Isotopic Exchange plus Substrate and Inhibition Kinetics of D-Xylose Isomerase Do Not Support a Proton-Transfer Mechanism†

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ABSTRACT: The D-xylose isomerase of *Streptomyces olivochromogenes* is a Mg^{2+}- or Mn^{2+}-dependent enzyme that catalyzes the aldose–ketose isomerization of xylose to xylulose or of glucose to fructose. Proton exchange into water during enzyme-catalyzed isomerization of C-2 tritiated glucose at 15, 25 and 55 °C shows <0.6% exchange (the loss of one proton in every billion turnovers). High concentrations of guanidine hydrochloride and extremes of pH had no effect on the amount of exchange detected. Such a low percentage of exchange is inconsistent with a proton-transfer mechanism as the main kinetic pathway for isomerization. ^{19}F NMR experiments showed no release of fluoride after incubation of the enzyme for 4 weeks with 800 mM 3-deoxy-3-fluoroglucose or 3-deoxy-3-fluoroallose (both are competitive inhibitors with K_i values of 600 mM). This result is also inconsistent with a proton-transfer mechanism. A hydride-shift mechanism following ring opening has been proposed for the isomerization. Enzyme-catalyzed ring opening was directly measured by demonstrating H_2S release upon reaction of xylose isomerase with 1-thioglucose. D-Xylose isomerase-catalyzed interconversion of glucose to fructose exhibited linear Arrhenius behavior with an activation energy of 14 kcal/mol from 0 to 50 °C. No change in rate-determining step occurs over this temperature range. ^{13}C NMR experiments with glucose show that enzyme-bound magnesium or manganese does not interact specifically with any one site on the sugar. These results are consistent with nonproductive binding modes for the substrate glucose in addition to productive binding.

D-Xylose isomerase (EC 5.3.1.5) is among the most widely used industrial enzymes. Although the physiological reaction catalyzed by the enzyme is the interconversion between the five carbon aldose sugar D-xylose and the five carbon ketose sugar D-xylulose, the enzyme will also isomerise glucose and fructose. Immobilized D-xylose isomerase, catalyzing the glucose–fructose isomerization, is used in the production of high-fructose corn syrup.

Despite the large amount of enzyme used commercially every year, detailed studies of its catalytic mechanism have only recently been carried out. Owing to the similarity between the xylose isomerase reaction and that catalyzed by the well-studied glycolytic enzyme triosephosphate isomerase, it was long assumed that their mechanisms must be similar. Triosephosphate isomerase interconverts the aldose D-glyceraldehyde 3-phosphate and the ketose dihydroxyacetone phosphate by a proton-transfer mechanism involving a single base on the enzyme and formation of an ene-diol (or ene-diolate) intermediate (Reider & Rose, 1959; Albery & Knowles, 1976). The three-dimensional structures of xylose isomerase, determined initially by Carrell et al. (1984) and shortly thereafter in several other laborates (Farber, 1987; Henrick et al., 1989; Rey et al., 1988; Carrel et al., 1989; Dauter et al., 1989; Whitlow et al., 1991), seemed at first to reinforce this similarity: like triosephosphate isomerase, D-xylose isomerase was found to be an eight-stranded, parallel β-barrel with connecting α-helices. The active sites of the two enzymes are also located in similar positions, at the C-terminal end of the β-barrel.

However, indirect evidence has long existed that D-xylose isomerase might not have a mechanism precisely analogous to that of triosephosphate isomerase. Whereas triosephosphate isomerase requires no cofactors for catalysis, D-xylose isomerase has an absolute requirement for a divalent metal ion, preferably Mg^{2+} or Mn^{2+}, for activity. The substrate for triosephosphate isomerase is an open chain, unhydrated triose phosphate; D-xylose isomerase binds the closed form α-anomers of its sugar substrates and appears to catalyze ring opening (Schray & Rose, 1971). In contrast to triosephosphate isomerase, which operates at the diffusion-controlled limit (Blacklow et al., 1988), D-xylose isomerase is a very slow enzyme with a turnover number for glucose of 1 s^{-1}. Finally, isotope exchange experiments from tritiated water into glucose failed to detect significant washing-in of label (Rose et al., 1969; Rose, 1981); observation of both exchange-in and exchange-out of label were essential in establishing a proton-transfer mechanism for triosephosphate isomerase and for determining its complete free energy profile (Albery & Knowles, 1976; Rieder & Rose, 1959).

