2). There has been some speculation about which reaction step—isomerization or ring opening—is reflected in the catalytic rate constant (k_{cat}). When glucose, specifically deuterated at the C2 carbon, is used as a substrate, a deuterium effect of about

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[‡] The coordinates of the structures have been deposited in the Brookhaven Protein Data Bank under the file names 1XIM, 2XIM, 3XIM, 4XIM, 5XIM, 6XIM, 7XIM, 8XIM, and 9XIM.

[*] Author to whom correspondence should be addressed.

[§] Plant Genetic Systems N.V.

[‖] Present address: Corvas International, N.V., J. Plateaustraat 22, B-9000 Gent, Belgium.

[⊥] Present address: LEGG/URA-CNRS 457, Université de Nancy I, Nancy, France.

[#] Present address: LCBD, KU Leuven, Celestijnenlaan 200D, B-3001 Heverlee, Belgium.

[○] Present address: LCCMB, Faculté de Médecine Nord, Boulevard Pierre Dramard, F-13326 Marseille 15, France.

[Δ] PGS/UCMB, Université Libre de Bruxelles.

[×] UCMB, Université Libre de Bruxelles.

[∇] CNRS UA1131.

[+] Present address: AFRC Institute of Food Research, Shinfield, Reading RG2 9AT, England.

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4 was observed on the catalytic rate constant for the wild-type and His 101 mutant enzymes (in *Clostridium thermosulfurogenes* xylose isomerase, corresponding with His 54 in the *A. missouriensis* sequence numbering used in this paper) (Lee et al., 1990). This clearly indicates that the hydride transfer is monitored by the catalytic rate constant.

In this paper we report on the properties of a series of active site mutant xylose isomerases from *A. missouriensis*, how these properties relate to observed and predicted changes in the structural model of the active site, and what the implications are for the catalytic mechanism.

EXPERIMENTAL PROCEDURES

Enzymes. The production and purification of wild-type and mutant forms of recombinant xylose isomerase will be published elsewhere (P. Stanssens and M. Lauwereys, personal communication). Bound cations were removed by dialysis against triethanolamine (TEA)[1] buffer containing 10 mM EDTA, followed by several buffer changes of 10 mM TEA, pH 7.2. No effort was made to remove EDTA that may be bound to the enzyme under these conditions (N. T. Mrabet, personal communication).

Activity Measurements and Kinetic Methods. The initial rates of xylose and glucose consumption were measured using sorbitol dehydrogenase as described by Callens et al. (1986). In some cases fructose concentrations were determined with a colorimetric test using cysteine and carbazole based on the procedure of Dische and Borenfreund (1951). Both methods gave essentially the same results. At relatively low cation concentrations ($[\mathrm{Mg}^{2+}] < 2$ mM; $[\mathrm{Co}^{2+}]$ and $[\mathrm{Mn}^{2+}] < 0.2$ mM), the experimentally measured cation and xylose dependence of the initial rate (v_i) can be adequately expressed by the following equation, which assumes a random order equilibrium mechanism:

$$v_i = k_{cat}E_{tot}/(1 + 1/K_4[\mathrm{M}] + 1/K_3[\mathrm{S}] + 1/K_1K_3[\mathrm{M}][\mathrm{S}]) \quad (1)$$

In this mechanism formally K_1 and K_4 are the affinity constants of the cation for the free enzyme and the enzyme–substrate complex, respectively, and K_2 and K_3 are the affinity constants of the substrate for the free enzyme and the enzyme–metal complex, respectively. $K_1K_3 = K_2K_4$. [S] and [M] are the total substrate and cation concentrations, E_{tot} is the total enzyme concentration, and k_{cat} is the catalytic rate constant. Double reciprocal plots (Lineweaver–Burk) of the initial rates vs the substrate concentration at various cation concentrations are straight lines intersecting in the upper left quadrant. Systematic variation of both substrate and cation concentrations allows values to be determined for k_{cat} and all four equilibrium constants. At the limit of infinite cation concentrations, the observed K_M (Michaelis constant) for the substrate becomes equal to $1/K_3$. At the limit of infinite substrate concentrations the cation concentration of half-maximal activity (hereafter called the metal ion activation constant or K_{act}) is $1/K_4$. At high cation concentrations the cations become inhibitory. These conditions were avoided as much as possible in this study. To determine the full set of steady-state parameters, xylose concentration was varied between 2 and 200 mM and that of magnesium ions between 0.025 and 2 mM. Except when indicated otherwise, experiments were carried out in 50 mM TEA buffer, pH 7.5, at 35 °C in the presence of xylose and at 60 °C in the presence of glucose. Routinely, steady-state kinetic parameters for xylose and glucose were determined in the presence of 10 mM $\mathrm{MgSO_4}$; the K_{act} for magnesium was determined in the presence of 100 or 200 mM xylose. These concentrations are saturating for the wild-type enzyme and for most of the mutant enzymes. However, when the K_{act} for Mg is high, the K_M for the substrate may contain a dependence on cation concentration, and when the K_M is high, the K_{act} may depend on xylose concentration. The pH–activity profiles were determined in the presence of 200 mM xylose, 10 mM $\mathrm{MgSO_4}$, and 25 mM each of MES and HEPES buffers of the required pH. Typical concentrations of wild-type xylose isomerase in the kinetic assays were 2.5 μg/mL in the presence of xylose and 10 μg/mL in the presence of glucose. The mutant concentrations were adjusted to obtain initial rates similar to those measured for the wild type under the same conditions.

