Protein Engineering of Xylose (Glucose) Isomerase from *Actinoplanes missouriensis*. 3. Changing Metal Specificity and the pH Profile by Site-Directed Mutagenesis[†,‡]

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ABSTRACT: Aldose–ketose isomerization by xylose isomerase requires bivalent cations such as Mg^{2+}, Mn^{2+}, or Co^{2+}. The active site of the enzyme from *Actinoplanes missouriensis* contains two metal ions that are involved in substrate binding and in catalyzing a hydride shift between the C1 and C2 substrate atoms. Glu 186 is a conserved residue located near the active site but not in contact with the substrate and not with a metal ligand. The E186D and E186Q mutant enzymes were prepared. Both are active, and their metal specificity is different from that of the wild type. The E186Q enzyme is most active with Mn^{2+} and has a drastically shifted pH optimum. The X-ray analysis of E186Q was performed in the presence of xylose and either Mn^{2+} or Mg^{2+}. The Mn^{2+} structure is essentially identical to that of the wild type. In the presence of Mg^{2+}, the carboxylate group of residue Asp 255, which is part of metal site 2 and a metal ligand, turns toward Gln 186 and hydrogen bonds to its side-chain amide. Mg^{2+} is not bound at metal site 2, explaining the low activity of the mutant with this cation. Movements of Asp 255 also occur in the wild-type enzyme. We propose that they play a role in the O1 to O2 proton relay accompanying the hydride shift.

Xylose isomerases have an absolute requirement for divalent cations (Danno, 1971; Callens et al., 1986). The enzyme from *Actinoplanes missouriensis* is most active with Mg^{2+}, but it also catalyzes isomerization of xylose to xylulose or glucose to fructose with Co^{2+} or Mn^{2+}. The cations bind to two sites with a higher affinity for Co^{2+} and Mn^{2+} than for Mg^{2+}. The metal sites are rich in acidic residues, the pK_a of which may govern the pH dependence of enzymic activity below neutral pH. Metal specificity and the pH dependence of activity are of interest in industrial processes using xylose isomerase to convert glucose into fructose, since cations have to be added and the pH has to be raised for the reaction to take place. These properties can be modified by site-directed mutagenesis of residues involved in metal binding. Mutant enzymes where metal ligands have been removed or replaced are described in an accompanying paper (Jenkins et al., 1992). Changes in metal specificity are observed in these mutants, but the residual activity is low.

This paper describes the effects of replacing Glu 186, an acidic residue that is not directly involved in metal binding but is sufficiently close to the active site to interfere with binding and catalysis. Glu 186 is strictly conserved in known xylose isomerase sequences. We report properties of enzymes where it is replaced with Asp (E186D) or Gln (E186Q). We find that the mutant enzymes have a low activity with Mg^{2+} as the activating cation. With Mn^{2+}, they perform like the wild type on xylose at neutral pH. At acidic pH, the E186Q enzyme is significantly better than the wild type, its pH optimum being downshifted by nearly 2 pH units. The deuterium isotopic effect is normal with Mn^{2+} and much reduced with Mg^{2+}, indicating a change in the rate-limiting step of the reaction.

X-ray structures were determined at 2.4-Å resolution for the E186Q enzyme in the presence of substrate and either Mg^{2+} or Mn^{2+}. Metal site 1 is occupied in both structures, and the substrate appears to bind normally. The two structures differ nevertheless at metal site 2 and in its immediate neighborhood. The side chain of Asp 255, part of site 2, has a different orientation with Mn^{2+}, where it binds to the metal as in the wild-type enzyme, and with Mg^{2+}. In this case, site 2 is not occupied, and Asp 255 hydrogen bonds to the mutated side chain of Gln 186. These structural differences explain the biochemical properties of the mutant. They also support a model of catalysis in the wild type: during aldose–ketose isomerization, a similar movement of the Asp 255 side chain may help in the O1 to O2 proton relay accompanying the hydride shift.

Materials and Methods

Site-Directed Mutagenesis and Purification of the Mutants. Cloning and overexpression in *Escherichia coli* of the *A. missouriensis* xylose isomerase gene will be described elsewhere (P. Stanssens and M. Lauwereys, personal communication). Oligonucleotide-directed mutation construction experiments were carried out by the gapped duplex DNA method with the pMa/pMc plasmid vectors of Stanssens et al. (1989). Re-

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[‡] The coordinates of the structures have been deposited in the Brookhaven Protein Data Bank under the file names 8XIM and 9XIM.