Earlier, we carried out medium-resolution studies (3 Å) of the binding of substrate to crystals of D-xylose isomerase from *Streptomyces olivochromogenes* (Farber et al., 1989). At low protein concentrations, the enzyme is a homodimer of 40 000 kDa subunits; at high concentrations it is a tetramer with 222 point group symmetry. The four active sites appear to be noninteracting. From the position of bound substrate with respect to the active-site residues, we concluded that a proton-transfer mechanism was unlikely (Farber et al.,

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1989): no base was observed in a reasonable location to catalyze proton abstraction; and it seemed most likely that any acid–base catalysis was solely concerned with ring opening. We suggested that alternative mechanisms, especially hydride transfer, should be considered for this enzyme. Our suggestion was based in part on the observation that the substrate was coordinated directly to one of the two metal sites, which suggested a possible analogy with metal-promoted hydride transfer reactions such as the Meerwein–Ponndorf–Verley–Oppenheimer reaction (Kemp & Vellaccio, 1980). Subsequently, high-resolution crystal structures of enzyme–substrate and enzyme–inhibitor complexes for D-xylose isomerases from various organisms have been interpreted in terms of a hydride-transfer mechanism and detailed roles for the metals and active site residues have been proposed (Collyer et al., 1990; Lee et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992; Rangarajan & Hartley, 1992; van Tilbeurgh et al., 1992). All of these structural data are indeed inconsistent with a proton-transfer mechanism [with one exception (Carrel et al., 1989); however, this interpretation has been challenged (Collyer & Blow, 1990)].

Structural data alone can never prove a mechanism. Although the available crystallographic data for D-xylose isomerase would appear to rule out a proton-transfer mechanism, they do not actually do so, nor do they establish a hydride-transfer mechanism. In this paper, we report the results of isotopic exchange and kinetic experiments designed to uncover a proton-transfer mechanism if one were to exist for this enzyme. Uncertainty about a proton-transfer mechanism results in large part from the reported absence of solvent isotope exchange in the direction of the incorporation of tritiated water into product (Rose et al., 1969). However, the possibility of a fast proton transfer in a shielded active site (Hall et al., 1976) can only be tested by observing the exchange at increased temperatures (Rose & O'Connell, 1961). We have performed the isotope-exchange experiments in the direction of exchange out of labeled substrate into solvent. We have also carried out these experiments at high temperature and in the presence of guanidine hydrochloride where the enzyme structure may be partially unfolded and the exchange rate between enzyme and solvent increased, to investigate the possibility of a shielded proton transfer. We have also carried out the exchange experiments at high (10.0) and low (5.5) values of pH. We have looked for elimination of fluorine from fluorine-substituted substrate analogs and have also demonstrated directly catalysis of ring opening by the enzyme. Experiments with glucal have been used to assess the possibility of acid-catalyzed ring opening. Finally, given some controversy regarding the binding site for sugar in the active site (Carrell et al., 1989; Collyer & Blow, 1990), we have used NMR to examine which groups on the sugar substrates are directly coordinated to the metal cofactor. Our results do not support a proton-transfer mechanism for this enzyme.

MATERIALS AND METHODS

Strains, Plasmids, and Molecular Biology Reagents. The *xylA* gene of *S. olivochromogenes* was the generous gift of Professor Gerard Tiraby. The gene was provided as a *Bam*HI insert in the pUC18 multiple cloning site of pUT638. Due to interference from DNA 5′ to the translation initiation codon, the isomerase could not be expressed in pUT638. Site-directed mutagenesis was undertaken on the gene cloned into M13mp18, to insert an additional *Eco*RI site directly 5′ to the ATG codon, allowing the removal of the 5′DNA between the *Eco*RI site of the multiple cloning site and the introduced *Eco*RI site. The resulting gene was excised as an *Eco*RI/*Hind*III fragment and transferred to the multiple cloning site of pKK223-3 (Pharmacia Biochemicals) resulting in plasmid pX15. Expression is controlled by the *tac* promoter from the pKK223-3 vector. The *Eco*RI/*Hind*III region was completely sequenced.

Purification of Xylose Isomerase. Xylose isomerase was purified from *Escherichia coli* HB101(pX15). Throughout the purification, 0.025 M HEPES–KOH buffer with 0.01 M $MgCl_2$, pH 7.5, was used and the temperature was 25 °C unless otherwise specified. Cells were grown in 700 mL of M9 medium with 25 mL of M9 supplement and ampicillin (100 mg/L) at 37 °C until OD_{600} 0.8 was reached. The cells were then induced with 30 mg isopropyl β-D-thiogalactopyranoside and incubated for an additional 12 h. The cells were harvested by centrifugation at 10000*g* for 10 min, washed in 40 mL buffer, pelleted at 12000*g* for 10 min, and resuspended in 80 mL of buffer. The cells were lysed by sonication at 0 °C using a Branson sonicator, Branson Instrument Co., Danbury, CT, for 10 min at 90% maximum power with the large probe. The cell debris was removed by centrifugation at 12000*g* for 10 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to 50% saturation, the solution was stirred for 10 min, and the precipitated proteins were removed by centrifugation at 12000*g* for 10 min. To the supernatant was added solid $(NH_4)_2SO_4$ to a final saturation of 90%, and the solution was stirred for 20 min. The enzyme pellet was collected by centrifugation at 12000*g* for 10 min, dissolved in 5 mL of buffer, and dialyzed 12 h against 2 L buffer at 4 °C. The enzyme was then loaded onto a DEAE cellulose, Whatman DE-52 column (2.5 × 25 cm) preequilibrated with buffer. The column was washed with 200 mL of buffer and eluted with a linear 500-mL NaCl gradient (0.0–0.7 M) in buffer. Fractions containing significant xylose isomerase activity (see enzyme assays, this section) were pooled, and the enzyme was precipitated by adding solid $(NH_4)_2SO_4$ to a final saturation of 90% and stirring for 20 min. The enzyme pellet was collected by centrifugation at 12000*g* for 10 min, resuspended in 5 mL of buffer, and applied to a Sephadex G-150 column (2.5 × 40 cm) preequilibrated with buffer. The column was eluted with 600 mL of buffer, fractions containing significant xylose isomerase activity were pooled, and the enzyme was concentrated using a Centricon-30 microconcentrator. This preparation typically yields 20 mg of enzyme per liter of culture, with purity by Coomassie staining of SDS/PAGE of >95%.