Deuterium isotope effects on the catalytic parameters were determined using glucose deuterated (97% atom D) at the C2 position, purchased from Sigma. The presence of deuterium on the fructose did not affect the sorbitol dehydrogenase reaction. The concentration of the deuterated glucose was varied between 25 mM and 1.2 M.

The following experiment was carried out to determine the anomeric specificity of the wild-type and the H54N enzymes. Crystalline 95% pure α-xylose (purchased from Sigma) was dissolved in water at time 0 (200 mM, 25 °C). Immediately after dissolution a sample was withdrawn and diluted in the xylose isomerase reaction mixture, and the initial rate of xylulose production was monitored during 2 min at 35 °C. Subsequently, samples were withdrawn and assayed at 2, 4, 7, 11, 15, 20, 25, and 30 min after the α-xylose was dissolved. The final concentrations in the assay mixture were 2 mM xylose, 2 mM $\mathrm{MgSO_4}$, and 8.9 μg/mL wild-type xylose isomerase or 188 μg/mL H54N enzyme.

X-ray Crystallography. Crystal structures were obtained and analyzed as described in the accompanying paper by Jenkins et al. (1992).

RESULTS

Interactions of the Protein with Bound Substrate and Substrate Analogues. The position of xylitol in the xylose isomerase active site is shown in Figure 1. Polar side chains with at least one atom within a distance of 3 Å of one of the hydroxyl groups of the substrate are His 220, Lys 183, Asp 255, Asp 292, Glu 181, Glu 217, Asp 245, and His 54. Trp 137 and Phe 26 are in van der Waals contact with the substrate. Table I summarizes the most prominent interactions between the protein and the substrate or inhibitor in a number of wild-type and mutant protein crystal structures. Note that in all these structures the substrate or inhibitor is bound in an open-chain extended conformation. The active site also includes a number of water molecules which appear to participate in substrate binding. The residues shown in Figure 1 and Table I were chosen as target sites for site-directed mutagenesis to investigate structure–function relationships in the xylose isomerase active site. The catalytic properties of some active site mutant xylose isomerases are discussed in the following paragraphs. Effects of mutations in residues that are also involved in cation coordination are described in an accompanying paper by Jenkins et al. (1992).

Mutations of Conserved Lys Residues. The mutations of Lys 183 into Ser, Gln, and Arg yield inactive proteins, suggesting that Lys 183 is important for catalysis. In the wild-type structure in the presence of Co^{2+} and xylitol (structure 1), the ε-amino group of Lys 183 is involved in hydrogen bonds with

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[1] Abbreviations: TEA, triethanolamine; MES, 4-morpholineethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

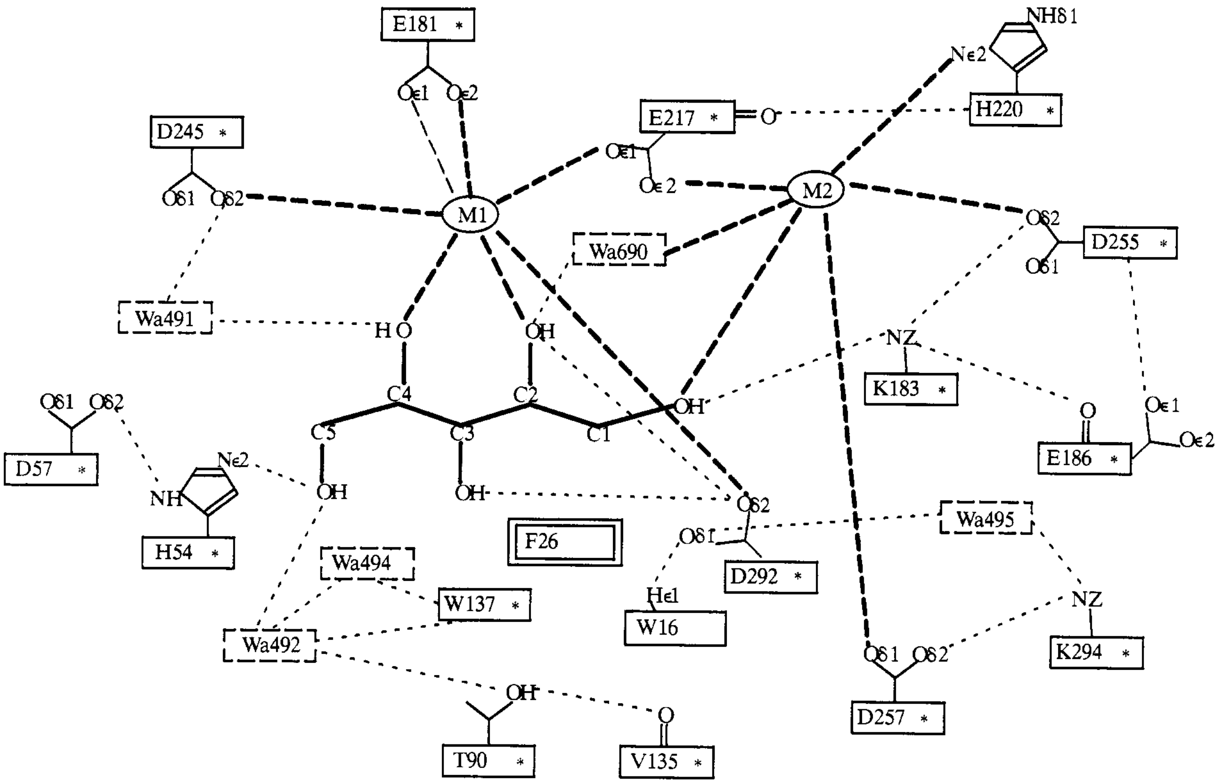

FIGURE 1: Schematic representation of the active site. In addition to residues interacting with metal ions (Jenkins et al., 1992) and with the substrate and substrate analogs (this paper), some of their immediate neighbors, such as Glu 186 (van Tilbeurgh et al., 1992), have been subjected to site-directed mutagenesis. Hydrogen bonds (light dashes) and metal bonds (heavy dashes) are indicated. Conserved residues are labeled with an asterisk.

