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Contribution of Second Metal Binding Site for Metal Specificity of D-Xylose Isomerase

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Abstract The metal specificity of D-xylose isomerase from *Streptomyces rubiginosus* was examined by site-directed mutagenesis. The activation constants for metal ion (Mg^{2+}, Mn^{2+}, or Co^{2+}) of wild-type and mutant enzymes were determined by titrating the metal ion-free enzyme with Mg^{2+}, Mn^{2+}, and Co^{2+}, respectively. Substitutions of amino acids either on coordinated or around the M2 site (His-220, Asn-185, Glu-186, and Glu-221) dramatically affected the activation constants as well as activity. A decrease of metal binding affinity was most significant in the presence of Mg^{2+}. When compared with the wild-type enzymes, the binding affinity of H220S and N185K for Mg^{2+} was decreased by 10-15-fold, while the affinity for Mn^{2+} or Co^{2+} only decreased by 3-5-fold. All the mutations close to the M2 site changed their metal preference from Mg^{2+} to Mn^{2+} or Co^{2+}. These altered metal preferences may be caused by a relatively weak binding affinity of Mg^{2+} to the enzyme. Thermal inactivation studies of mutants at the M2 site also support the importance of the M2 site geometry for metal specificity as well as the thermostability of the enzyme. Mutations of other important groups hardly affected the metal preference, although pronounced effects on the kinetic parameters were sometimes observed. This study proposes that the metal specificity of D-xylose isomerase can be altered by the perturbation of the M2 site geometry, and that the different metal preference of Group I and Group II D-xylose isomerases may be caused by nonconserved amino acid residues around the M2 site.

Key words: Xylose isomerase, site-directed mutagenesis, metal specificity, metal binding

D-Xylose isomerase catalyzes the interconversion of D-glucose and D-xylose to D-fructose and D-xylulose, respectively. D-Xylose isomerase has an absolute requirement for the divalent metal ions, Mg^{2+}, Mn^{2+}, or Co^{2+}, for its activity [2, 9, 11]. The x-ray crystallographic structures of xylose isomerase from a variety of bacterial sources have already been elucidated and found to be almost identical [5, 8, 10, 13, 15, 16, 20, 26]. In these crystallographic structures, two distinct metal binding sites have been observed [5, 10, 26]. One metal ion site (the M1 site) is structurally more rigid than the second site (the M2 site) and stabilizes the transition state of the substrate, while the M2 site is relatively flexible and appears to be important for catalysis. The binding of the two metals at the active site has also been previously demonstrated by kinetic and spectroscopic studies, further suggesting the presence of two separate binding sites with different affinities [1, 2, 3]. Each site has a different binding affinity for Mn^{2+}, Co^{2+}, and Mg^{2+}. However, the strength of the binding affinity is in the same order for $Mn^{2+} > Co^{2+} >> Mg^{2+}$ in both sites [21]. The metal selectivity of an enzyme depends on the type of substrate and the bacterial sources [9]. The metal-activating properties differ, Mg^{2+} being superior to Co^{2+} and Mn^{2+} with D-xylose isomerases from Actinomycetaceae (designated as Group I) [4, 7, 14], and Mn^{2+} or Co^{2+} being superior to Mg^{2+} with other D-xylose isomerases (designated as Group II) [11, 27]. The difference in metal specificity of Group I and Group II D-xylose isomerases dictates their industrial use because Co^{2+} is not acceptable for food additives. D-Xylose isomerases from Group I are more effective in isomerization of D-glucose and thus are used in the production of high-fructose corn syrup. In contrast, D-xylose isomerases from Group II are more effective in isomerization of D-xylose into D-xylulose and are potentially useful in biomass conversion of xylose, which can be ultimately fermented to ethanol.