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Table I: Steady-State Reaction Parameters[a]

substrate	cation	pH	WT k_{cat} (s^{-1})	WT K_M (mM)	E186D k_{cat} (s^{-1})	E186D K_M (mM)	E186Q k_{cat} (s^{-1})	E186Q K_M (mM)
xylose	Mg^{2+}	7.5	17.3	5	6.1	7	0.6	1.8
	Co^{2+}	7.5	5.9	7	5.1	27	0.6	2.2
	Mn^{2+}	7.5	6.1	13	8.4	52	3.9	3.2
	Mn^{2+}	6.4					10.3	7.8
glucose	Mg^{2+}	7.5	24.9	290	27.1	740	1.5	60
	Co^{2+}	7.5	12.5	1350	7.9	4200	0.8	45
	Mn^{2+}	7.5	4.7	1540	15.0	1800	16.2	390
fructose	Mg^{2+}	7.5	25.7	240	6.7	200	0.4	16

[a] D-Xylose isomerization was followed at 35 °C and D-glucose and D-fructose at 60 °C. Cation concentration was 10 mM for Mg^{2+} and 1 mM for Co^{2+} and Mn^{2+}.

combinant protein accumulated to about 20% of the total cellular protein content. Overproduced wild-type xylose isomerase and the mutants discussed here were prepared and purified with high yield by immobilized copper-affinity chromatography as will be described elsewhere (N. T. Mrabet, personal communication).

Enzymic Assays. Enzyme activity was determined using sorbitol dehydrogenase as an auxiliary enzyme (Kersters-Hilderson et al., 1987). Steady-state parameters for xylose were measured at 35 °C in 50 mM triethanolamine hydrochloride buffer, pH 7.5, and those for glucose in the same buffer at 60 °C. The metal cation concentration was 10 mM for Mg^{2+}, 1 mM for Mn^{2+}, and 1 mM for Co^{2+}. D-Xylose concentration was varied between 1 and 100 mM and D-glucose concentration between 0.01 and 1 M for K_M and k_{cat} determinations. pH–activity profiles were determined in 25 mM HEPES buffer of the required pH in the range 6–8. Steady-state parameters for fructose were determined under the same conditions as for glucose, isomerization being followed with a commercial kit containing glucose oxidase, horseradish peroxidase and *o*-dianisidine (Sigma). Deuterium isotopic effects were checked using glucose 97% deuterated on the C2 position (Sigma). The presence of deuterium had no observable effect on the coupling enzyme.

Crystallization, Data Collection, and Structure Refinement. The E186Q enzyme was crystallized in 30 mM phosphate buffer, pH 7.0, in drops hanging over pits containing 1.2 M ammonium sulfate. The crystals were isomorphous to the wild-type crystals (space group $P3_221$, $a = b = 143.45$ Å, $c = 231.5$ Å). Before data collection, they were soaked overnight in 1.7 M ammonium sulfate and 100 mM MOPS buffer, pH 6.8, with 0.5 M D-xylose and either 25 mM $MgSO_4$ or 10 mM $MnCl_2$. Two almost complete 2.4-Å data sets (over 95% of expected reflections) were collected on films using the synchrotron X-ray source at LURE (Orsay, France) using monochromatic radiation near 1.4 Å. The crystals were kept at 4 °C during exposure. Data collection and processing were performed as described in Jenkins et al. (1992).

Structural changes seen in $3F_o - 2F_c$ electron density maps, calculated using observed amplitudes and phases from the refined model 1 of wild-type *A. missouriensis* xylose isomerase (Jenkins et al., 1992) omitting residue 186, metal ions, and the substrate, were introduced with FRODO (Jones, 1985). The modified model was then subjected to PROLSQ refinement (Hendrickson, 1985). The two models of the E186Q enzyme were refined to *R* factors of 14.6% (Mg^{2+}) and 14.4% (Mn^{2+}) to 2.4-Å resolution with excellent stereochemistry. Statistics on data collection and refinement are quoted in Table I of Jenkins et al. (1992).

RESULTS

Steady-State Kinetics. The E186D and E186Q mutant enzymes are active on xylose and on glucose (Table I).

