Role of the Divalent Metal Ion in Sugar Binding, Ring Opening, and Isomerization by D-Xylose Isomerase: Replacement of a Catalytic Metal by an Amino Acid†,‡

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ABSTRACT: The distinct roles of the two magnesium ions essential to the activity of D-xylose isomerase from *Streptomyces olivochromogenes* were examined. The enzyme–magnesium complex was isolated, and the stoichiometry of cation binding determined by neutron activation analysis to be 2 mol of magnesium per mole of enzyme. A plot of Mg^{2+} added versus Mg^{2+} bound to enzyme is consistent with apparent K_D values of ≤0.5–1.0 mM for one Mg^{2+} and ≤2–5 mM for the second. A site-directed mutant of D-xylose isomerase was designed to remove the tighter, tetracoordinated magnesium binding site (site 1, Mg-1); Glu180 was replaced with Lys180. The stoichiometry of metal binding to this mutant, E180K, is 1 mol of magnesium per mole of enzyme. Ring-opening assays with 1-thioglucose (H_2S released upon ring opening) show E180K catalyzes the opening of the sugar ring at 20% the rate of the wild-type, but E180K does not catalyze isomerization of glucose to fructose. Thus, the magnesium bound to Glu180 is essential for isomerization but not essential for ring opening. The X-ray crystallographic structures of E180K in the absence of magnesium and in the presence and absence of 250 mM glucose were obtained to 1.8-Å resolution and refined to *R* factors of 17.7% and 19.7%, respectively. The wild-type and both E180K structures show no significant structural differences, except the ϵ-amino group of Lys180, which occupies the position usually occupied by the Mg-1. Other active-site residues usually bound to Mg-1 are only slightly changed in position, with a magnesium ion occupying the second metal-binding site (site 2, Mg-2). Structurally, the lysine residue has successfully replaced Mg-1. Europium(III) excitation spectroscopy shows that when Eu^{3+} is bound to E180K at site 2 (in the absence of substrate), one to two water molecules is coordinated to Eu^{3+}. The X-ray crystallographic structure of the E180K enzyme shows that one hydroxide molecule is coordinated to Mg-2. This result is consistent with the apparent pK_a of 7.2 from a plot of pH versus log V_{max}/K_m for the wild-type enzyme. Hydroxide coordinated to Mg-2 could act to deprotonate the glucose O2 and protonate O1 in a step concomitant with the hydride-transfer step of isomerization.

D-Xylose isomerase (EC 5.3.1.5), one of the most widely used industrial enzymes, catalyzes the interconversion of aldose and ketose sugars and has broad substrate specificity. Although the physiological substrate appears to be xylose, the commercial importance of the enzyme arises from its ability to interconvert glucose and fructose. Catalysis by D-xylose isomerase requires divalent metal cations. A variety of crystallographic studies (Carrell et al., 1989; Collyer et al., 1990; Whitlow et al., 1991) have located two metal binding sites per subunit, both at the active site. Several metals give some activity, but magnesium and manganese are preferred and magnesium appears to be the physiological cofactor under most conditions (Suekane et al., 1978).

Xylose isomerase binds the closed form of its sugar substrates. NMR studies have shown that only the α-anomer of glucose is bound (Feather et al., 1970; Schray & Rose, 1971). The enzyme catalyzes ring opening to generate the productive open-chain conformation of the substrate (Collyer et al., 1990; Whitlow et al., 1991; Lambeir et al., 1992; Jenkins et al., 1992) which is directly coordinated to one of the metal sites. Isomerization appears to proceed via a hydride-transfer mechanism. Since non-metallo sugar isomerases employ a proton-transfer mechanism, we and others have proposed that the use of a metal to bind the substrate imposes hydride-transfer chemistry on this enzyme (Farber et al., 1989; Collyer et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992; van Tilbeurgh et al., 1992; Lee et al., 1990).

In this paper, we examine the roles of the two distinct metal ions in the active site of D-xylose isomerase from *Streptomyces olivochromogenes*. Neutron activation analysis was used to determine the stoichiometry of metal binding and the relative affinities of the two sites. We find the two sites to differ by approximately 1 order of magnitude in their affinity for magnesium. To probe the role of the tetracoordinated site (site 1, Mg-1), we have replaced one of its ligands with a lysine residue by site-directed mutagenesis. The mutant enzyme, E180K, contains only one Mg^{2+} per active site as determined by neutron activation analysis. We find that E180K is able to bind substrate and catalyze ring opening with near wild-type efficiency, but is devoid of isomerase activity. The X-ray crystallographic structure of E180K,

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‡ The coordinates of the structures have been deposited in the Brookhaven Protein Data Bank under filenames 1XYL and 1XYM.

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solved to 1.8-Å resolution, confirms that one metal binding site remains intact while the second is occupied by the ε-amino group of Lys180. Europium(III) excitation spectroscopy shows that one to two waters is coordinated to the metal bound in the remaining, octahedrally coordinated metal site (site 2). pH rate studies of the wild-type enzyme are consistent with a possible role for a water as a catalytic base.