Van Tilbeurgh *et al.* [23] reported that the metal specificity of *Actinoplanes missouriensis* D-xylose isomerase could be changed by site-directed mutagenesis [23]. The E186Q and E186D mutants exhibited the highest activity with Mn^{2+}, while the wild-type enzyme was most active with Mg^{2+}. The altered metal specificity of E186Q was further studied by resolving the both structures conjugated

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with either Mg^{2+} or Mn^{2+}. The M1 site in both structures was occupied and the substrate was bound normally; however, the geometry of the M2 site was completely different. In the presence of Mg^{2+}, the carboxylate of Asp-255 that coordinated to the M2 site was displaced and formed a hydrogen bond with the side chain of Glu-186. Mg^{2+} was not found at the M2 site. The structure of the Mn^{2+}-activated enzyme revealed a Mn^{2+} bound at the M2 site with Asp-255 coordinated to it as in the wild-type enzyme. Our earlier studies of the His-220 mutants of *Streptomyces rubiginosus* xylose isomerase also showed a metal specificity different from the wild-type enzyme [7]. The three mutants, H220N, H220S, and H220E, showed the highest activity with Co^{2+} instead of Mg^{2+}.

The role of metal ions in catalysis and stabilization of D-xylose isomerase has been well studied, however, the factors that influence metal specificity are still poorly understood. This study focused on the M2 site and examined the amino acid residues around this site to investigate their roles in the metal specificity of D-xylose isomerase.

MATERIALS AND METHODS

Materials

D-xylose, $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, and $CoCl_2 \cdot 6H_2O$ were obtained from Merck, Darmstadt, Germany. Other reagents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All compounds were reagent grade. Buffers were prepared in Millipore Milli-Q water and stored in acid-washed plastic containers.

Bacterial Strains and Vectors

Streptomyces rubiginosus (ATCC 21175) was obtained from the American Type Culture Collection. *Escherichia coli* TG1 [*supE hsdD5 thi* Δ(*lac-proAB*) F'(*traD36 proAB*$^+$ *lacI*^{q} *lacZ* DM15)] was used to propagate the plasmid and M13. M13mp19 was used as a cloning vector. *E. coli* BL21 [F^- *ompT* rB^- mB^- λ(DE3)] was used as a host for the pET11-d derivative to express the recombinant xylose isomerase.

Site-Directed Mutagenesis and Purification of D-Xylose Isomerase Mutants

The cloning and overexpression of the *S. rubiginosus* xylose isomerase gene in *E. coli* was described previously [7, 25]. The site-directed mutagenesis was conducted according to the method of Vandeyar *et al.* [24]. Asn-185 was changed to Lys, and Glu-186 was changed to Gln. The specific primers used were as follows (changed nucleotides are underlined): N185K, 5'-GAGCCCAAGCCAAAGGAGCCGCG-3; E186Q, 5'-GAGCCCAAGCCAAACCAGCCGCG-3'. The other mutants used in this study have been described previously [6, 7, 25]. The wild-type and mutant D-xylose isomerases were purified from cells of recombinant *E. coli* according to the method previously described [7].

Enzyme Assays and Kinetic Analyses

D-xylose isomerase was assayed using D-xylose as a substrate as previously described [7]. The assays were carried out in a 10 mM triethanolamine (TEA) buffer, pH 7.3, at 37°C with 50 mM D-xylose in the presence of 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CoCl_2$, respectively. The amount of D-xylulose produced was determined using the cysteine-carbazole method [12]. For the kinetic analysis, the steady-state parameters for D-xylose were determined at 37°C in 10 mM TEA, pH 7.3, in the presence of 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CoCl_2$, respectively. The substrate concentrations ranged from 1×10^{-6} to 1×10^{-4} M, and the enzyme concentrations in the assay mixture ranged from 1×10^{-6} to 1×10^{-5} M. The kinetic parameters, K_m and k_{cat}, were determined by a Lineweaver-Burk plot using the ENZFITTER program. The protein concentrations were determined either by Bio-Rad protein assays using a bovine serum albumin as the protein standard or spectrophotometrically at 280 nm using an extinction coefficient of 1.06 $cm^{-1}mg^{-1}ml$.