Substrates and Materials. 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 β-D-fructose 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 compounds 3-deoxy-3-fluoro-D-glucopyranose (**1**) and 2-deoxy-2-fluoro-D-glucopyranose were obtained from PCR Inc., Florida. D-(U-$^{13}C_6$,26–30%)glucose was purchased from Cambridge Isotope Labs. D-(2-^{3}H)glucose, 1 mCi, was purchased from NEN Research Products. D-glucal was synthesized by the method of Whistler and Wolfram (1963) by the deacetylation of 3,4,6-tri-*O*-acetyl-D-glucal (Sigma Chemical Company). All other organic compounds used were reagent grade or better.

Synthesis of 3-Deoxy-3-fluoroallose (**2**). Methyl 6-*O*-trityl-β-D-glucopyranoside was synthesized (Chaudhary & Hernandez, 1979) and used to prepare methyl 3-deoxy-3-fluoro-6-*O*-trityl-β-D-*allo*-pyranoside (Card & Reddy, 1983),

1.5 g of which was deprotected with 1 M sulfuric acid in 60 mL of 1:1 dioxane/water at 105 °C for 26 h, neutralized with Dowex HCO_3^{2-}, and extracted with CH_2Cl_2 (3 × 80 mL), and the aqueous layer was evaporated to dryness to give 420 mg of (**2**), a white solid, in 67% yield for the deprotection step. Final purification was done by HPLC using a Rainin Dynamax-60A column eluting with 1:1 water/acetonitrile, affording a mixture of α and β anomers in a ratio of about 9:1, respectively: ^{19}F NMR (D_2O) δ −216.5 (m, α) −214.3 (m, β); ^{13}C NMR (D_2O, α anomer) δ 63.8 (s, C-6), 68.8 (d, C-2, $J_{c-2,f-3}$ = 17.7 Hz), 73.4 (d, C-4, $J_{c-4,f-3}$ = 17.0 Hz), 76.7 (s, C-5), 96.3 (d, C-3, $J_{c-3,f-3}$ = 174.3 Hz), 96.5 (d, C-1, $J_{c-1,f-3}$ = 3.9 Hz).

Analytical Procedures. Xylose isomerase assays were performed in 0.025 M HEPES–KOH, 0.01 M $MgCl_2$ buffer, pH 7.5, at 25 °C unless otherwise stated. Xylose isomerase (8.3 × 10^{-6} M) was incubated with α-D-(+)-glucose (0.04–0.08 M) in buffer. At various time points, aliquots were diluted 40-fold into buffer, and the amount of fructose was determined using cysteine–carbazole reagent (Diesche & Boreufreund, 1951). All spectrophotometric assays were performed on a Hitachi U-2000 UV/vis spectrophotometer using 1-cm quartz cells.

Alternatively, xylose isomerase (8.3 × 10^{-6} M) was assayed in 0.025 M HEPES–KOH, 0.01 M $MgCl_2$ buffer, pH 7.5, with the substrate 1-thio-β-D-glucose (**3**) (0.3 M) for production of H_2S. Sometimes, 100 mM xylitol (a competitive inhibitor) was added to the identical incubation mixture. The incubation mixture was sealed in a scintillation vial containing a polypropylene cup filled with 0.4 mL of 1 M KOH. After 96 h, the contents of the polypropylene cup were transferred to a tube containing 0.4 mL of 1 M HCl and immediately stoppered. (Note that the tube is quickly and securely stoppered after each addition in the assay procedure to prevent escape of H_2S.) A 0.2 M solution of *N*-ethylmaleimide (0.5 mL) is added and the mixture incubated 10 min at 37 °C. Next, 1.5 mL of 2 M Na_2CO_3 is added and the mixture incubated 10 min at 37 °C. The OD_{520} is read immediately; a brilliant red color indicates the presence of H_2S. The amount of H_2S produced was standardized using a stock solution of 0.56 M Na_2S in 0.5 M HEPES–KOH, 0.01 M $MgCl_2$ buffer, pH 7.5 at 25 °C, in which the amount of free sulfide was determined by titration with lead(II) perchlorate trihydrate using a Radiometer America silver/sulfide combination ion selective electrode to detect the end point as per the electrode instruction manual.