MATERIALS AND METHODS

Strains, Plasmids, and Molecular Biology Reagents. Site-directed mutagenesis was performed using the Mutagene Kit (BioRad) which is based on the method of Kunkel (1985). The *Escherichia coli* strains MV1190 and CJ236 were used in producing and selecting for the E180K mutation. The mutant gene was transferred to the vector PKK223-3 (Pharmacia), where expression is controlled by the *tac* promotor. Restriction and DNA modifying reagents were purchased from New England Biolabs and U.S. Biochemicals. DNA sequencing was performed using Sequenase v 2.0 (U.S. Biochemicals). DNA primers were prepared by Gensys.

Site-Directed Mutagenesis. Primer-directed site-directed mutagenesis was carried out using an oligonucleotide complementary to the region of the gene surrounding the codon for E180. The 20 base oligonucleotide, 5′-GGCTTGGGCTTGATCGCGAA-3′, contains one mismatch. In addition to directing the mutation of the GAG codon for E180 to an AAG codon for K180, it also results in the loss of a *PvuI* restriction site which aided in screening for the mutation. After preliminary screening, the E180K mutation was confirmed by sequence analysis, and the mutant gene was cloned into PKK223-3 (pX15-E180K) for expression in the *xyl*$^-$ *E. coli* strain HB101.

Purification of Xylose Isomerase. Wild-type xylose isomerase and E180K were purified as described in the preceding paper. In the case of E180K, Mg^{2+} was excluded from the buffers used in the purification procedure. Fractions from column chromatography were examined for E180K by SDS/PAGE.

Substrates, Materials, and Analytical Procedures. The substrates and inhibitors α-D-(+)-glucose, β-D-(−)-fructose, 1-thio-β-D-glucose (sodium salt), and xylitol were purchased from Sigma Chemical Company and used without further purification. In the case of 1-thio-β-D-glucose, stock solutions were allowed to incubate >12 h to allow equilibration of the α- and β-forms before use as a standard in enzyme assays. The metal complexes magnesium chloride hexahydrate and manganese(II) chloride tetrahydrate were purchased from Aldrich Chemical Company at the highest purity available. The inhibitor xylitol was purchased from Sigma Chemical Company. All other organic compounds used were reagent grade or better.

Xylose isomerase assays using α-D-glucose as substrate and ring-opening assays using the substrate 1-thio-glucose were performed as previously described (Allen et al., 1994) except where otherwise stated.

Neutron Activation Analysis. Demetalized wild-type xylose isomerase or E180K [see Materials and Methods of previous paper (Allen et al., 1994)], at 4.3×10^{-4} M was incubated > 1 h with 0.1, 0.5, 1.0, 2.0, 5.0, or 10 mM $MgCl_2$ in 0.025 M HEPES–KOH buffer, pH 7.5, in a total volume of 0.15 mL at 25 °C. As a control sample, the 10 mM $MgCl_2$ concentration was also repeated in the absence of enzyme. The samples were then placed on centrifugation columns [described by Penefsky (1979) as modified by Allen and Abeles (1989)], and 0.5 mL of the eluent was placed in metal-free plastic bags (plastic bags soaked in 1:3 nitric acid/H_2O for 2 h). The remaining eluent was analyzed for protein. The sample bags were placed in a desiccator in metal-free plastic beakers (note that no desiccant was present to avoid sample contamination, also labels were placed only on the beakers, not the plastic bags). The desicator was cooled to −20 °C for >3 h to freeze samples, and placed under vacuum (<1 mmHg), and the samples were lyophilized for >12 h. The samples were then subjected to neutron activation analysis for the presence of magnesium at the Nuclear Reactor Facility, Massachusetts Institute of Technology.

Europium(III) Excitation Spectroscopy. Europium(III) was used as a luminescence probe to investigate the nature of the remaining metal binding site (site 2) in the E180K mutant. Following the work of Horrocks et al. (1979), $EuCl_3$ was added to a final concentration of 2×10^{-4} M in a solution of E180K (2×10^{-4} M active sites) in 0.025 M HEPES–KOH buffer, pH 7.6, and the mole fraction of D_2O was varied between 0 and 0.3. A 0.02-mL drop was suspended from a capillary and irradiated with light at 394 nm from a pulsed (20 Hz) dye laser pumped by a nitrogen laser. Emission was monitored with a monochromator set to accept the $^5D_0 \rightarrow {}^7F_0$ emission at 592 nm. Photomultiplier tube output was coupled to a terminating resistance of 4.7 kΩ. Data were combined and averaged from 256 pulses. The excited-state lifetime was measured by plotting the natural logarithm of the emission intensity versus time.

X-ray Crystallographic Data Collection. Crystals of the E180K mutant were grown by the sitting drop vapor diffusion method, under conditions identical to those used to obtain crystals of wild-type xylose isomerase (Farber et al., 1987) with the exception that Mg^{2+} was excluded from the crystallization solutions. Crystals of E180K did not grow from conditions which included 10 mM $MgCl_2$. For the structure in the presence of glucose, crystals were soaked in 0.25 M α-D-glucose in 65% ammonium sulfate, 0.025 M HEPES–KOH buffer, pH 7.5. Diffraction data for the E180K crystals with glucose were collected to 1.5 Å resolution at −5 °C on a FAST area detector at the Brookhaven National Laboratories X-12 beam line. Data reduction using the program MADNES (Pflugrath & Messerschmidt, 1987) PROCOR and XSCALE (Kabsch, 1988) resulted in a total of 57 060 unique reflections with $I > 0$ to 1.8 Å resolution, which represents 63% of the unique data. (Data from 1.5 to 1.8 Å were excluded due to low completeness.) Diffraction data for E180K in the absence of glucose were collected to 1.8-Å resolution at 4 °C on a Siemens area detector. Data reduction was performed using the program XDS and XSCALE (Kabsch, 1988) resulting in a total of 167 580 reflections with $I > 0$ from 5.0- to 1.8-Å resolution, of which 67 901 were unique reflections (75% of the unique data).