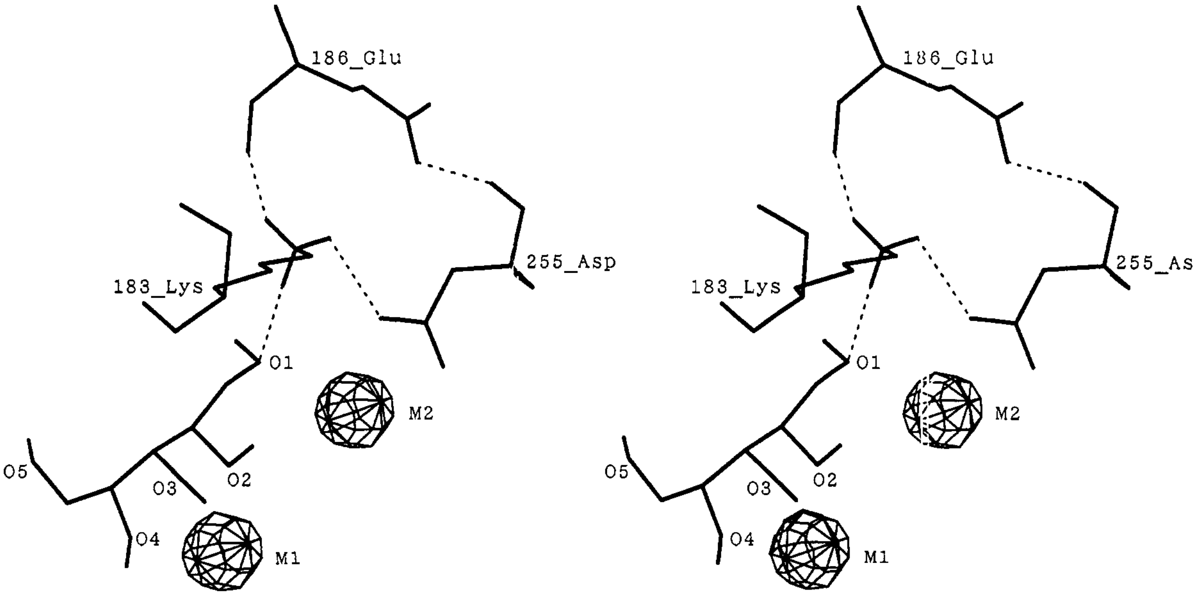

FIGURE 2: Interactions of the Lys 183 side chain with the surrounding atoms in the wild-type–Co–xylitol complex. Amino acids and xylitol are shown with heavy lines, and the metal ions are represented as polygons labeled M1 and M2. The amino hydrogens of the Lys 183 side chain are involved in hydrogen bonds (dashed lines) with the carboxylate of Asp 255, the main-chain carbonyl oxygen of Glu 186, and the O1 oxygen of the inhibitor. Introduction of a different residue at position 183 is expected to disrupt this well-structured environment, resulting in an inactive enzyme.

the carboxylate of Asp 255, the main-chain carbonyl oxygen of Glu 186, and the O1 oxygen of the inhibitor (Figure 2). Such a well-defined structural arrangement may prevent the introduction of any other type of side chain at this site without thoroughly disturbing the structural environment.

The ε-amino group of Lys 294 interacts with Asp 257, which is a ligand of metal 2 [see Jenkins et al. (1992)], and with two water molecules. One of these water molecules form a hydrogen bond with the O3 of xylitol, and the other interacts with one of the metal 1 ligands (Asp 292). In the K294Q mutant, where the positive charge of Lys 294 is eliminated, thereby disturbing the charge balance near the cation binding site, we observe an increase in the K_{act} for Mg and, concomitantly, in a shift of the pH–activity optimum to the alkaline side (by 0.6 pH unit). The catalytic rate constant and the K_M of the substrates are also affected. The positive charge at position 294 thus appears to be important for proper functioning of the enzyme, perhaps by influencing the ionization

Table I: Protein to Substrate Contacts[a]