Table II: Metal Cation Activation[a]

cation	pH	WT k_{cat} (s^{-1})	WT K_{act} (μM)	E186D k_{cat} (s^{-1})	E186D K_{act} (μM)	E186Q k_{cat} (s^{-1})	E186Q K_{act} (μM)
Mg^{2+}	7.5	18.2	80	6.4	210	0.6	200
	6.0	12.7	3600	4.9	7300	1.9	5400
Mn^{2+}	7.5	7.3	4.8	6.3	5.4	4.0	10
	6.4					10.4	<30
Co^{2+}	7.5	8.4	8.4	5.4	90	0.6	<50

[a] Substrate is 0.2 M D-xylose. K_{act} is the cation concentration that yields half of the maximum activity on 0.2 M D-xylose at 35 °C.

Steady-state parameters of the E186D enzyme are similar to those of the wild-type enzyme. With Mg^{2+} as the activating cation, it has 35% of the maximum activity on xylose and full activity on glucose. With Co^{2+} and Mn^{2+}, the maximum rate of xylose isomerization is about the same as for the wild type, but the K_M increases by less than a factor of 4.

Steady-state parameters of the E186Q enzyme differ more significantly. With Mg^{2+}, it is a rather poor catalyst, having only 3–5% of the maximum activity on xylose and glucose at pH 7.5, albeit with a lower K_M. The drop in efficiency is accompanied with a loss of the isotopic effect observed with deuterated glucose (Table III). In the wild type, the isotopic effect is about 3, consistent with the idea that breaking the CH bond during the C1 to C2 hydride shift is rate limiting. With Mg^{2+} as the activating cation, the E186Q enzyme has an isotopic effect near unity, implying a change in the rate-limiting step. With Mn^{2+}, it is a better catalyst with a higher k_{cat} and a lower K_M on glucose than those of the wild-type enzyme, and the isotopic effect is fully recovered. The E186Q enzyme also has 60% maximum activity on xylose and a K_M 4-fold less than that of the wild type. In addition, its thermostability at 84 °C is similar to that of the wild type in the presence of Mn^{2+}, while it is significantly reduced with Mg^{2+}: the inactivation rate constant increases more than 200-fold (data not shown).

The most striking property of the E186Q xylose isomerase is its pH–activity profile (Figure 1). The pH optimum of the wild-type enzyme for xylose isomerization is 7.3 with Mg^{2+} and 7.9 with Mn^{2+}. It is shifted in the E186Q mutant to about 6.25 with either activating cation. At pH 6.25, the mutant enzyme is about twice as active as the wild type with Mn^{2+}. The pH effect is on k_{cat}, as the apparent affinity for the cations, estimated as the concentration that yields half of the maximum activity, changes by less than a factor of 2 (Table II).

Structure of the E186Q Mutant Enzyme. The substitution at position 186 has no observable effect on the structure outside its immediate neighborhood. Main-chain movements from wild-type *A. missouriensis* xylose isomerase X-ray structure are insignificant: less than 0.1 Å except at the N-terminus which is mobile anyway. Residue 186 has an unusual main-chain conformation with a positive ϕ angle ($\phi = 75°$, $\psi =$

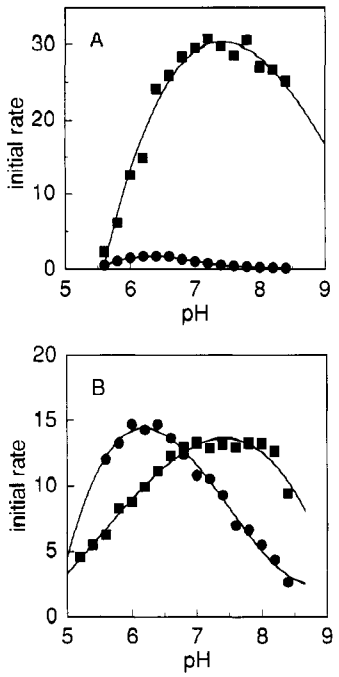

FIGURE 1: pH–activity profiles of wild-type (■) and E186Q (●) xylose isomerases. Panel A: pH profile determined in the presence of 10 mM $MgSO_4$ and 0.2 M xylose using 22 μg of wild type (■) or 317 μg of E186Q (●) per milliliter. Panel B: pH profile determined in the presence of 1 mM $MnCl_2$ and 0.2 M xylose using 22 μg of wild type (■) or 63 μg of E186Q (●) per milliliter. The initial rate is expressed in micromoles per minute per milligram.