Crystallographic Refinement. Crystals of E180K and E180K plus glucose were isomorphous with those of the wild-type xylose isomerase, allowing the structure of E180K to be solved by using the phases from the wild-type enzyme. (An 1.81 Å resolution structure of xylose isomerase plus Mg^{2+} refined to an R factor of 16.1 was solved recently in this lab (Lavie, unpublished results).) Note that there is a dimer in the asymmetric unit and that each monomer was manually rebuilt independently. The phases and structure factors were calculated from the wild-type structure in which the magnesium ions were omitted but the residue Glu180 was not changed to lysine (this was to avoid bias from the residue of interest). This calculation and an initial molecular dynamics refinement was accomplished using the program XPLOR

Table 1: Data Collection and Refinement Statistics for the Structure of the E180K Mutant of Xylose Isomerase with and without the Substrate α-D-Glucose

	E180K	E180K, 0.25 M glucose
	Data Collection	
space group	$P2_12_12$	$P2_12_12$
unit cell	a = 87.7 Å	a = 88.1 Å
	b = 99.5 Å	b = 99.5 Å
	c = 94.2 Å	c = 94.8 Å
	$\alpha = \beta = \gamma = 90.0°$	$\alpha = \beta = \gamma = 90.0°$
molecules per asymmetric unit	2	2
resolution (Å)	∞ to 1.8	∞ to 1.5
	Refinement	
no. of unique reflections	59 731	46 227
resolution range (Å)	10.0–1.8	5.0–1.8
completeness	78%	63%
final R factor	17.7	19.7
restraints (rms observed)		
bond length (Å)	0.014	0.015
bond angles (Å)	2.8	2.7
dihedral angles (deg)	22.8	22.8
improper angles (deg)	1.2	1.2
average B factor		
main chain	13	21.5
side chain	17	25
water	30	34
total no. of protein atoms	6048	6048
total no. of waters	476	473

(Brünger, 1987). The initial R factor after individual B factor refinement was 23.8% for the glucose structure, 22.7% for the structure without glucose. The electron-density map was calculated using the coefficents $(3F_o - 2F_c)$. The side chain of Lys180 was easily positioned into the observed density. Molecular dynamics refinement alternating with manual refitting to the $(2F_o - F_c)$ electron-density map was performed for more than two cycles. Water molecules were added after the first cycle of refinement in two rounds using the program WATERHUNTER (S. Sugio, unpublished results) to fit waters to electron density calculated using the coefficients $(F_o - F_c)$. Finally, two cycles of molecular dynamics refinement were performed, with the linear form of the substrate D-glucose added to the E180K plus glucose structure after the first cycle. The final R factors were 19.7% and 17.7% for the E180K with and without glucose respectively, with good geometry for the models (see Table 1).

RESULTS

Characterization of E180K. Site-directed mutagenesis (see Materials and Methods) was performed to replace Glu180 with Lys180 to test whether Mg-1 could be replaced structurally and functionally by the ε-amino group of lysine. The E180K mutant, purified to homogeneity, migrates on SDS/PAGE identically to wild-type xylose isomerase.

E180K was assayed for isomerase activity with α-D-glucose (0.25 M) as substrate and in the presence of 0.001, 0.010, and 1 M $MgCl_2$ in 0.025 M HEPES–KOH buffer at 37 °C [see Materials and Methods (Allen et al., 1994)]. After 3 weeks of incubation, no product (α-D-fructose) was detected at any of the magnesium concentrations utilized. Thus, E180K does not catalyze the isomerization of α-D-glucose to α-D-fructose even at concentrations of magnesium exceeding by 100-fold that required for isomerase activity by the wild-type enzyme. Therefore, the mutation of E180K did not simply weaken the affinity of site-1 for magnesium but completely eliminated the site.