In xylose isomerase assays using D-glucal as substrate, D-glucal (1 M) was incubated with xylose isomerase (2.5 × 10^{-4} M) for 2 weeks and the incubation mixture subjected to an assay for 2-deoxyglucose (the expected product) using the enzyme glucose oxidase as per the Worthington Enzymes Catalog instructions.

Protein determination was performed by the method of Bradford (1976). Liquid scintillation counting was performed with a Beckman LS100C scintillation counter using Amersham ACS fluid. All NMR data were acquired on a Varian XL300, 300-MHz NMR spectrometer. The standard to which ^{19}F NMR signals were referred was $CClF_3$ (0 ppm). The ^{19}F NMR spectra were not proton decoupled.

Xylose isomerase samples for circular dichroism were prepared by incubating 1 mg/mL xylose isomerase with guanidine hydrochloride (0–4 M) for 1 h (a total of 18 samples were prepared to span the denaturant concentration range). Spectra were collected and analyzed in the laboratory of Dr. Gerald Fasman, Brandeis University, on a Jobin Yvon Auto Dictograph-Mark 5.

Kinetic Parameters. For the fluorinated sugars, values for K_i were determined from Lineweaver–Burk plots using initial velocities. Inhibitor concentrations used were $0.2K_i$ to $5K_i$. Product formation was determined using cysteine-carbazole reagent with α-D-glucose (0.04–0.8 M) as substrate. The assays were initiated by the addition of xylose isomerase (8.3 × 10^{-6} M).

For D-glucal, the K_i value was determined from Lineweaver–Burk plots using initial velocities. Inhibitor concentrations used were $0.8K_i$ to $6.8K_i$. The inhibition assays were performed using the same procedure as for the fluorinated sugars.

Substrate–Solvent Tritium Exchange. The radiopurity of the D-(2-^{3}H)glucose was checked by TLC (3:2:1 ethyl acetate/2-propanol/H_2O, R_f = 0.66, as visualized by 30% H_2SO_4/water and charring) in which >0.95% of the radiolabeled material comigrated with unlabeled α-D-glucose. Xylose isomerase (8.3 × 10^{-5} M), D-(2-^{3}H)glucose (0.1 MCi), and α-D-glucose (0.08 M) as cold carrier were incubated in a total volume of 1 mL of 0.025 M of HEPES–KOH buffer, 0.01 M $MgCl_2$, pH 7.5, at 10, 25, and 50 °C, for 210, 105, and 55 min, respectively. Additionally, the 25 °C incubation was repeated with demetalized enzyme (see Materials and Methods) in 0.025 M HEPES–KOH buffer, pH 7.5, at pH 5.5 in 0.025 M MES–KOH, 0.01 M $MgCl_2$, at pH 10.0 in 0.025 M CHES–KOH, 0.01 M $MgCl_2$, and at pH 7.5 in 2 M guanidine hydrochloride, 0.025 M HEPES–KOH, 0.01 M $MgCl_2$. In the case of the guanidine hydrochloride and extremes of pH the enzyme was incubated for 1 h with the buffer in order to allow for full unfolding or equilibration prior to introduction of labeled substrate. All conditions were repeated in the absence of xylose isomerase as control. In all cases the incubations were initiated by the addition of enzyme. The incubation times were sufficient to allow for equilibrium to be reached (predetermined by assays with unlabeled substrate). The incubation mixtures were transferred to one-piece, Pyrex, bulb-to-bulb distillation tubes and plunged into dry ice/acetone. While the sample remained at −70 °C, the tubes were evacuated under vacuum (<1 mmHg) and sealed. The collection side of the tube was then placed in dry ice/acetone while the sample was warmed to room temperature to allow distillation of the solvent. The seal was broken and 1 mL of distilled water was added to the sample side of the tube and the distillation procedure repeated to insure complete distillation of all solvent from the sample. The distillate was subjected to liquid scintillation counting.

Preparation of Demetalized Xylose Isomerase. The enyzme (1 mL, 40 mg/mL) was dialyzed >12 h against 1 L 0.025 M HEPES–KOH buffer, pH 7.5, with 0.1 mM EDTA and then >3 h against 1 L 0.025 M HEPES–KOH buffer, pH 7.5. Distilled, deionized water was run on a 200-mL Chelex-100 (Bio-Rad) column to remove residual metals before use.

RESULTS

Cloning and Sequencing. The *Bam*HI fragment including the *xylA* gene from *S. olivochromogenes* was mutagenized so as to allow direct control of transcription and translation from the pKK223-3 vector, where expression is controlled by the *tac* promotor. The resulting gene fragment was completely sequenced. The procedure used to purify xylose isomerase from *S. olivochromogenes* was modified to permit efficient isolation from *E. coli*. The cloned gene product behaves identically to the enzyme from the native source, as judged by SDS/PAGE electrophoresis and X-ray crystallography. Furthermore k_{cat} and K_m for the substrates xylose and glucose were unchanged for the cloned enzyme.