105°) in the wild type, and the following residue is a cis proline. These features, already noted by Collyer et al. (1990) for equivalent residues of *Arthrobacter* xylose isomerase, are unchanged in the mutant.

The active site of the E186Q–xylose–Mn^{2+} complex is shown in Figure 2 superimposed on that of the wild-type enzyme–xylitol–Co^{2+} complex [model 1 of Jenkins et al. (1992)]. Both metal sites are fully occupied by Mn^{2+} and have octahedral coordination. At site 1, Mn^{2+} coordinates to the substrate O2 and O4, and it is less than 0.1 Å from the Co^{2+} position in the wild-type–xylitol complex (model 1) and from the Mg^{2+} position in the xylose complex (model 5). Moreover, the substrate is in open conformation and overlaps exactly with xylitol (Figure 2). At site 2, Mn^{2+} does not coordinate directly to O1 of the substrate as Co^{2+} does in the wild-type structure. The cation forms six bonds to Glu 217, His 220, Asp 255, Asp 257, and a water molecule (W690). These bonds are characteristic of site 2_0 (Jenkins et al., 1992). This is confirmed by the position of Mn^{2+} only 0.13 Å from that of Co^{2+} in model 2 (wild type, no substrate) which defines site 2_0. It is 0.5 Å away from the position of Co^{2+} in model 1 and 0.5–0.7 Å from that in other examples of site 2_1 listed in Table III of Jenkins et al. (1992). Otherwise, the mutant structure is indistinguishable from that of the wild type.

In contrast, the active site of the E186Q–xylose–Mg^{2+} complex deviates from others in a number of ways. Site 1 is the same as in wild type and so is the xylose moiety bound to it. Electron density at the expected metal 2 position is weak and suggests that the metal is largely replaced with either a water or an ammonium ion, which could hydrogen bond to Glu 217, to His 220, and to both O1 and O2 of the substrate. Figure 3 compares the two mutant structures. With Mg^{2+}, it can be seen that the carboxylate of Asp 255 has turned away from site 2 and points now toward Gln 186. Its side chain changes from the trans conformation ($\chi 1 = -178°$) to g^+ ($\chi 1 = 44 \pm 8°$, average and standard deviation taken over the four subunits). Its carboxylate hydrogen bonds to the amide of the mutated side chain, with an Oδ···Nε distance of 2.85 ± 0.35 Å. The amide carbonyl of Gln 186 in the Mn^{2+} structure and the carboxylate of Glu 186 in the wild-type enzyme hydrogen bond to the main-chain NH of Asp 255 instead of its carboxylate.

Table III: Deuterium Isotopic Effect[a]

		WT		E186Q	
cation	pH	$k_{cat}(H)/k_{cat}(D)$	$K_M(H)/K_M(D)$	$k_{cat}(H)/k_{cat}(D)$	$K_M(H)/K_M(D)$
Mg^{2+}	7.5	3.3	1.2	0.87	1.1
Mn^{2+}	8.0			3.0	1.5
Mn^{2+}	6.7			2.7	1.7

[a] Steady-state parameters for glucose labeled with deuterium on C2 in the presence of 10 mM Mg^{2+} or 1 mM Mn^{2+} in TEA buffer at 60 °C.

Asp 255 also interacts with the ε-amino group of Lys 183, an essential residue and a catalytically important group (Lambeir et al., 1992) as it binds O1 of the substrate in all our structures. In the E186Q–xylose–Mg^{2+} complex, one of the Asp 255 carboxylate oxygens is still within hydrogen-bond distance (2.75 ± 0.4 Å) of Nζ of Lys 183, yet the lysine maintains its position and conformation. Other small movements can be seen in Figure 3, notably that of a loop containing residues 25 and 26 from a neighboring subunit. The side chain of Phe 26, which comes close to Asp 255, moves away as a result of steric hindrance when Asp 255 turns toward it.

Discussion

Glu 186 was chosen as a target for site-directed mutagenesis because it is a conserved residue near the active site but is not directly involved in substrate or metal binding. Removing its negative charge could be expected to change the affinity for divalent cations and the pH dependence of enzymic activity. The actual result is a changed metal specificity and a large shift in pH dependence. The wild-type enzyme is most active with Mg^{2+} and near pH 7.5. The E186Q enzyme has a strong preference for Mn^{2+} as the activating cation and an optimum pH near 6.25. With Mn^{2+} and at pH below neutrality, it is significantly more active than the wild-type enzyme, and it has a lower K_M for pentose and for hexose substrates. The E186D enzyme, where the negative charge is displaced but not removed, has similar but less pronounced features.