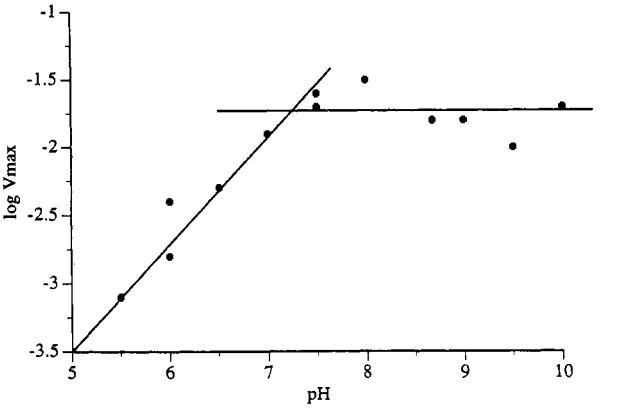

FIGURE 1: log V_{max} for isomerization of α-D-glucose to α-D-fructose at various pH values. V_{max} was derived from Lineweaver–Burk plots using the initial velocities. The reactions contained 8.3×10^{-6} M xylose isomerase, α-D-glucose (0.04–0.25 M), 0.01 M $MgCl_2$, and 0.025 M of the following buffers: pH 5.5 MES–KOH, pH 6.0 MES–KOH or PIPES–KOH, pH 6.5 PIPES-KOH, pH 7.0 HEPES–KOH, pH 7.5 HEPES–KOH or TES, pH 8.0 HEPES–KOH, pH 8.68 CHES, pH 9.0 CHES or BICINE, pH 9.5 CHES, pH 10.0 CAPS. Reactions were performed at 25 °C and were initiated by enzyme addition. At various time points, aliqots were diluted 40-fold into buffer, and the amount of fructose was determined using cysteine–carbazole reagent (Diesche & Borefreund, 1951). Product formation was measured at 560 nm. All spectrophotometric assays were performed on a Hitachi U-2000 UV/vis spectrophotometer using 1-cm quartz cells.

In order to determine whether E180K does catalyze ring opening, which is believed to precede the isomerization step in the normal catalytic mechanism, a ring-opening assay was performed with 1-thioglucose as substrate [see Materials and Methods (Allen et al., 1994)]. As in the assay with wild-type xylose isomerase, 0.010 M $MgCl_2$ was included in the buffer. Ring opening, as determined by the amount of H_2S release, was determined to be 20% of that of the wild-type enzyme; 0.05 μmole H_2S per 96 h produced by E180K at identical protein and 1-thioglucose concentrations as those utilized for the wild-type. This provides direct evidence that E180K catalyzes the ring-opening reaction. Note that the enzyme-catalyzed rate of ring opening for 1-thioglucose is slow compared to the rate of ring opening of glucose (Allen et al., 1994). However, the rate for 1-thioglucose is a lower limit since not every ring-opening event necessarily results in an elimination event. Also, the slow rate obtained is comparable to rates obtained for slow substrates of other enzymes (Bender & Kezdy, 1965).

Ionizable Residues of Wild-Type Xylose Isomerase. The dependence upon pH of the kinetic constants of wild-type xylose isomerase with glucose as substrate was determined at 25 °C. The pH range covered was from 5.5 to 10.0 and the measurements at pH 6.0, 7.5, and 9.0 were repeated in different buffers to eliminate the possibility of buffer catalysis. The resulting constants at the same pH with different buffers were not significantly different. The K_m did not vary over the pH range studied, thus plots of V_{max} versus pH and V/K versus pH did not differ significantly. Figure 1 shows the change in log V_{max} versus pH. The shape of the V_{max} plot is consistent with a single ionizable group with a pK_a value of 7.2.

Quantification of Mg^{2+} in Wild-Type Xylose Isomerase and E180K. In order to establish the number of magnesium ions bound to wild-type xylose isomerase, the enzyme–magnesium complex was isolated (from solutions containing various concentrations of magnesium) and subjected to neutron activation analysis (see Figure 2 and accompanying legend). The protein concentration as determined by Bradford (1979)

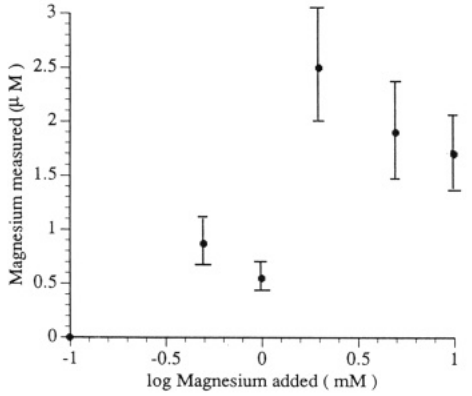

FIGURE 2: Total magnesium bound to wild-type xylose isomerase at various $MgCl_2$ concentrations. Demetalized wild-type xylose isomerase was incubated with various concentrations of $MgCl_2$ (0–0.01 M), and the xylose isomerase–magnesium complex (4.3×10^{-4} M) was isolated and subjected to neutron activation analysis as detailed in Materials and Methods. The total magnesium concentration determined per enzyme sample is plotted versus the log concentration $MgCl_2$ in the incubation.

protein assay was used to calculate the total number of moles of magnesium bound per mole of xylose isomerase. An identical sample in the absence of protein was also submitted so that magnesium not bound to enzyme which bled through the column could be subtracted from each sample. In the case of the wild-type xylose isomerase, at concentrations of 0–0.001 M $MgCl_2$, 0.8 mol of Mg^{2+}/mol of enzyme were detected, and at concentrations of 0.002–0.01 M $MgCl_2$, 1.9 mol of Mg^{2+}/mol of enzyme were detected. In the case of the E180K mutant, only 1.2 ± 0.1 mol of Mg^{2+}/mol of enzyme was detected in the presence of 0.01 M $MgCl_2$. Therefore, there are two magnesium-binding sites present in wild-type xylose isomerase while there remains only one magnesium-binding site in the E180K mutant. Furthermore, the binding constants of the two magnesium ions for the wild-type xylose isomerase are approximately measured by this experiment, with $K_{D\ app}$[1] ≤ 0.5–1 mM for one magnesium and $K_{D\ app}$ ≤ 2–10 mM for the second.