Temperature Dependence of the Isomerization Rate. The dependence of the observed rate constant upon temperature

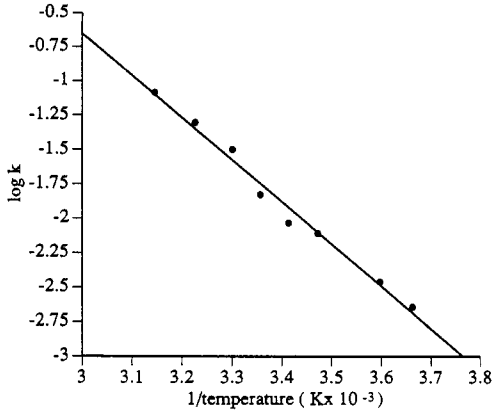


FIGURE 1: Arrhenius plot of log k_{obs} versus $1/T$ (K). The activity of xylose isomerase was measured at 0, 5, 15, 20, 25, 30, 37, 45, and 50 ± 2 °C. Xylose isomerase (8.3×10^{-6} M) was incubated with α-D-glucose (0.3 M) in 0.050 M HEPES–KOH, 0.01 M $MgCl_2$ buffer, pH 7.5. Total incubation times ranged from 180 min at 0 °C to 14 min at 50 °C. Aliquots were removed at regular intervals and product formation measured using the cysteine–carbazole reagent (Diesche & Boreufreund, 1951) and the observed rate at each temperature was determined.

Table 1: Exchange of Tritium out of the C-2 Position of Glucose Bound to Xylose Isomerase into Solvent[a]

	temperature (°C)	exchange (%)
+0.01 M $MgCl_2$	15	0.58
	25	0.58
	50	0.13
$-MgCl_2$	25	0.05
pH 5.5	25	0.60
pH 10.0	25	0.58
+2 M guanidine hydrochloride	25	0.60

[a] The exchange reaction was performed as described in Materials and Methods. The percentage exchange was calculated from the total counts per minute per enzyme sample minus the counts per minute per sample without enzyme divided by the total counts per minute of glucose originally added.

for D-xylose isomerase with α-D-glucose ($2K_m$) was determined from 0 to 50 °C. Figure 1 shows an Arrhenius plot of k_{obs} versus the inverse of the temperature in Kelvins. This plot is linear over the range of temperatures used and was fitted to the Arrhenius equation (Cornish-Bowden, 1979), yielding an activation energy (E_a) of 14.0 kcal/mol. A linear, continuous, Arrhenius plot indicates that there was no change in the rate-limiting step over the temperature range examined.

Substrate–Solvent Tritium Exchange. The incorporation of solvent protons (tritium) into the fructose product of D-xylose isomerase has been reported as <0.4% (Rose et al., 1969). Although such a low exchange rate has led to the suggestion that the mechanism of D-xylose isomerase proceeds via a hydride shift (Farber et al., 1989; Collyer et al., 1990; Lee et al., 1990; Whitlow et al., 1991) it has been pointed out for the enzyme glyoxylase I that such results do not rule out a fast proton transfer taking place in a shielded active site (Hall et al., 1976). However, this possibility can be tested by observing the exchange at increased temperatures (Rose & O'Connell, 1961). The percentage exchange of tritium out of D-(2-^{3}H)-glucose into solvent at 15, 25, and 50 °C was determined; the results are shown in Table 1. In all cases, the amount of exchange under the same conditions in the absence of xylose isomerase was subtracted from the observed exchange. The extremely low rate of proton loss to solvent is nearly constant over all temperatures examined. It also agrees well with the percent tritium incorporation from solvent determined by Rose et al. (1969). In order to test whether the small amount of exchange measured was magnesium dependent, the experiment

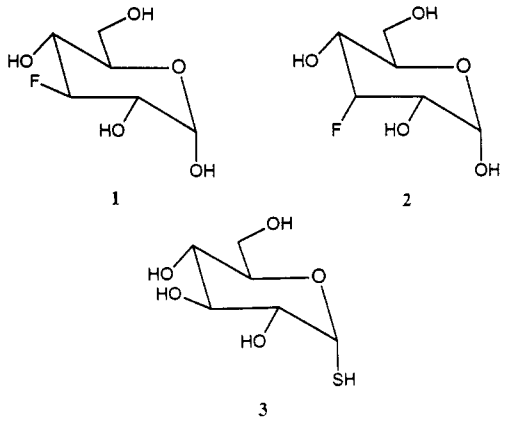


FIGURE 2.

was repeated at 25 °C with demetalized D-xylose isomerase in the absence of magnesium. There is a 10-fold decrease in the amount of exchange in the absence of magnesium. Therefore the observed exchange is dependent upon both the presence of enzyme and magnesium. The total amount of the exchange in the absence of magnesium is equivalent to the amount of nonenzymatic conversion of glucose to fructose expected over the same time period. To increase the probability of observing exchange (i.e. to address the possibility of an active site shielded from water), the experiment was repeated at pH 5.5 and pH 10.0 and in 2 M guanidinium hydrochloride. Circular dichroism spectra show a loss of secondary structure of ~50% in 2 M guanidinium hydrochloride. The amount of exchange observed is not significantly different from that observed at pH 7.5 without denaturant (see Table 1). Under all conditions examined, therefore, the measured percentage of exchange was less than 0.60%.