distance (Å) from	distance (Å) to	data set 1 Co–xylitol (WT)	data set 9 Co–sorbitol (triple)	data set 10 Mg–xylose (K253R)
O1	Lys 183 Nζ	3.05 (0.11)	3.00 (0.17)	2.99 (0.07)
	His 220 Nε2	3.19 (0.06)	3.43 (0.03)	3.24 (0.08)
	Asp 255 Oδ1	3.26 (0.11)	3.21 (0.13)	3.38 (0.14)
	water 690	3.32 (0.08)	3.41 (0.19)	3.34 (0.07)
C1	Trp 137 Cη2	3.70 (0.12)	3.74 (0.11)	3.75 (0.16)
	Phe 26 Cε1	3.92 (0.07)	3.92 (0.10)	3.81 (0.15)
O2	Glu 181 Oε2	3.10 (0.18)	2.96 (0.11)	2.83 (0.11)
	Glu 217 Oε2	3.10 (0.12)	3.01 (0.09)	3.12 (0.06)
	His 220 Nε2	3.64 (0.15)	3.33 (0.09)	3.60 (0.07)
	Asp 292 Oδ1	3.00 (0.05)	3.04 (0.12)	2.91 (0.04)
	water 690	2.92 (0.10)	2.98 (0.09)	3.16 (0.10)
C2	Trp 137 Cδ2	3.60 (0.06)	3.65 (0.07)	3.58 (0.03)
O3	Trp 16 Nε1	3.49 (0.06)	3.48 (0.02)	3.43 (0.05)
	Asp 292 Oδ1	2.92 (0.06)	2.66 (0.13)	2.75 (0.15)
	water 899	2.60 (0.10)	2.75 (0.07)	3.44 (0.40)
C3	Trp 137 Cε3	3.63 (0.03)	3.83 (0.03)	3.78 (0.03)
O4	Glu 181 Oε1	2.52 (0.05)	2.47 (0.10)	2.50 (0.06)
	Glu 181 Oε2	3.07 (0.20)	3.02 (0.14)	2.87 (0.12)
	Asp 245 Oδ2	3.40 (0.10)	3.17 (0.05)	3.26 (0.02)
	Asp 292 Oδ1	3.15 (0.03)	2.93 (0.14)	3.04 (0.11)
	water 491	2.95 (0.02)	2.91 (0.05)	2.95 (0.10)
C4	Trp 137 Cβ	3.63 (0.11)	3.74 (0.07)	3.72 (0.10)
O5	His 54 Nε2	2.63 (0.13)	2.65 (0.12)	2.67 (0.11)
	water 492	2.63 (0.08)	2.72 (0.06)	2.61 (0.06)
C5	His 54 Nε2	3.18 (0.09)	3.30 (0.09)	3.21 (0.08)
O6	Thr 90 Cγ2		3.46 (0.09)	
	Val 135 Cγ1		3.40 (0.10)	
	water 491		2.52 (0.13)	
C6	Thr 90 Oγ1		3.43 (0.14)	
	Val 135 Cγ1		3.48 (0.14)	

[a] Contacts shorter than 4 Å between bound substrate or inhibitor and protein atoms. Only the nearest atom within each residue is listed. Distances are given as averages of the four subunits, with standard deviations in parentheses. Residue Phe 26 is from an adjacent subunit.

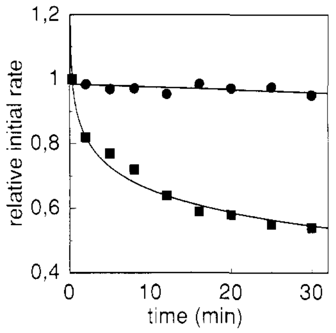

FIGURE 3: Anomeric specificity of wild-type xylose isomerase and the corresponding H54N mutant. α-Xylose is dissolved in water at time 0. A sample is withdrawn immediately after dissolution, and the initial rate is recorded during 2 min. Subsequently, samples are withdrawn and assayed at various time points after dissolution. In the presence of wild-type xylose isomerase (■) the initial rate decreases with time as the concentration of α-xylose decreases. H54N (●) does not show anomeric specificity. The conditions are described under Experimental Procedures.

Table II: Xylose and Magnesium Binding Parameters[a]

		Mg		xylose		
enzyme	k_{cat} (s^{-1})	K_1 ($\times 10^3$ M^{-1})	K_4 ($\times 10^3$ M^{-1})	K_2 (M^{-1})	K_3 (M^{-1})	activation coeff
wild type	17.3	2.1	9.9	43	200	5
H54Q	1.6	1.6	12.1	12	96	8
H220Q	0.6	1.0	9.0	7	63	9

[a] K_1 and K_4 are the association equilibrium constants of Mg extrapolated to zero and infinite substrate concentrations, respectively. K_2 and K_3 are the equilibrum constants of xylose binding at zero and infinite Mg concentrations. The activation coefficient ($K_1/K_4 = K_2/K_3$) is a measure of the increase in affinity of the cation by the presence of bound substrate and vice versa.

state of the cation ligands. Introduction of an Arg at position 294 restores practically all parameters to wild-type values. In addition, disruption of the interaction with Asp 257, in the K294Q mutant, may influence the relative stability of the metal 2 subsites (Jenkins et al., 1992) or interfere with the proton abstraction from the substrate's hydroxyl group as suggested by Whitlow et al. (1991) and in the accompanying paper by van Tilbeurgh et al. (1992).

Mutations of Conserved Histidines. In the wild-type structures of *A. missouriensis* xylose isomerase, His 54 interacts with the O5 of xylitol via its Nε2 (Table I). This residue has been suggested by several authors to act as a catalytic base for ring opening (Collyer & Blow, 1990; Collyer et al., 1990; Whitlow et al., 1991). We find that the mutant enzymes H54A, H54N, and H54Q generally display very similar behavior. All three mutant enzymes have a k_{cat} for xylose of about 10% of that in the wild type. The K_M for xylose is increased with factors ranging between 2 and 4, while the K_{act} for Mg is increased 1–3-fold. The three His 54 mutant enzymes seem to obey the same catalytic mechanism as the wild type, as judged by the following criteria: (1) the mutants and the wild-type enzymes used the closed sugar as a substrate, (2) in both the mutants and the wild type, Lineweaver–Burk plots obtained at different Mg concentrations intersect in the upper left quadrant, (3) the pH profiles of the mutant enzymes are similar to that of the wild type, and (4) the deuterium isotope effect on the catalytic rate constant is identical to that in the wild type.