The pH profile of xylose isomerase is bell-shaped with an optimum between pH 7 and pH 8. Vangrysperre et al. (1990) have shown that the drop in activity at acidic pH is due to an increase in K_M, k_{cat} being constant between pH 6.0 and pH 8.0. As the substrate directly interacts with the metal ions, K_M values also reflect the affinity for divalent ions. The pK_a that affects K_M on the acidic side may therefore belong to one or, more likely, to several of the many carboxylic acids involved in metal binding. In the absence of metal, there is no major rearrangement of the active site region (Collyer et al., 1990; Jenkins et al., 1992). When present, the metal ions compete with protons for the carboxylates of four aspartates (Asp 245, Asp 255, Asp 257, Asp 292) and two glutamates (Glu 181, Glu 217) and the imidazole of His 220.

The carboxylate of Glu 186 is only 7 Å away from metal site 2 in the wild-type structure. This is near enough for its negative charge to affect metal binding through the pK_a of the ligands. Replacing Glu 186 with a glutamine should lower these pK_a and raise the metal affinity at low pH. Such effects have been observed in calbindin when charges surrounding the Ca^{2+} binding site are changed by mutagenesis (Linse et al., 1988). The pH profile of the E186Q enzyme with Mn^{2+}

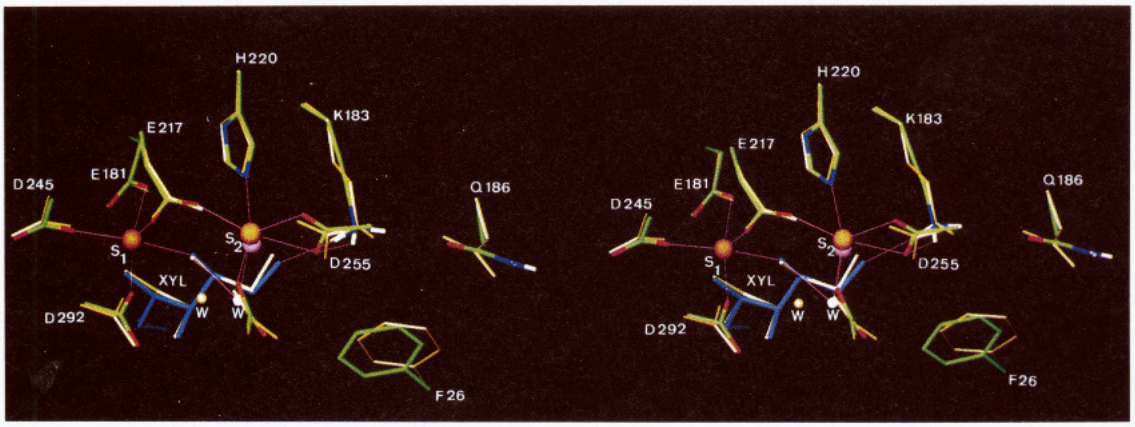

FIGURE 2: Active site of the E186Q–Mn–xylose complex. The structure of the wild-type–Co–xylose complex is superimposed on that of the E186Q–Mn–xylitol complex. Active site residues and the sugar ligand in the wild-type complex are displayed in gold with the bound Mn^{2+} shown in red/brown. The corresponding residues in the E186Q structure are displayed using conventional bond coloring (bonds to oxygens are in red, those to nitrogens in blue, and those to carbons in green). The bound Co^{2+} is shown in purple, and the open form of xylose is displayed in blue. For the sake of clarity, metal coordination (purple bonds) is depicted for Co in the wild-type structure; metal bindings are labeled S_1 and S_2 for sites 1 and 2, respectively.