Determination of Metal Ion Activation Constants

The binding affinity of each metal ion on the enzyme was evaluated as an activation constant, K_d, which was the metal ion concentration that yielded half of the maximum activity. The activation constant was determined by typical titrations [21] carried out at a fixed enzyme concentration ($1 \times 10^{-7} - 1 \times 10^{-6}$ M for the wild-type enzyme and $5 \times 10^{-7} - 1 \times 10^{-5}$ M for the mutant enzymes). The protein-metal solutions were allowed to equilibrate before the enzyme reaction was started by the addition of 50 mM D-xylose. The metal ion concentrations ranged between 1×10^{-6} and 5×10^{-2} M. A metal-free enzyme was prepared by extensive dialysis at 4°C against 10 mM TEA and 1 mM EDTA, followed by dialysis against TEA without EDTA. Concentrated stock solutions of $MgCl_2 \cdot 6H_2O$ (500 mM), $MnCl_2 \cdot 4H_2O$ (50 mM), and $CoCl_2 \cdot 6H_2O$ (100 mM) were prepared in Millipore Milli-Q water and stored at 4°C until used.

Thermal Inactivation Measurements

The thermal inactivation kinetics were determined in 10 mM TEA, pH 7.3, in the presence of 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CoCl_2$, respectively. The enzyme solution (1×10^{-6} M – 1×10^{-5} M) was incubated at 82°C and then stopped after various time periods by chilling on ice. The residual activity was then measured using 50 mM D-xylose at 50°C, as described previously [7]. A first-order rate constant, k, for the irreversible thermal inactivation was

obtained using the linear regression of a semilogarithmic plot. The half-life, $t_{1/2}$, of the enzyme was calculated from the equation: $t_{1/2} = (\ln 2)/k$.

RESULTS

Effect of Mutants on the Metal Specificity of D-Xylose Isomerases

The amino acid residues that were located in the substrate binding site, in the metal binding site, and/or on the surface were substituted to examine their effects on the metal specificity of D-xylose isomerase. All the mutant enzymes were well expressed and produced in a soluble form. The purified mutant enzymes showed an activity ranging from 0.5-100% of the wild-type enzyme. The activities of each mutant with the three different metal ions (Mg^{2+}, Mn^{2+}, and Co^{2+}) were measured and the relative activities were compared (Table 1; Fig. 1). The wild-type enzyme was most active with Mg^{2+}, and the mutations in the substrate binding site (H54N and F94S) and on the surface (D65A) exhibited the metal preference similar to the wild-type enzyme. The other surface mutants, D81A and D163N/E167Q, showed the same pattern with the D65A mutant (data not shown). In contrast, substitutions around the M2 site altered the metal specificity. The three His-220 mutants, H220S, H220N, and H220E, showed a strong preference for Co^{2+}, while Mn^{2+} was the best activator for E186Q and E221Q. No metal exhibited a strong preference for the activity of N185K. The results reveal that the amino acid residues near the M2 site are important for metal specificity.

Table 1. Metal specificity of the mutant D-xylose isomerases.

Enzyme	Relative activity*		Metal specificity
	Co^{2+}/Mg^{2+}	Mn^{2+}/Mg^{2+}	
Wild-type	0.66	0.55	$Mg^{2+} > Co^{2+} > Mn^{2+}$
H54N	0.57	0.40	$Mg^{2+} > Co^{2+} > Mn^{2+}$
F94S	0.79	0.31	$Mg^{2+} > Co^{2+} > Mn^{2+}$
D65A	0.78	0.70	$Mg^{2+} > Co^{2+} > Mn^{2+}$
D56N	0.52	0.50	$Mg^{2+} > Co^{2+} > Mn^{2+}$
E221A	0.90	0.65	$Mg^{2+} \cong Co^{2+} > Mn^{2+}$
E221Q	1.02	1.32	$Mn^{2+} > Co^{2+} \cong Mg^{2+}$
E186Q	1.06	2.05	$Mn^{2+} > Co^{2+} \cong Mg^{2+}$
H220S	2.80	0.75	$Co^{2+} > Mg^{2+} > Mn^{2+}$
H220N	1.80	1.10	$Co^{2+} > Mn^{2+} \cong Mg^{2+}$
H220E	1.75	0.65	$Co^{2+} > Mg^{2+} > Mn^{2+}$
N185K	1.16	1.01	$Co^{2+} \cong Mn^{2+} \cong Mg^{2+}$