Some evidence that the H54N enzyme uses the closed sugar form as a substrate is provided by the experiment shown in Figure 3. Crystalline α-xylose is dissolved and immediately used in an enzymatic assay as described under Experimental Procedures. In water, α-xylose is slowly converted to the β-form until the equilibrium mixture is reached (about 40% α- and 60% β-xylose at 30 °C). The rate of this process is determined by the rate of ring opening in solution and is measured by the mutarotation rate constant, which is 0.14 min^{-1} in 50 mM TEA, pH 7.5, at 35 °C. Therefore, since ring opening is slow, immediately upon dissolution, all the xylose is present in the cyclic form. If only the open form were used as a substrate, the initial rate would be expected to increase as the xylose solution is allowed to stand until equilibrium is reached. This is, however, not observed. On the contrary, for wild-type xylose isomerase, the initial rate decreases with time after dissolution, indicating that the enzyme uses the α-form more efficiently than the β-form of the substrate. The initial rate at every time point is a composite of the catalytic efficiencies of both anomeric forms (α and β) proportional to their relative concentrations. At substrate concentrations below the K_M, the following relationship holds:

$$v_i/E_{tot} = (k_{cat}/K_M)_\alpha[\alpha] + (k_{cat}/K_M)_\beta[\beta] \qquad (2)$$

It has been shown that xylose isomerase uses only the α-form of xylose and that the decrease in initial rate seen in an experiment such as shown in Figure 3 is entirely due to an increase in apparent K_M with a factor that parallels the decrease in concentration of the α-xylose (Kersters-Hilderson et al., 1976). We find that, unlike the wild type, the H54N enzyme is not specific for the α-form of the substrate since the initial rate does not appreciably change upon time after dissolution (Figure 3).

As mentioned above, there is also evidence that the His 54 mutant enzymes obey the same initial rate equation as the wild type. The H54Q kinetic parameters (determined by varying both Mg and xylose concentrations as outlined under Exper-

Table III: Deuterium Isotope Effect[a]

enzyme	$k_{cat}(H)/k_{cat}(D)$	$K_M(H)/K_M(D)$
wild type	3.3	1.2
H54N	3.2	1.1
H54Q	2.7	1.8
H220N	3.9	1.1

[a] Glucose specifically deuterated on the C2 position was used as a substrate. Kinetic parameters were determined in 50 mM TEA buffer, pH 7.5, at 60 °C in the presence of 10 mM $MgSO_4$.

imental Procedures) are listed in Table II. From these data it is clear that the effect of the mutation is expressed mainly in the k_{cat} value (10-fold reduced) and less in the affinity constants for xylose (2–4-fold reduced). The activation coefficient ($K_4/K_1 = K_3/K_2$), which is equal to the factor by which the substrate affinity is increased due to the bound metal ion, is increased by less than a factor of 2. The magnesium parameters are in the same range as those of the wild type. Therefore, steady-state kinetics do not reveal major changes in the mechanism of this mutant enzyme.

This conclusion is supported by the data presented in Table III, showing a deuterium effect of around 3 on the k_{cat} of the H54N and H54Q enzymes when the C2 carbon of glucose is specifically deuterated. A deuterium effect of this magnitude is expected for the rate constant of hydrogen transfer but not on ring opening, since this latter does not involve the breaking of the C2–H bond (Lee et al., 1990).

To further probe into the role of His 54, the mutant D57N was made and analyzed. Asp 57 is a conserved residue that interacts via its carboxylate group with the Nδ1 of His 54 and has been suggested to assist in the catalysis of ring opening. Removal of the negative charge (D57N), with the possibility of maintaining the hydrogen bond, was expected to have an effect on the ionization constant of the histidine and hence on the rate of ring opening. We find that the steady-state kinetic properties of the D57N mutant enzyme are not dramatically altered, relative to those of the wild type. The k_{cat} values are reduced by a factor 2 or 3 compared to those of wild type. The K_M of the substrates is similar to that of the wild type's; the K_{act} for Mg is increased with a factor of 2. The pH–activity profile is very similar to that of the wild type's, with its acidic side moved by 0.1 pH unit toward lower pH.

His 220 functions as a ligand for metal 2, moving with the metal when it takes alternative positions, as discussed in paper 1 of this series (Jenkins et al., 1992). However, the close proximity of His 220 to the chemically active centers of the substrate warrants further investigation. The H220N and H220Q mutant proteins were found to have reduced activities of 0.3% and 4% relative to those of the wild type. In the H220Q enzyme the K_M and K_{act} for Mg are also increased by a factor of 3 or 4. This contrasts with the results obtained for xylose isomerase containing mutations in the carboxylate metal ligands, where the effects on K_M and the magnesium binding were generally much larger. The His 220 mutant enzymes obey the same catalytic mechanisms as in the wild type. Lineweaver–Burk plots of the H220Q mutant obtained at different magnesium concentrations intersect in the upper left quadrant, the pH–activity profile of H220Q is similar to that of the wild type, and the deuterium isotope effect on the catalytic rate constant measured for H220N is identical to that of the wild type.

The catalytic parameters shown in Table II clearly indicate an effect of the His 220 mutations on the catalytic rate constant and the xylose binding, but Mg binding in the presence of xylose is not significantly affected. The activation coefficient ($K_4/K_1 = K_3/K_2$) is increased by almost a factor of 2, indicating that magnesium binding partially remediates the deleterious effect of the mutation on the substrate binding.

The magnitude of the deuterium effect on the catalytic rate constant (Table III) again indicates that the isomerization reaction is seriously affected by the H220N mutation.