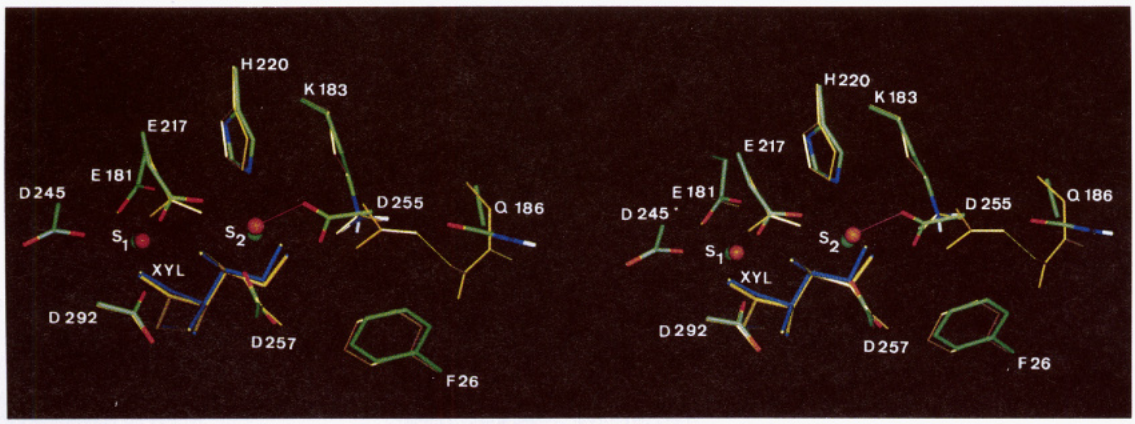

FIGURE 3: Superimposed active site structures of the E186Q–xylose complexes with bound Mn^{2+} and Mg^{2+}, respectively. Residues of the Mn^{2+} complex are displayed using conventional bond coloring (bonds to oxygens are in red, those to nitrogens in blue, and those to carbons in green). The Mn ions are displayed in brown, the open-chain xylose is shown in blue, and the bound water molecule (W) is in white. Active site residues and the sugar ligand in the Mg^{2+} complex are displayed in gold, and the bound water molecule in yellow. Major changes occur at residue Asp 255. In the E186Q–Mn^{2+}–xylose complex, which is wild type like, it is coordinated (purple bond) to the Mn ion bound at site 2 (S_2). In the E186Q–Mg^{2+}–xylose complex, this residue rotates away to form an H-bond with the Q186 side chain (yellow bond).

(Figure 1) is compatible with an acidic pK_a shift.

The enzymic activity is governed on the alkaline side by another pK_a. Near 9 in the wild-type enzyme, it is shifted to below 7.5 in the mutant. Possible candidates for this pK_a are a water molecule interacting with metal 2 (W690, see below) and the ϵ-amino group of Lys 183. This lysine, which cannot be replaced (by serine, glutamine, or arginine) without a total loss of activity, interacts with O1 of the substrate and with Asp 255 when it is in position to bind to the metal (Lambeir et al., 1992). The specific effect of the E186Q mutation, discussed below, on Asp 255 may indirectly lower the pK_a of Lys 183 on the alkaline side as well as that of the aspartate on the acidic side.

The E186Q enzyme has a lower K_M than the wild-type enzyme. The factor is about 4 at pH 7.5 with either Mg^{2+} or Mn^{2+} as the activation cation (Table I). This suggests another possible electrostatic effect on the bound substrate. The carboxylate of Glu 186 is less than 10 Å from both O1 and O2 of the substrate, and its charge should raise the pK_a of the hydroxyl group at O2. If the metal ions bind the hydroxylate better, removing the negative charge of Glu 186 would improve the enzyme affinity for the open-chain substrate.

The high activity of the mutant enzyme with Mn^{2+} is easily understood from the X-ray structure of the E186Q–xylose–Mn^{2+} complex, which shows essentially no effect attributable to the mutation: both metal sites are occupied and the substrate binds normally. That of the E186Q–xylose–Mg^{2+} complex explains equally well the low activity of the mutant enzyme with Mg^{2+}. The cation binds weakly at site 2, and the new conformation of the Asp 255 side chain suggests that the metal competes with the amide of Gln 186 for interaction with the carboxylate of Asp 255. With Mn^{2+}, the competition is in favor of the metal and a wild-type-like structure is restored.