Activity was measured in 10 mM TEA, pH 7.3, using 50 mM D-xylose at 37°C in the presence of 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CoCl_2$, respectively. The amount of D-xylulose produced was determined by the cysteine-carbazole method [10].

*The relative activity was the ratio of the activity of each mutant in the presence of Co^{2+} or Mn^{2+} over Mg^{2+}.

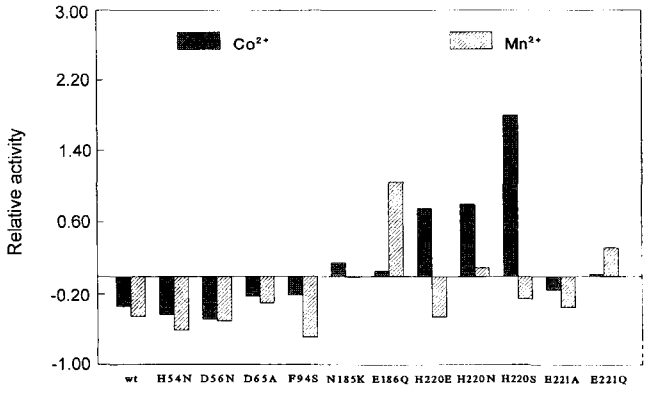

Fig. 1. Relative activity of mutant D-xylose isomerases for Co^{2+} and Mn^{2+} compared to Mg^{2+}.

Steady-State Kinetics of H220S, N185K, and E221A Mutants

Three mutants (H220S, N185K, and E221A) were chosen and further characterized to examine the altered metal specificity of the M2 site mutants. Table 2 shows the kinetic parameters of the three mutants when xylose was used as a substrate. The order of the metal specific activity of the wild-type enzyme was $Mg^{2+} > Co^{2+} > Mn^{2+}$. The k_{cat} value for E221A was similar to the wild-type enzyme when Mg^{2+} or Mn^{2+} was added, however, it increased 1.3-fold with Co^{2+}. However, Mg^{2+} was still the best activator for E221A. Previously, it was observed that the enzyme activity of E221A was the highest with Co^{2+} when glucose was used as a substrate instead of xylose [6]. Nevertheless, E221Q showed better activity with Mn^{2+} than with Mg^{2+} (Table 1). This would suggest that the metal

Table 2. Kinetic parameters of the mutant D-xylose isomerases for D-xylose.

Enzyme	Metal	k_{cat}(sec^{-1})	K_m (mM)	k_{cat}/K_m(s^{-1}/mM)
Wild-type	Mg^{2+}	3.36±1.10	3.03±2.74	1.11
	Mn^{2+}	2.10±0.03	3.83±0.41	0.55
	Co^{2+}	2.26±0.58	3.66±2.37	0.62
E221A	Mg^{2+}	3.38±1.90	14.2±12.0	0.24
	Mn^{2+}	2.13±0.71	30.0±12.4	0.07
	Co^{2+}	2.86±1.04	26.5±12.4	0.11
H220S	Mg^{2+}	0.012±0.003	144±56.4	8.3×10^{-5}
	Mn^{2+}	0.009±0.001	167±41.9	5.4×10^{-5}
	Co^{2+}	0.027±0.006	182±70.9	1.5×10^{-4}
N185K	Mg^{2+}	0.46±0.16	18.4±9.18	2.5×10^{-2}
	Mn^{2+}	0.46±0.13	18.3±7.48	2.5×10^{-2}
	Co^{2+}	0.50±0.18	16.0±7.82	3.1×10^{-2}

Enzyme activity was measured in 10 mM TEA, pH 7.3, at 37°C with 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CoCl_2$, respectively. The amount of D-xylulose produced was determined by the cysteine-carbazole method [10].

preference of Glu-221 mutants may be affected by the type of substrate.