Mutations of the Active Site Tryptophans and Phe 26. There are two tryptophans in the active site, Trp 137, which is conserved in all known sequences, and Trp 16, conserved only in the *Streptomyces* sequences. Trp 16 does not interact directly with the cations, but it forms a hydrogen bond with one of the metal 1 ligands (Asp 292). In most structures the bound substrate or inhibitor is "sandwiched" between the two tryptophan side chains, as revealed by extensive van der Waals contacts, especially with Trp 137 (Table I).

Replacing Trp 16 by Ala, Leu, or Phe causes a drop in activity ranging from 20% (W16L) to 50% (W16F) of the wild-type value. The K_{act} for Mg is not seriously affected (factors of 1–3), but the K_M for the substrates is significantly increased by factors of 4–30.

Of the Trp 137 mutant proteins only W137F was expressed in high enough amounts to allow detailed characterization. In this mutant enzyme the k_{cat} is reduced to 10% of the wild-type value, there is an increase in K_M by a factor of 4, and metal binding occurs more or less as in the wild type. It has been noticed by us and other investigators (Collyer et al., 1990; Farber et al., 1989) that the side chain of Trp 137 in xylose isomerases occupies a position equivalent to that of the catalytic base in triose-phosphate isomerase. A naive attempt to introduce a carboxylate group at that site by engineering the W137E mutant does not yield an active enzyme in our hands. The results of the tryptophan mutations imply that both Trp 16 and Trp 137 are involved in substrate binding rather than catalysis, presumably by maintaining the architecture of the active site mostly by the mere bulk of their side chain.

Residue Phe 26 extends from one subunit into the next across the interdimer interface coming quite close to its active site (Rey et al., 1988). Together with Trp 137 it provides a hydrophobic binding surface for the C1–C2 aliphatic portion of the substrate backbone. In an attempt to obtain a variant of xylose isomerase with a reduced pH optimum for catalysis, residue 26 was substituted by a Lys. This, however, resulted in a thermolabile and inactive enzyme. Molecular modeling based on the wild-type crystal structure in which the Phe 26 side chain has been replaced by Lys suggests that this may be due to electrostatic repulsion resulting from spatial proximity of the engineered Lys to the side chain of Lys 183 in the active site of the neighboring subunit. Moreover, the engineered Lys, lacking an aromatic character, cannot interact with the Trp 137 indole ring as phenylalanine does in the wild-type structure. Engineering a histidine at that position (F26H) produces an active enzyme (80% of wild type). The K_M is increased by a factor of 2–3 compared to that of wild type, and magnesium binding is not significantly affected. The pH–activity profile in the presence of magnesium and xylose is, however, not altered (data not shown), suggesting that the substituted histidine is not protonated between pH 6 and pH 9.

Mutations in the Active Site Alter Substrate and Cation Specificity. Values of the steady-state kinetic parameters of the wild-type and the mutant enzymes discussed in the preceding paragraphs are listed in Table IV. We see that all the mutations in the active site cause a decrease in the catalytic efficiency (k_{cat}/K_M) for both xylose and glucose. However, the relative specificity for glucose versus xylose, defined as the ratio of $(k_{cat}/K_M)_{glucose}$ over $(k_{cat}/K_M)_{xylose}$, increases for most

Table IV: Kinetic Parameters of Xylose Isomerase Mutants in the Xylitol Binding Site

enzyme	xylose[a]		glucose[b]		rel specificity[d]	Mg^{2+} K_{act}[c]	rel activity	
	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})	K_M (M)	(glucose/xylose)	(mM)	Co/Mg	Mn/Mg
wild type	17.3	4.8	24.9	0.29	1	0.08	0.46	0.40
K183Q	no activity							
K183R	no activity							
K183S	no activity							
K294L	no expression							
K294Q	4.1	32	6.9	0.43	7.4	0.25	0.36	0.37
K294R	9.9	4.5	19.7	0.31	1.2	0.08	0.51	0.65
H54A	1.3	8.6	1.2	0.27	1.2	0.29	1.16	0.23
H54K	no expression							
H54N	2.4	21	2.0	0.34	2.1	0.11	0.71	0.28
H54Q	1.6	12	0.9	0.21	1.7	0.08	1.06	0.34
D57N	10.1	4.7	7.0	0.21	0.7	0.19	0.54	0.52
F26H	13.4	11	14.5	0.76	0.7	0.12	0.24	0.18
F26K	no activity							
W16A	5.5	160	2.5	1.2	2.6	0.08	0.41	0.12
W16F	8.1	21	11.6	0.72	1.8	0.11	0.70	0.19
W16L	3.6	120	10.4	7.8	1.8	0.21	0.54	0.10
W137A	no expression							
W137E	no activity							
W137F	1.4	21	1.2	0.79	1.0	0.12	0.74	0.10

[a] Kinetic parameters for xylose are determined at 35 °C in the presence of 10 mM $MgSO_4$. No expression: no overexpression of the mutant in *E. coli*. No activity: less than 0.5% activity of the wild type. [b] Kinetic parameters for glucose were determined at 60 °C in the presence of 10 mM $MgSO_4$, except for K294Q where it was 20 mM. [c] K_{act} is the metal ion concentration that yields 50% activity with 0.2 M xylose (or 0.1 M xylose for F26H) at 35 °C. The relative activity is the ratio of maximum activity achieved with Co and Mn compared to Mg. [d] The relative specificity of glucose vs xylose is calculated by dividing the $(k_{cat}/K_M)_{glucose}/(k_{cat}/K_M)_{xylose}$ value of the mutant by the wild-type value. A number higher than one indicates that the mutant is more selective for glucose than the wild type.

mutants. The H54A and K294R mutant enzymes have the same relative specificity as the wild type, and the mutants F26H and D57N have an improved specificity for xylose. The factors by which the relative specificities have changed as compared to wild type are given in Table IV.