Fluoride Elimination. The compounds 3-deoxy-3-fluoroglucose (**1**) and 3-deoxy-3-fluoroallose (**2**) (Figure 2) were determined to be competitive inhibitors from Lineweaver–Burk plots using α-D-glucose as substrate. The K_i = 0.64 M for 3-deoxy-3-fluoroglucose and K_i = 0.61 M for 3-deoxy-3-fluoroallose. Both fluoro sugars bind to the active site of xylose isomerase in the same binding site as glucose. In order to test if fluoride ion elimination from the 3-deoxy-3-fluoro sugars is catalyzed by the enzyme, 3-deoxy-3-fluoroglucose or 3-deoxy-3-fluoroallose (0.8 m) was incubated with 2 mg of xylose isomerase and 15% total D_2O and subjected to ^{19}F NMR (see Materials and Methods). The ^{19}F NMR signals were observed at –199 (multiplet) and –194.1 ppm (multiplet) for the α-3-deoxy-3-fluoroglucose and β-3-deoxy-3-fluoroglucose (ratio α/β = 40/60), respectively, and at –216.4 (multiplet) and –213.2 ppm (multiplet) for the α-3-deoxy-3-fluoroallose and β-3-deoxy-3-fluoroallose (ratio α/β = 18/92), respectively. No changes in the chemical shifts were observed nor were any new peaks in the ^{19}F NMR spectra observed at 4 min, 8 h, 24 h, 19 days, and 40 days of incubation. The identical results were observed for the spectra obtained under the same conditions in the absence of enzyme. Thus, there is no elimination of F^- (–117.0 ppm) from either 3-deoxy-3-fluoro sugar nor is there any activity of either sugar as substrate of D-xylose isomerase. The same concentration of enzyme incubated under identical conditions with 250 mM glucose produces 0.1 mmol of fructose in one hour. Therefore, if they were poor substrates, the turnover rate of the 3-deoxy-3-fluoro sugars would be at least 10^6-fold less than that of glucose.

Assay for Sugar Ring Opening. The catalysis of sugar ring opening by xylose isomerase was directly observed by measuring the amount of H_2S released from 1-thio-β-D-glucose

(3). Xylose isomerase and 1-thio-β-D-glucose (0.3 M) were incubated for 96 h (to allow anomerization of the 1-thio-β-D-glucose to 1 thio-α-D-glucose and also vapor diffusion into the trapping KOH, see Materials and Methods). The amount of H_2S released in the presence of xylose isomerase, corrected for the amount released in the identical incubation with no enzyme (0.04 μmol of H_2S released) equals 1 μmol of H_2S per 96 h. A competitive inhibitor for the glucose to fructose isomerization reaction, xylitol, also inhibits the production of H_2S with 1-thioglucose. The presence of xylitol (competitive K_i = 0.3 mM, Collyer et al., 1990) decreases the amount of H_2S observed to 0.2 μmol per 96 h. Thus, the enzymic reaction with 1-thioglucose occurs at the same active site as the reaction with glucose. If the amount of H_2S is equated with the amount of 1-thio sugar ring opening, at least 1 μmol of sugar has undergone ring opening in 96 h (since not every ring opening event releases H_2S). This amount of ring-opened sugar is 1600-fold less than the amount of glucose turnover with the same enzyme concentration in the same period of time. Therefore, the measured 1-thioglucose ring-opening rate cannot be assumed to be the same as the rate of ring opening for glucose. However, it should be noted that such a 1600-fold lower activity is within the velocity limits of a slow substrate and unlikely to be a side reaction of xylose isomerase. For example, the k_{cat} of chymotrypsin with amide substrates is 1000-fold less than k_{cat} with ester substrates (Bender & Kezdy, 1965).

D-Glucal Is Not a Substrate. The compound D-glucal was determined to be a competitive inhibitor from Lineweaver–Burk plots with K_i = 7.4 mM using α-D-glucose as substrate. Hydration of D-glucal is catalyzed by α- and β-glucosidases to produce 2-deoxy-D-glucose by protonating the C1–C2 double bond from above or below the double bond, respectively. Incubation of 1 M D-glucal with xylose isomerase for 2 weeks produced no 2-deoxy-D-glucose as detected using the glucose oxidase assay method (see Materials and Methods). It should also be noted that incubation of xylose isomerase (2 mg) with D-glucal (0.15 M) in 15% D_2O and buffer produced no product detectable by ^{13}C NMR after 10 days. Thus, although D-glucal occupies the same binding site as α-D-glucose, there is no group present in the correct orientation to act as a general acid donor.