The H220S and N185K mutants showed a significantly reduced activity compared to the wild-type enzyme. The k_{cat} values for H220S and N185K were 0.35-1.1% and 13.6-22% of the wild-type enzyme, respectively, and the K_m values were 43-50-fold and 5-6-fold higher than the wild-type enzyme, respectively. In addition, both mutants had different metal preferences for their enzyme activity. The H220S showed the highest activity with Co^{2+} and its k_{cat} value was two times higher than the Mg^{2+}-activated enzyme. Other His-220 mutants, H220E and H220N, also displayed the highest activity with Co^{2+}. The k_{cat} values of N185K with the three different metals were not significantly different. When Mg^{2+} was used as an activator, the k_{cat} values for H220S and N185K were 0.3% and 10% of the wild-type enzyme, respectively, whereas the k_{cat} values for Co^{2+} were 1% and 20%, respectively. These results imply that alteration of the metal preference for the H220S and N185K mutants is due to extremely low activity of the Mg^{2+}-activated enzyme caused by the weak binding affinity of Mg^{2+} to the enzyme compared to Co^{2+} or Mn^{2+}.

Metal Binding Affinity Studies of D-Xylose Isomerase Mutants

To confirm a hypothesis that the altered metal specificity of the mutants near the M2 site was related to weak binding of Mg^{2+} to the enzyme, the binding affinities of N185K, H220S, and E221A with three different metal ions were investigated and compared with the wild-type enzyme (Table 3). The activation constants of the wild-type enzyme and E221A with Co^{2+} and Mn^{2+} were

Table 3. Metal ion activation constants, K_d, of the mutant D-xylose isomerases.

Enzyme	Mg^{2+} (μM)	Mn^{2+} (μM)	Co^{2+} (μM)
Wild-type	57	7.4	6.8
E221A	38	2.1	2.7
H220S	743	8.5	34.0
N185K	896	22.0	26.0

Enzyme activity was measured in 10 mM TEA, pH 7.3, using 50 mM D-xylose at 37°C with metal ion concentrations ranging from 1×10^{-6} to 5×10^{-2} M.

The activation constant, K_d, was determined by the metal ion concentration that yielded 50% of the maximum activity.