A plausible explanation for these observations is that many of the mutant xylose isomerases analyzed here, introducing residues of the same size or smaller than the wild-type residue, produce additional flexibility in the active site which enables it to accommodate the larger glucose substrate. Meng et al. (1991) reported a change in substrate specificity in favor of glucose for a number of mutations introduced near the sorbitol O6 binding pocket in xylose isomerase from *C. thermosulfurogenes*. The mutated positions are, however, located at a certain distance from the catalytic center determined by the cations and explored in the present study. Most effective is the replacement of Trp 139 with the smaller phenylalanine. This residue is equivalent to Met 87 in the *Arthrobacter* sequence and to Met 88 in the *Actinoplanes* structures studied here. Additional evidence for increased flexibility correlated with altered substrate specificity comes from the crystal structures of mutant enzymes H290N and M88S/H243N [structures 14 and 19 as described in Jenkins et al. (1992)]. These mutations appear to affect the shape of the TIM barrel (De Maeyer, unpublished results). Since the residues mutated in these enzymes are not directly involved in substrate binding, their properties were not included in this paper.

It is noteworthy that many of the mutants described here and in paper 1 of the series (Jenkins et al., 1992) have an altered cation specificity even when the mutation site is relatively far from the cation binding sites (e.g., H54Q). Presumably the effect of the mutation is transmitted via the open-chain substrate intermediate to metal 2. It has indeed been observed that the conformation of metal 2 is very sensitive to local differences in the environment (Jenkins et al., 1992). Hence the apparent cation affinity and specificity determined by steady-state kinetics most probably reflect binding of the cation at site 2.

DISCUSSION

This study presents results of site-directed mutagenesis experiments performed in the active site region of xylose isomerase. Together with the accompanying studies that focus on detailed crystallographic analysis of mutant proteins, it has allowed us to improve our understanding of the functional role of specific residues, thereby gaining further insight into the catalytic mechanism of this enzyme.

The Role of Lys 183. From the finding that none of the attempted substitutions yields active enzyme, not even the K183R mutant where the positive charge is conserved, we deduce that the ϵ-amino group of Lys 183 is important for catalysis. This group hydrogen bonds to O1 of the substrate and of linear inhibitors (Table I). The $N\zeta$–O1 distance is 2.9–3.1 Å in all our structures except in the (inactive) D255A–Mg–xylose complex, where it is 3.4 Å. Since this H-bond cannot be made with the substrate in the cyclic form, the role of Lys 183 seems to be that of maintaining O1 in place and stabilizing the open substrate conformation. Thus, Lys 183 should at least in part by responsible for driving the ring-opening reaction which in turn is essential for the catalytic mechanism discussed below. It is noteworthy that ring opening and the interaction of O1 with Lys 183 are not affected by the position of metal 2, or for that matter by its absence. Indeed, the H220N–Mg–xylose complex [model 13 of Jenkins et al. (1992)] shows no bound metal at site 2, yet xylose is bound in an open form and the $N\zeta$–O1 distance is 2.90 Å. We also find that the affinity of wild-type enzyme for cyclic inhibitors is low (A. M. Lambeir, unpublished results).

The charge carried by the ϵ-amino group of Lys 183 may also contribute to polarize the C=O bond of aldose substrates and to lower the pK_a of the hydroxyl group of ketoses. The $N\zeta$–O1 hydrogen bond could even play a direct role in catalysis, by enabling Lys 183 to take or give a proton from O1 during isomerization. There is, however, good reason to believe that this function is performed by a water molecule bound to metal 2 rather than by a protein group. A proton shuttle involving a water molecule has been proposed by Whitlow et al. (1991). We have evidence from the study of the E186Q mutant enzyme that Asp 255 is part of this shuttle (van Tilbeurgh et al., 1992). In summary, we observe that, in the structure of the active ternary complex, E186Q–Mn–xylose, the carboxylate of Asp 255 forms an H-bond with the Lys 183 ϵ-amino group, as it does in most of the structures we have determined. On the other hand, in the structure of the complex E186Q–Mg–xylose, which displays low activity, the Asp 255 side chain is rotated away and this H-bond is not observed. Furthermore, the observed shift in the pH–activity profile of the E186Q mutant suggests that the pK_a of Lys 183 governs the pH dependence on the alkaline side in the wild type. Hence, the role of Lys 183 in catalyzing the isomerization step in xylose isomerase may be the important (though indirect) one of maintaining the integrity of the proton shuttle. Substitution of Lys 183 by smaller side chains (Gly, Ala, Cys) might give more information about the role of this side chain. The K183S mutant protein, however, was not active in our hands.

The Role of His 54. On the basis of the position of His 54 in crystal structures containing closed sugar forms or cyclic inhibitors, it was postulated that His 54 acts as a catalytic base for ring opening, shuttling a proton between the O1 and O5 oxygens of the substrate (Collyer & Blow, 1990; Collyer et al., 1990; Whitlow et al., 1991). In the extended open-chain intermediate, as in the xylitol complex, this residue is seen to form H-bonding interactions with O5 of the sugar ligand. Mutating the equivalent of His 54 in the *Escherichia coli* xylose isomerase into Arg has been reported to yield an inactive enzyme (Batt et al., 1990). Our His 54 mutant enzymes, where the histidine side chain is either removed or replaced by sterically similar but smaller side groups, show significant activity, as also observed by other investigators (Lee et al., 1990). The effect of the His 54 mutations on the K_M of xylose is only a 2–4-fold increase. From the deuterium effect on k_{cat}, we have to conclude that in the His 54 mutant enzymes ring opening occurs faster than isomerization. The mechanism of ring opening may, however, be different than in the wild-type reaction. The decrease in k_{cat} of the His 54 mutant enzymes observed here suggests a role of this residue in transition-state stabilization of the isomerization reaction. Furthermore, the data for the H54N mutant enzymes indicate that in the wild type His 54 is responsible for the anomeric specificity of the enzyme. This provides additional evidence that His 54 is able to interact with the O1H of the cyclic substrate but does not support the hypothesis that His 54 is a catalytic base for ring opening. On the whole, our site-directed mutagenesis experiments thus strongly suggest that His 54 cannot be a catalytic base in a rate-limiting step of the enzymatic reaction in the wild type.