Ligands of the E180K Metal Binding Site. It has been shown previously (Farber et al., 1987; Sudfeldt et al., 1990) that Eu^{3+} binds to both Mg-1 and Mg-2 sites of wild-type xylose isomerase. Figure 3 shows the linear dependence of the inverse of the excited-state lifetime of Eu(III) bound to E180K versus the mole fraction of D_2O in solution. The slope of this line corresponds to 1.7 waters bound to the Eu(III). The uncertainty inherent in the method coupled to the experimental uncertainty gives rise to the possibility that either one or two water molecules could be bound to Eu(III) at site-2 (Horrocks & Sudnick, 1979). Crystallographic evidence (Whitlow et al., 1991) has pointed to a single water molecule bound to this position in the wild-type enzyme. This result confirms that the metal-binding capacity of the Mg-2 site has not been dramatically altered by the replacement of Glu180 with Lys, and the coordination geometry has been maintained. Addition of 80 mM xylitol to E180K containing Eu(III) does not alter the excited-state lifetimes (data not shown), indicating that the coordination remained constant as is to be expected from crystallographic characterization of site 2 in the wild-type enzyme (Whitlow et al., 1991) and in the E180K enzyme in the presence of glucose (see below).

The Structure of E180K. The 1.81-Å E180K structures with and without glucose were refined to R factors of 19.7%

[1] Abbreviations: $K_{D\ app}$, apparent K_D.

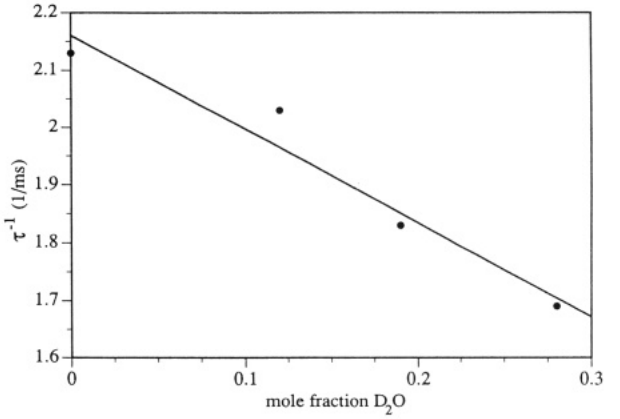

FIGURE 3: Plot of the reciprocal lifetimes of Eu(III) bound to E180K in solutions containing varying mole fractions of D_2O. The slope of the line fit to the data (−1.63) can be related to the number of ligated waters by the multiplier, A_{Eu}, equal to 1.05 (Horrocks & Sudnick, 1979).

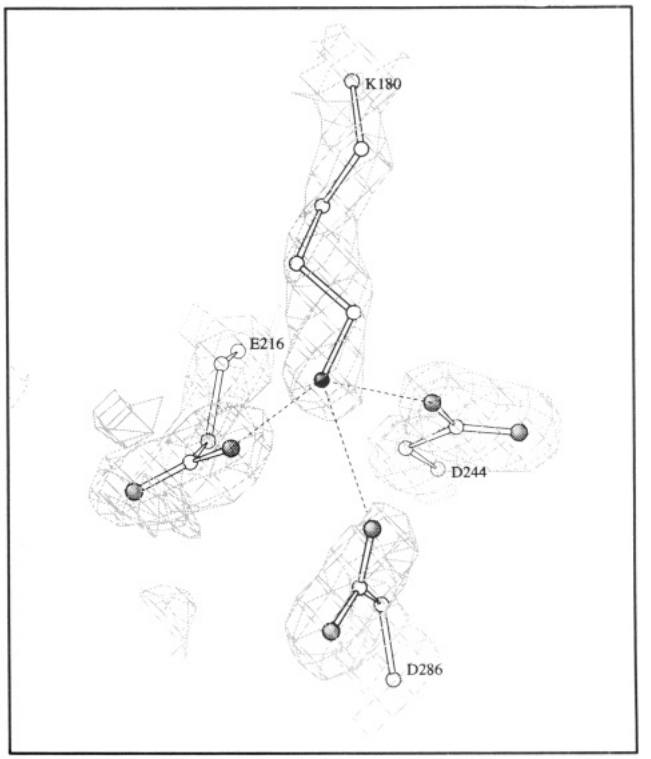

FIGURE 4: Fit of Lys180 into the 1.8-Å resolution electron-density map calculated with coefficients ($2F_o - F_c$) at the 1.5σ contour level. Also depicted are the molecular model and electron density for those residues which are within a 3.0-Å sphere of the ϵ-N of Lys180. This figure was rendered using the program Molscript (Kraulis, 1991).

and 17.7%, respectively, with only minor changes to the original native xylose isomerase model. The overall structure of E180K is identical to the structure of the wild-type isomerase. The rms deviation for α carbons between the wild-type enzyme and E180K in the presence of glucose is 0.18 Å and in the absence of glucose is 0.13 Å. (Note that the rms deviation for α carbons between the two E180K structures themselves is 0.17 Å.) The position of lysine 180 (the site of the mutation) was determined from an electron density map calculated from a model with the native Glu180 still in place in order to exclude the possibility of model bias from the key residue. A lysine was easily fit into the electron density calculated with coefficients ($2F_o - F_c$). Figure 4 depicts the fit of the Lys180 residue into this electron density and the surrounding ligands for the structure of E180K without glucose.