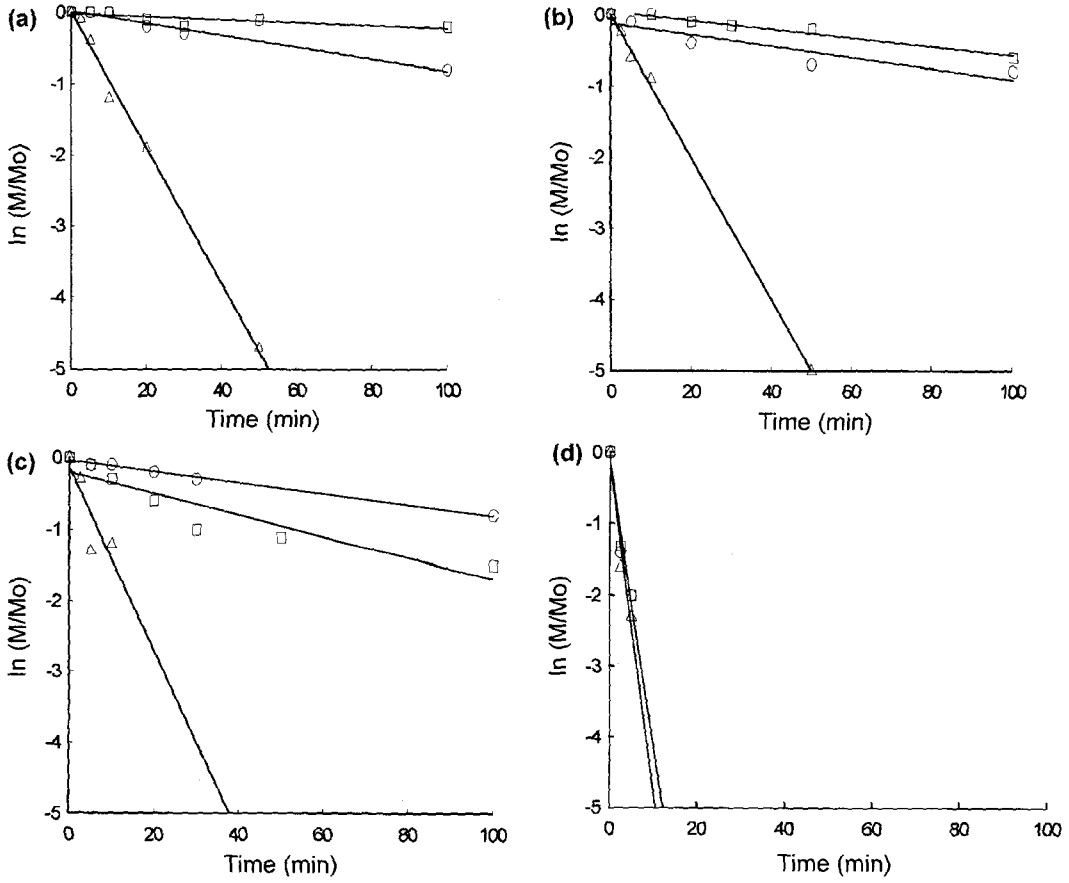

Fig. 2. Thermal inactivation of mutant D-xylose isomerases in the presence of 10 mM Mg^{2+} (△), 1 mM Mn^{2+} (○), or 1 mM Co^{2+} (□) at 82°C.

(a) Wild-type, (b) E221A, (c) H220S, and (d) N185K. M/Mo is the ratio of the enzyme activity before and after heat treatment.

approximately 10 times lower than that for Mg^{2+}. The order of the metal binding strengths to the enzyme was $Mn^{2+} \cong Co^{2+} > Mg^{2+}$. In H220S and N185K, the activation constant for Mg^{2+} was 50-100-fold higher than that for Mn^{2+}, and 20-40-fold higher than that for Co^{2+}. When both mutants were compared with the wild-type enzyme for metal binding affinity, the binding affinity of Mn^{2+} or Co^{2+} decreased 1.1-5-fold while the binding affinity of Mg^{2+} decreased 13-16-fold. The higher K_d values of both mutants for Mg^{2+} than for Mn^{2+} or Co^{2+} reflect the weaker binding of Mg^{2+} to the M2 site. These results are consistent with the studies of His-220 mutants that exhibit the highest activation constant in the presence of Mg^{2+} [7].

Thermal Inactivation Studies of Mutant Enzymes

The heat stability of the mutant D-xylose isomerases was measured in the presence of three metals. The loss of activity followed first-order kinetics with a single rate constant (Fig. 2). This was accompanied by a significant loss of enzyme solubility. The initial concentration of the enzyme did not affect the inactivation rate constant (data not shown). With Co^{2+} or Mn^{2+}, the activity loss was slow, while the Mg^{2+}-enzyme was rapidly inactivated. The thermal inactivation pattern of the E221A mutant was very similar to the wild-type enzyme. In contrast, H220S and N185K were inactivated relatively fast even with Co^{2+} and Mn^{2+}.