A similar competition may well occur in the wild-type enzyme. When no substrate is present, Asp 255 forms a bidentate bond to metal 2. The bond becomes long or monodentate in the presence of an open-chain substrate or inhibitor, and it is broken altogether in two structures [models 14 and 19 in Jenkins et al. (1992)], so that the side chain is free to adopt two alternative conformations. In all structures of *A. missouriensis* xylose isomerase, we built Asp 255 in the trans side-chain conformation, even though there was sometimes evidence for multiple conformations and disorder in the electron density map. In the E186Q–xylose–Mg^{2+} complex, the conformation is clearly g^+. The g^+ conformation is also observed for the equivalent residue Asp 254 in at least two

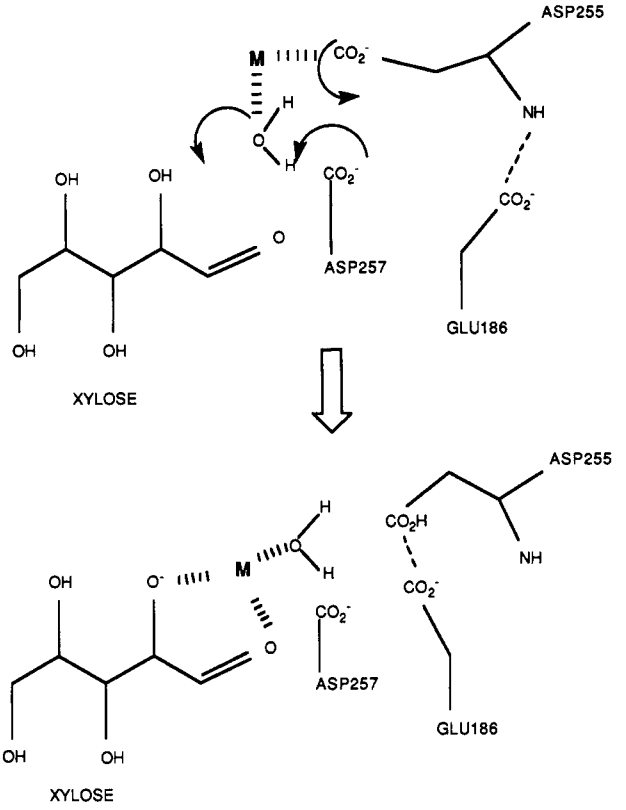

FIGURE 4: Mechanism for the O2 to O1 proton shuttle in the wild-type enzyme. The proton on O2 of xylose or glucose is transferred onto a water molecule bound to metal 2 and then onto Asp 257 and Asp 255. The protonated Asp 255 side chain rotates about the Cα–Cβ bond and hydrogen bonds to Glu 186 (in the wild type) or Gln 186 (in the E186Q mutant), letting the metal free to approach O1, which will carry a negative charge after the hydride transfer from C2 to C1. Hydride and proton transfer may be concerted.

of the X-ray structures of *Arthrobacter* xylose isomerase: the Mn^{2+} complex (Collyer et al., 1990) and the sorbitol–Mg^{2+} complex (Henrick et al., 1989; file 4XIA in the Brookhaven Protein Data Bank). The active site of the latter is very similar to that of our E186Q–xylose–Mg^{2+} complex. The two carboxylates of residues equivalent to Asp 255 and Glu 186 are in close contact (O···O distances less than 2.5 Å), which implies that one of them is protonated at the pH used for these studies, which is lower (6 instead of 7) than the one we used here. Glu 186 can then donate a hydrogen bond just as Gln 186 does in E186Q. In other structures of *Arthrobacter* and of *Streptomyces rubiginosus* xylose isomerases, multiple or alternative conformations of Asp 255 are noted (Collyer et al., 1990; Whitlow et al., 1991).

We suggest that the rotation of the Asp 255 side chain is an integral part of the xylose isomerase mechanism. First, it allows the metal to move from site 2_0 in the absence of substrate, to site 2_1 when it binds O1, and then probably to site 2_2 when the hydride shift occurs. Second, Asp 255 may play a major role in shuttling a proton between O2 and O1 (Figure 4). While the C2 to C1 hydrogen transfer does not involve base catalysis, that from O2 to O1 should do so. Whitlow et al. (1991) point out that a water molecule tightly bound to metal 2 (W690 in our structure) is ideally positioned for this purpose. It is at hydrogen-bonding distance from both oxygen atoms of the substrate, and its pK_a is expected to be low as a result of binding the cation. The carboxylate of Asp 257 is in position to abstract a proton from W690, which in turn does so with O2 of an aldose substrate. The proton of Asp 257 is transferred to Asp 255, which is no longer a good metal ligand when protonated. The metal is then free to approach O2, while Asp 255 turns away and hydrogen bonds to Glu 186.

This study therefore adds new information on the catalytic mechanism of aldose–ketose isomerization by xylose isomerases, while demonstrating that a single amino acid substitution can shift the pH optimum of the enzyme by more than 1 pH unit.

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