Table 2: Metal Ligands and Distances[a]

	E180K			E180K + glucose		
		distance (Å)			distance (Å)	
ion	ligand[b]	Mon1	Mon2	ligand	Mon1	Mon2
ϵN-Lys180	Glu216	2.7	2.8	Glu216	2.6	2.7
	Asp244	3.1	2.8	Asp244	2.9	2.9
	Asp286	3.1	3.3	Asp286	2.8	3.3
				sugar O2	2.6	2.6
				sugar O4	2.6	2.7
Mg-2	Glu216	2.5	2.5	Glu216	2.4	2.4
	His219	2.6	2.9	His219	2.7	2.8
	Glu254	2.4	2.4	Glu254	2.4	2.3
	Glu254	2.5	2.6	Glu254	2.7	2.7
	Glu256	2.5	2.5	Glu256	2.4	2.4
	H_2O	1.9	1.9	H_2O	1.9	

[a] All distances are ±0.25 Å. [b] Ligands are to side chain carboxylates of Asp and Glu or side chain Nϵ2 of histidine.

In the E180K structures with and without glucose, the position of the ϵ-amino group of Lys180 is 0.7 and 0.9 Å, respectively, from that of the Mg^{2+} bound to Glu180 (Mg-1) in the wild-type structure. Table 2 lists the distances between the ϵ-amino group and the groups normally coordinated to Mg-1. The terminal amino group of Lys180 is within hydrogen-bond distance of Glu216, Asp244, and Asp286.

Additionally, the crystal structure reveals the presence of a cation bound to site 2 of E180K. This cation was first located after the initial round of refinement when the electron density calculated with the coefficients $(F_o - F_c)$ showed a density at site 2 at the 9σ contour level. Such a strong density precludes the possibility that the ion is ammonium, therefore we modeled it as magnesium. Although magnesium was not added to the buffers during the purification or crystallization procedure, the enzyme may have bound contaminating magnesium. Table 2 lists the distances between this magnesium (Mg-2) and its ligands. An important ligand is the OH 1700 (OH 1800 in the second monomer) which has been refined with a distance of 1.95 Å from the Mg-2. This ligand–magnesium distance is short for a water molecule and the ligand has therefore been assigned as a hydroxide ion. Such a hydroxide ligand has also been observed by us in the wild-type enzyme without glucose (Lavie et al., unpublished results) and as a water molecule with a 2.4 Å metal–water distance in the wild-type enzyme plus glucose, by us (Lavie et al., unpublished results) and others (Collyer et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992; Lambeir et al., 1992; van Tilbeurgh et al., 1992).

Figure 5 depicts the glucose-bound E180K active site including Lys180, and the second metal-binding site, which contains a magnesium ion. The glucose is observed in the open-chain form, with O2 and O4 hydrogen bonded to the ϵ-N of Lys180, O1 within hydrogen-bond distance (3.1 Å) of Lys182 and O5 within hydrogen-bond distance (2.9 Å) of Nϵ2 of His53. This is the same configuration of bound glucose as is seen in the active site of the wild-type xylose isomerase by others (Collyer et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992; Lambeir et al., 1992; van Tilbeurgh et al., 1992) and in this lab (Lavie et al., unpublished results). This is consistent with the kinetic results, which show that E180K is capable of catalyzing the ring opening of glucose. It should be noted that the sugar density is weak (fitted in the $(F_o - F_c)$ electron-density map at the 1.6σ contour level), indicating a low occupancy (<1 glucose bound/active site).

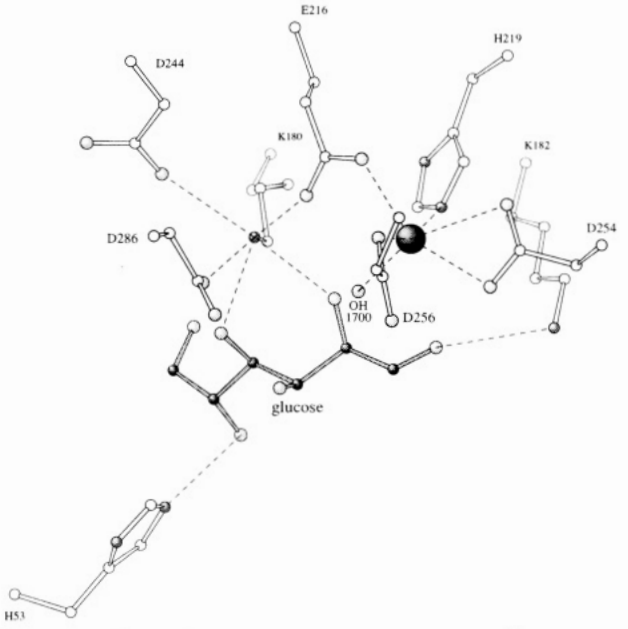

FIGURE 5: The active site of E180K from the 1.8-Å resolution structure determined in the presence of the substrate α-D-glucose. The bonds of the glucose molecule are shaded in gray and the magnesium ion bound in the remaining Mg-2 site is represented as a black sphere. Dashed lines depict those bonds which are ≤2.7 Å in length to Mg-2 and ≤3.0 Å in length to Lys180. This figure was rendered using the program Molscript (Kraulis, 1991).