Table 4 summarizes the half-lives calculated from the first-order rate constants for heat inactivation with different metal conditions. The half-lives of the wild-type enzyme with Co^{2+} or Mn^{2+} were 10-30 times longer than for Mg^{2+}, and those of E221A with all three metals were similar to the wild-type enzyme. H220S displayed the lowest thermostability, except for N185K, with half-lives of 4.3 and 31 min for Mg^{2+} and Co^{2+}, respectively. These data on the mutants indicate that both Mn^{2+} and Co^{2+} provide significant stabilization, whereas Mg^{2+} does not. The higher k_{cat} value for Co^{2+} than that of Mg^{2+} indicates an altered metal specificity of the His-220 mutants, probably caused by a greater structural stabilization at the M2 site of the H220S mutants in the presence of Co^{2+} versus Mg^{2+}. In contrast, the N185K lost the activity and precipitated immediately after heating at 82°C. The half-life of this mutant was 0.8-1.1 min regardless of the metals. This short half-life indicated that the metal was either not bound to the M2 site or completely displaced from the M2 site (see discussion).

Table 4. Half-life, $t_{1/2}$, of the mutant D-xylose isomerases.

Enzyme	$t_{1/2}$ (min)*		
	Mg^{2+}	Mn^{2+}	Co^{2+}
Wild-type	6.9	86	230
E221A	6.0	69	153
H220S	4.3	69	31
N185K	0.8	1.1	1.1

The irreversible thermal inactivation was measured as mentioned in Materials and Methods.

*The half-life, $t_{1/2}$, of the enzyme was calculated from the equation: $t_{1/2} = (\ln 2)/k$, in which k is the first-order rate constant of the thermal inactivation obtained by the linear regression of a semilogarithmic plot.

DISCUSSION

D-xylose isomerases from Actinomycetaceae (Group I) and more divergent bacteria (Group II) have distinct catalytic and physicochemical properties such as metal-activation, thermostability, and pH optima. One of the major differences between the two groups is their different metal specificity for enzyme activity. It has been recognized that the binding of metal to D-xylose isomerase is important for activity and stability [4, 14, 17, 18, 19]. Accordingly, the different metal specificity of the two groups should reflect different geometry of the metal binding site of the enzyme. A comparison of the amino acid sequences and crystallographic data for these two groups revealed that the amino acid residues directly coordinated to the metals were conserved. Several crystallographic structures of D-xylose isomerases bound with metals have been identified and revealed the geometry of the metal binding sites [5, 10, 13, 15, 20, 26]. The relative greater flexibility of the M2 site than the M1 site suggested by these structural studies has also been demonstrated by site-directed mutations of the metal-coordinating residues [7, 16, 22]. Therefore, it was assumed that the metal specificity of D-xylose isomerase is mediated by the M2 site geometry.

The mutants around the M2 site altered the metal specificity of D-xylose isomerase, whereas the mutants in other areas did not. Four amino acid residues in the M2 site, Asn-185, Glu-186, His-220, and Glu-221, were mutated. Asn-185 and Glu-186 are in the peptide chain at the 181th-187th (Glu-Pro-Lys-Pro-Asn-Glu-Pro) position which is close to the M2 site, including the *cis*-Pro-187, which is highly conserved. This conserved peptide containing Lys-183, Asp-255, and His-220 is catalytically important and coordinates the M2 site. The altered metal specificity of the Asn-185 and Glu-186 mutants is related to their effect on His-220 and Asp-255 which maintain the M2 site geometry. The strong preference of E186Q for Mn^{2+} in the *S. rubiginosus* enzyme agrees with the results of the same mutation in *Actinoplanes missouriensis* D-xylose isomerase [23]. This suggests that the altered metal specificity of E186Q may be due to the maintenance of the M2 site geometry by Mn^{2+}. The geometry of the M2 site of E186Q with Mg^{2+} is thus disrupted by dislocation of Asp-255 which is hydrogen bonded with Glu-186 and its weak binding affinity for Mg^{2+}. Site-directed mutagenesis studies revealed that the

removal of one of the four carboxylates coordinated with the M1 site yielded an enzyme with little or no activity [16]. Only one mutant that had 10% of the activity of the wild-type enzyme exhibited the same Mg^{2+} preference as the wild-type enzyme. From the results of Jenkins *et al.* [16] and the present study, it is proposed that the major factor controlling metal specificity is the M2 site geometry.