The Role of His 220. Mutations in His 220 appear to reduce mainly the rate of isomerization, presumably by interfering with the metal 2 binding site, in agreement with the study by Batt et al. (1990). Replacing His 220 by the sterically similar Gln and Asn residues does not affect the substrate and magnesium binding properties as severely as the mutations in the carboxylate ligands described in the accompanying paper (Jenkins et al., 1992). This could be ascribed to the charge conservation in the His 220 mutant proteins. The observation that the pH–activity profile of the H220Q enzyme is not significantly different from that of the wild type supports the assumption that His 220 is not protonated between pH 6 and pH 9 in the presence of magnesium. The improved performance of H220Q (where the amide group of the side chain can be considered as isosteric to the Nϵ2 atom of the imidazole), relative to H220N (where the side-chain amide group is considered isosteric to the Nδ1 atom of the imidazole), illustrates the importance of the Nϵ2 atom in the wild type, which is seen coordinating to metal 2 in many crystal structures. Moreover, His 220 is also directly engaged in polar and nonpolar interactions with the O1 and O2 of the substrate. Our results confirm the most likely importance of these interactions in stabilizing the transition state of the isomerization reaction.

Different Steps in the Catalytic Mechanism and the Role of the Cations. The simplest mechanism for xylose isomerase summarized in eq 3 must include the following steps: (1) binding of the cyclic sugar (S_{CYC}) to the enzyme; (2) ring opening to form an extended intermediate ($ES_{CYC} \rightarrow ES_{OPEN}$) containing two bound cations, similar to the one observed in most crystal structures; (3) isomerization by a hydride shift mechanism accompanied by a proton shuttle between O2 and O1 ($ES_{OPEN} \rightarrow EP_{OPEN}$); (4) ring closure ($EP_{OPEN} \rightarrow EP_{CYC}$); (5) dissociation of the product (P_{CYC}).

$$E + S_{CYC} \rightarrow ES_{CYC} \rightarrow ES_{OPEN} \rightarrow EP_{OPEN} \rightarrow EP_{CYC} \rightarrow E + P_{CYC} \quad (3)$$

Replacing the proton on the C2 carbon of glucose by a deuterium atom causes a 3-fold decrease in k_{cat} while leaving the K_M unaffected (within the experimental error which is about 15%). A deuterium isotope effect of this magnitude is consistent with hydride transfer (Lee et al., 1990). Consequently, the ring opening contributes to the K_M parameter, and the k_{cat} represents the rate constant for isomerization. This conclusion holds for wild-type and His 54 and His 220 mutant enzymes.

We also observe that mutations which affect the catalytic rate constant most are the ones that interfere with binding of the activating cations. This observation confirms the hypothesis that the metals act as catalysts for the hydride shift. The site-directed mutagenesis experiments indicate that both cations are necessary for catalysis. Moreover, interfering with metal binding at site 1 affects the parameters for cation binding to site 2. Crystallographic and kinetic studies have so far identified the cation at site 2 as the primary catalytic metal (Collyer et al., 1990; Collyer & Blow, 1990; Whitlow et al., 1991). Our results suggest that the cation at site 1 is of equal importance. From the crystal structures it is obvious that the cation at site 1 is involved in binding of the cyclic sugar and in stabilization of the open-chain intermediate. Moreover, the O2 of the substrate is a ligand of metal 1 throughout the catalytic cycle. As discussed in the accompanying paper by Jenkins et al. (1992), metal 2 is also involved in stabilization of the open-chain intermediate and is able to coordinate both O1 and O2 during the catalytic cycle. The site-directed mutagenesis results presented here indicate that interactions that are important for proper substrate binding are also important for catalysis, emphasizing the dual role of both cations in this respect.

CONCLUSION

The production and analysis of a number of site-directed mutations in the active site of xylose isomerase from *A. missouriensis* on the whole confirm the catalytic mechanism of this enzyme proposed primarily on crystallographic data of natural isolates. The metal ions act as catalysts in the isomerization reaction. Proton transfer between C2 and C1 of the substrate occurs via a hydride shift mechanism which is facilitated by the bound cations with no involvement of a protein side chain as a catalytic base. The proton shuttle between the oxygens of the substrates (O2 and O1) involves a water molecule coordinated to the metal ion bound in site 2. Our results show that both metals are important for catalysis and not only the metal at site 2 as previously suggested and provide evidence that the proton shuttle requires the assistance of Asp 255 and Lys 183. It is also confirmed that His 220 is not a catalytic base but plays a role in metal coordination and in positioning of the substrate. As to His 54, our results challenge its role as a catalytic base for ring opening despite ample structural evidence that it interacts with the O1H of the cyclic substrate and the O5H of the open-chain intermediate. On the other hand, our data suggest that this residue most probably governs the anomeric specificity of the enzyme.

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