DISCUSSION

The Role of Mg-1 in Xylose Isomerase. D-Xylose isomerase has an absolute requirement for the divalent metal cations magnesium or manganese. X-ray crystallographic structures of xylose isomerase from *S. olivochromogenes* (Lavie et al., unpublished results) and from a number of other bacterial sources reveal the presence of two divalent cations per enzyme active site (Collyer et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992; Lambeir et al., 1992; van Tilbeurgh et al., 1992). The results of neutron activation analysis confirm the presence of 2 mol of Mg^{2+}/active site and establish a 10-fold difference in the binding affinity of the two cations. Schray and Mildvan (1972) originally proposed that there exists a weak and a strong metal-binding site. The lack of a residue which might act as a catalytic base in a proton-transfer mechanism in high-resolution enzyme–substrate crystal structures (Lavie et al., unpublished results) combined with the lack of proton–solvent exchange from the C2 position of glucose under extremes of temperature, denaturant and pH (Rose, 1969; Allen et al., 1994) argues against a proton-transfer mechanism for isomerization. We and others (Farber et al., 1989; Collyer et al., 1990; Lee et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992; Lambeir et al., 1992; van Tilbeurgh, 1992; Rangarajan & Hartley 1992) have proposed a metal-mediated hydride-transfer mechanism for xylose isomerase similar to the Meerwein–Ponndorf–Verley–Oppenheimer reaction (Kemp & Vellaccio, 1980). Such a mechanism for the enzyme intimately involves metal cation in the chemistry of the reaction. It therefore becomes imperative to prove that metal cannot be replaced with a similarly charged and/or hydrogen bonded ligand and to dissect the roles of the two magnesium ions bound in the xylose isomerase active site. Are both magnesium ions used in the chemistry of catalysis of isomerization, or does one serve to bind substrate or provide a catalytic purpose other than to promote the hydride transfer? The E180K mutant was prepared in order to determine if the ϵ-N of lysine could structurally or chemically replace Mg-1, the tetracoordinated magnesium ion (assumed to be the more tightly bound of the two).

The X-ray crystallographic structure of E180K was determined to 1.8-Å resolution and refined to an *R* factor of

19.7% and 17.7% in the presence and absence of glucose as substrate. The crystal structure clearly shows that the ϵ-amino group of lysine assumes (within <1 Å) the position normally occupied by Mg-1 in the wild-type enzyme. The overall structure of the E180K mutant does not differ significantly (≤0.175 Å rms deviation of α carbons) from that of the wild-type enzyme. Therefore, the lysine ϵ-amino group has structurally replaced Mg-1. However, Lys180 has not chemically replaced Mg-1. The E180K mutant is completely devoid of isomerase activity as shown by kinetic analysis using D-glucose as the substrate and concentrations of magnesium of up to 1 M (for wild-type xylose isomerase the apparent K_D < 10 mM for either magnesium). Thus, Mg-1 is essential for the isomerization activity of xylose isomerase. The fact that high concentrations of magnesium do not restore isomerase activity indicates that the presence of Lys180 has not simply lowered the affinity of site 1 for magnesium but that we have completely abolished Mg binding at that site.

Kinetic studies using E180K and 1-thioglucose to measure the rate of ring opening by elimination of H_2S demonstrate that the mutant catalyzes ring opening at 20% the rate of the wild-type enzyme. (It should be noted that the rate of ring opening of 1-thioglucose by the wild-type enzyme is 1600-fold lower than the rate of isomerization of glucose to fructose and therefore this rate cannot be used as a measurement of the rate of ring opening of the substrate glucose.) The fact that E180K catalyzes ring opening of 1-thioglucose with near wild-type efficiency demonstrates that Mg-1 is not essential for the ring-opening reaction. Lys180 is capable of donating hydrogen bonds and in the E180K crystal structure, does hold the surrounding carboxylate ligands in a conformation similar to that in the wild-type enzyme. Therefore, any of these roles played by Mg-1 in the ring-opening reaction may have been substituted for adequately by Lys180.

The Role of Mg-2 in Xylose Isomerase. The second metal-binding site of xylose isomerase was not altered by the E180K mutation. Neutron activation analysis shows that in the presence of 10 mM $MgCl_2$ E180K binds 1 mol of magnesium/mole of enzyme; thus the affinity of site 2 for magnesium was not significantly altered. Furthermore, magnesium was observed to occupy site-2 in the X-ray crystal structures of E180K in the presence and absence of glucose. Its position was not significantly displaced from that of Mg-2 in the wild-type enzyme (0.3 and 0.5 ± 0.25 Å in E180K and E180K plus glucose, respectively). The results of the europium(III) excitation spectroscopy confirm that the metal binding capacity of site 2 in E180K has not been drastically altered and that the coordination geometry has been maintained. It is interesting to note that Eu^{3+} binds tightly to site 2 and requires a 10000-fold excess of Mg^{2+} ions to substantially displace the bound Eu^{3+}.