Binding of Glucose to Magnesium. In order to examine which hydroxyl groups of the sugar substrate glucose interact with the divalent metal cation, the enzyme–substrate complex was titrated with magnesium and observed by NMR. D-Xylose isomerase (5 mM) was incubated with (U-C^{13})glucose (0.25 M) in 10% D_2O and 0.025 M PIPES–KOH buffer, pH 7.5. Aliquots of $MgCl_2$ were added to the NMR tube and spectra obtained at concentrations of 0.5, 1.0, 2.0, 5.0, 8.0 and 10 mM $MgCl_2$. The expected shift in the ^{13}C signal is 0.25 ppm (the average of the bound and unbound species) for a carbon bonded to a hydroxyl interacting with Mg^{2+}. However, this was not observed for any one carbon atom of glucose, instead a shift of ≤0.05 ppm was observed for all carbon nuclei observed. Similar results were obtained when the identical experiment was performed using the divalent metal cation manganese. The binding of paramagnetic manganese to the enzyme-bound glucose hydroxyls is expected to produce line broadening of the peaks corresponding to the attached carbon atoms. Titration of the enzyme–(U-C^{13})glucose complex with up to 0.01 M $MnCl_2$ produced a broadening of the peaks of all six carbons of the glucose ring. These results are consistent with the presence of several binding modes for the substrate, each with different contacts of the hydroxyl groups with the divalent metal cation(s).

DISCUSSION

Isotope Exchange. Although most of the crystallographic studies of substrate and inhibitor complexes of D-xylose isomerase show no enzymic base positioned to catalyze proton transfer, the only mechanistic evidence against a proton-transfer mechanism has been the original solvent isotope incorporation experiments of Rose et al. (1969). Less than 0.4% exchange of tritium into fructose was observed when glucose was incubated with the enzyme at 25 °C. However, Rose has suggested that "hidden" proton transfers can be detected by carrying out exchange experiments at higher temperatures (Rose & O'Connell, 1961); if proton abstraction occurs in a site shielded from solvent, elevated temperatures may permit solvent access. Additionally, if exchange is not observed because of competing rates of transfer between sites on the substrate and protonated enzyme with solvent, raising the temperature should affect those two processes unequally, leading to increased incorporation of label. Hall et al. (1976) employed this method to establish that the glyoxylase I reaction involved base catalyzed proton abstraction. Thus, it is clear that simple exchange experiments such as those previously employed for D-xylose isomerase can fail to detect a proton-transfer step. Therefore, we carried out isotope-exchange experiments from substrates labeled in the H2 position into solvent over a wide range of temperatures and pH values. We also used guanidine hydrochloride to "loosen" the structure of the enzyme and measured proton loss to solvent under these conditions.

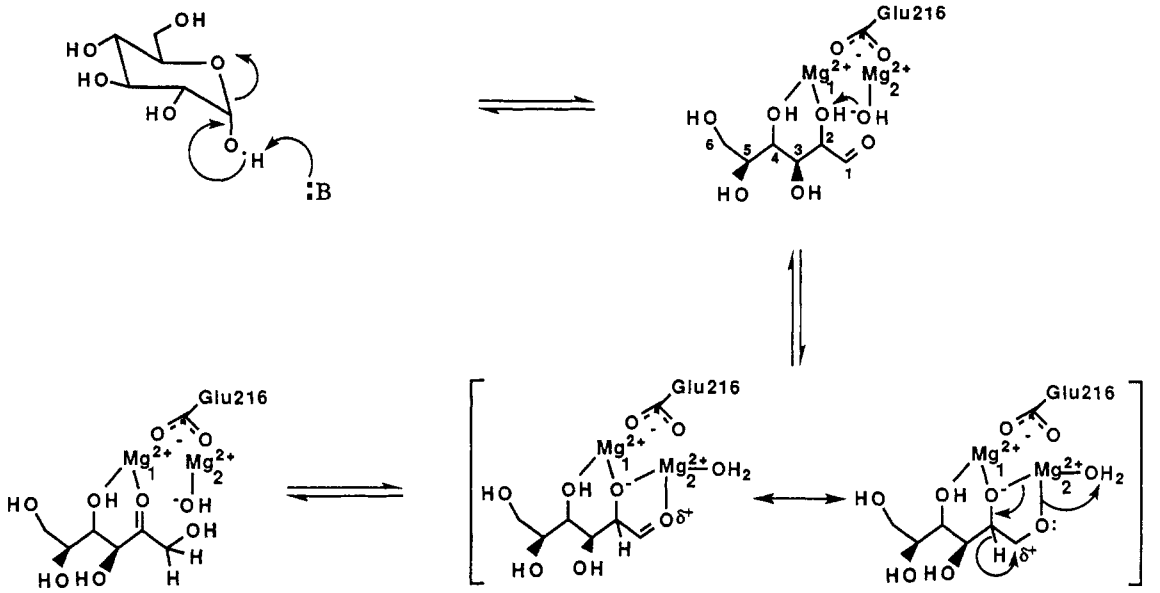
In all cases, the measured percentage of exchange was less than 0.6%. This value was independent of temperature, pH, or the presence of denaturant, but was found to depend on the presence of both metal and enzyme. An exchange value of 0.6% corresponds to the loss of one tritium to solvent every 1 billion turnovers. We conclude therefore that proton transfer to a solvent-exchangeable base cannot be involved in the normal turnover of D-xylose isomerase.