In order to confirm the role of the M2 site in metal specificity, the metal binding to the M2 site mutants was examined. Both H220S and N185K altered the metal specificity ($Co^{2+} > Mg^{2+}$) and had a lower apparent affinity for all three metals compared to the wild-type enzyme. The metal binding affinity for Mg^{2+} decreased 13-16-fold while the affinity for Mn^{2+} or Co^{2+} decreased only 1.1-5-fold. The high activation constant for Mg^{2+} over Mn^{2+} or Co^{2+} was not unexpected. A previous spectroscopic study suggested that the M1 site has a high affinity for Mg^{2+} while the M2 site has a high affinity for Co^{2+} [3]. Therefore, the weak binding affinity for Mg^{2+} in both mutants would be due to a perturbation of the M2 site geometry. Thermal inactivation studies of H220S and N185K indicated that both mutants are thermolabile. The half-life of the N185K mutant was less than 1 min at 82°C. The substitution of Asn-185, which is 3.48 Å away from the catalytically important Lys-183, with a positively charged lysine may repel the Lys-183 and disturb the charge balance around the M2 site. These results are also consistent with the authors' previous data indicating a weaker binding and greater flexibility of the metal at the M2 site compared to the M1 site affected thermostability [7].

This altered metal specificity of N185K is interesting. Asn-185 and Glu-221 are conserved in all D-xylose isomerases in Group I, while they are replaced by lysine and alanine, respectively, in the Group II enzymes. The different metal preferences relative to the activities between the two groups are shown in Table 5. The higher catalytic activity of the Group II enzymes for Co^{2+} or Mn^{2+} than for Mg^{2+} is similar to the metal specificity of N185K. Although E221A showed a higher activity with Mg^{2+} than with Co^{2+} or Mn^{2+} with xylose as a substrate, the highest activity was observed with Co^{2+} when glucose was the substrate. A crude extract of an another mutant, E221Q, exhibited a higher activity with Co^{2+} or Mn^{2+} than with Mg^{2+} (Table 1). This mutant, however, was extremely unstable and could not be purified. The instability of E221Q supports the structural role of Glu-221 in maintaining the local stability of the enzyme through hydrogen bonding with two water molecules [6]. Bogumil *et al.* [1] examined the geometry of the two metal binding sites using X- and Q-band EPR spectroscopy. Substituting Co^{2+} with the larger Cd^{2+} or Pb^{2+} at the M2 site led to a drastic change in the M1 site environment and thereby significantly decreased the catalytic activity. The catalytic role of the M2 site geometry identified by EPR spectroscopy was consistent with the kinetic data of the His-220 and Asn-185 mutants that showed 0.5-10% of the activity of the wild-type enzyme. In conclusion, this study suggests that the metal specificity of D-xylose isomerase can be altered by perturbation of the M2 site geometry, and the differences in metal specificity between group I and group II may be caused by nonconserved residues around the M2 site.

Table 5. Comparison of metal specificity between Groups I and II D-xylose isomerases.

	Enzyme	Activator	
		Xylose	Glucose
Group I	Wild-type (N185, E221)	Mg^{2+}	Mg^{2+}
	N185K	Mn^{2+}or Co^{2+}	Co^{2+}
	E221A	Mg^{2+}	Co^{2+}
	E221Q	Mn^{2+}	ND*
Group II	Wild-type (K185, A221)	Mn^{2+}	Co^{2+}

ND: not determined.

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