Thus, under the conditions utilized for activity assays using glucose as substrate, magnesium is expected to be bound at site 2 and is observed there crystallographically. Since no isomerization activity was detected for E180K, Mg-2 is not *sufficient* to catalyze isomerization. This does not mean, however, that it may not be integrally involved in the catalysis of isomerization. The role of Mg-2 in ring opening cannot be determined from these results.

Magnesium-Bound Hydroxide as a Catalytic Base. In the X-ray crystal structures of E180K, a water molecule (1700 in monomer 1 or 1800 in monomer 2) was observed to coordinate to Mg-2. However, it could not be refined into the electron density without fixing the bond distance between this water and Mg-2 as 1.95 Å. This distance is short for a water–magnesium bond and seems more consistent with a hydroxide–Mg bond. The pK_a of 7.2 obtained from the pH versus log V_{max}/K_m is consistent with the pK_a of a metal-bound water. A water in the environment of two magnesium ions would be expected to possess a depressed pK_a. The pK_a of 7.2 is probably not the pK_a of His219 (which is coordinated to Mg-2), since a much more depressed pK_a would be expected for a histidine bound to magnesium. This conclusion is consistent with the observation by Lambeir et al. (1992) that a mutation of His219 to Gln does not result in a significantly different pH-rate profile. The function of hydroxide 1700 (1800 subunit 2) could be to act as a base during catalysis. O2 may be deprotonated and the proton moved to O1 concomitant with hydride transfer as suggested previously (Whitlow et al., 1991; van Tilbeurgh et al., 1992). The resultant deprotonated O2 may be stabilized by the metals. Structures determined in this laboratory with a potent inhibitor of xylose isomerase (Allen et al., unpublished results) and with substrates (Lavie et al., unpublished results) are consistent with such a deprotonation, with the resultant deprotonated O2 stabilized by the bridged dimetallic center.

Substrate Binding in E180K. The mode of substrate binding as determined X-ray crystallography of E180K is identical to that of the wild-type xylose isomerase. The electron density for substrate observed in the E180K crystal structure represents the average of all species bound at equilibrium. The D-glucose observed in E180K is in the open chain form. Although E180K does not catalyze the glucose to fructose isomerization, it is possible that the electron density observed in the enzymic active site could represent the product fructose if the E180K enzyme bound fructose produced from the uncatalyzed isomerization reaction in solution. At this resolution, we cannot differentiate the open-chain form of glucose from fructose in the electron density. The open-chain glucose makes two hydrogen bonds to Nϵ of Lys180 through O2 and O4 (both 2.6 ± 0.25 Å in length). These hydrogen bonds do not differ significantly in length from those between Mg-1 and O2 and O4 of sugar in the wild-type enzyme. Neither do those between Lys182 and O1 of sugar (3.1 Å) and His53 and O5 of sugar (2.9 Å).

CONCLUSIONS

The apparent binding constants for the two magnesium binding sites in wild-type xylose isomerase from *Streptomyces olivochromogenes* are determined to be $K_{D\ app} \leq 0.5$–1.0 mM and ≤ 2.0–10.0 mM by neutron activation analysis. Since a hydride-shift mechanism for isomerization is dependent upon the chemistry of the divalent cation, the mutant E180K was designed to dissect the roles of the two magnesiums by replacing Mg-1 with the ϵ-amino group of Lys180 (the side-chain carboxylate of Glu180 normally is coordinated to Mg-1). The X-ray crystal structure of the E180K mutant enzyme was determined to 1.8-Å resolution in the presence and absence of glucose, refined to an R factor of 19.7% and 17.7%, respectively. The structure of the E180K mutant enzyme reveals that the side-chain amino group of lysine binds in the Mg-1 binding site (site 1) and that the overall structure is identical with that of the wild-type isomerase. Therefore, structurally, we have succeeded in replacing Mg-1 with Lys180. E180K shows no isomerase activity with the substrate glucose at a wide range of magnesium concentrations, but shows only a 5-fold decrease compared to wild-type enzyme in the rate of ring opening with the substrate 1-thioglucose. Thus, Mg-1 is essential for isomerization but not for ring opening.

The affinity of site 2 for magnesium has not been significantly altered as shown by the results of the neutron

activation analysis, europium(III) excitation spectroscopy, and X-ray crystallography which together establish that site 2 binds a magnesium with the same affinity as wild-type enzyme and that the coordination geometry of the ligands surrounding site-2 have not changed. This also indicates that any synergistic effects of the binding of Mg-1 on site 2 can be mimicked by the binding of the Nε-amino group of lysine.

Since the E180K mutant catalyzes ring opening but not isomerization, the mutant enzyme provides the first step in the path of reengineering the mechanism of isomerization to a proton-shift mechanism like that of triosephosphate isomerase instead of hydride transfer. The next step is the addition of a base in the correct position to remove the proton from C2 and donate it to C1. Efforts are currently underway in this laboratory to produce a xylose isomerase with such an altered mechanism.

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