It would seem unlikely that these results could be misleading. It is possible that a very fast proton transfer could go undetected by experiments of this kind, but k_{cat} for D-xylose isomerase is extremely slow (1 s^{-1}). Triosephosphate isomerase, which has a proton-transfer rate faster than the diffusion-controlled limit, is easily shown to exchange tritium from labeled substrate into solvent in similar experiments (97% exchange after 66% conversion to product at 30 °C) (Nickbarg & Knowles, 1988). Finally, steric exclusion of solvent from the active site would appear to be ruled out on two grounds; one, that the three-dimensional structure of the enzyme–substrate complex shows an open active-site pocket (Farber et al., 1989), and two, that partial unfolding of the structure by heat or denaturant has no effect on the amount of exchange observed.

The small amount of exchange detected is clearly due to a process involving enzyme and metal but distinct from normal turnover. It may be the result of an alternate mechanistic path of significantly higher activation energy, or could arise from a nonproductive side reaction. Metal complexed with glucose in solution could react with a base anywhere on the enzyme to produce the small amount of exchange observed. This small percentage of exchange could also result from the presence of a contaminating enzyme.

Fluoride Elimination. If proton abstraction from the C2 of glucose is required for isomerization, fluorine should eliminate from 3-deoxy-3-fluoro analogs of the substrate. Kozarich et al. (1981) employed this method to detect proton transfer. To test this possibility, we synthesized 3-deoxy-3-fluoroallose in which the fluorine is *trans* to the putative abstracted hydrogen and purchased 3-deoxy-3-fluoroglucose

Scheme 1: Proposed Hydride-Transfer Mechanism for D-Xylose Isomerase with α-D-Glucose as the Substrate (See Discussion)



in which the fluorine occupies the *cis* position. Extensive incubation with high concentrations of both fluoro sugars showed no elimination of fluorine. Competitive inhibition was demonstrated for both compounds with respect to glucose, indicating that they were both bound to the active site. No one piece of evidence can distinguish between the hydride-transfer and proton-transfer mechanisms. However, in conjunction with the tritium exchange experiment, the lack of fluoride elimination strengthens the case against a proton transfer mechanism.

Enzyme-Catalyzed Ring Opening. ^{13}C NMR experiments have shown that only the α-anomer of glucose and fructose are bound to the enzyme and that the open-chain form of glucose is produced in the active site (Farber et al., 1989). It seems reasonable to assume that the enzyme must catalyze ring opening since the observed kinetics are inconsistent with preferential binding of the open chain form from solution. However, direct evidence for enzyme-catalyzed ring opening has not yet been obtained. We realized that the ring-open form of 1-thioglucose would tend to eliminate H_2S and that, if the enzyme catalyzed this reaction, we should observe detectable amounts of H_2S released upon incubation of D-xylose isomerase with this substrate analog. Chemical trapping showed that the enzyme catalyzes the elimination of 1 μmol of H_2S from 1-thioglucose over a 3-day period, a rate enhancement of at least 10-fold over the uncatalyzed rate of H_2S release. Since every ring-opening event for 1-thioglucose does not necessarily result in an elimination event, this technique cannot be used to determine the rate of ring opening of glucose itself, but it does demonstrate directly that enzyme-catalyzed ring opening occurs. Furthermore, this technique allows one to detect ring opening, or its absence, in any mutant D-xylose isomerase.

Ring opening would be facilitated by acid catalysis in which a proton is donated by the enzyme to the O5 (ring oxygen) leaving group. Hydration of D-glucal requires an acid catalyst to protonate the C1–C2 double bond to produce 2-deoxy-D-glucose. The results of assays with D-glucal show that there is no acid catalyst in xylose isomerase in the proper orientation to provide a proton at the double bond of D-glucal. Thus, there is no evidence for the existence of an active-site residue which can act as a donor in general-acid-catalyzed ring opening.

Temperature Dependence. Since a wide range of temperature has been employed in these studies, we felt it prudent to ascertain whether the rate-determining step of the enzyme catalyzed reaction was the same at all temperatures studied. D-Xylose isomerase catalyzed interconverison of glucose to fructose exhibits linear Arrhenius temperature dependence with an activation energy of 14 kcal/mol from 0 to 50 °C. Since no change in slope is observed in the Arrhenius plot, we conclude that the rate-determining step is unchanged over this temperature range. Unfortunately, the magnitude of the activation energy does not distinguish among ring opening, hydride transfer, or proton transfer as the step in question. A similar energy of activation of 12.7 kcal/mol was determined for the enzyme lactate dehydrogenase from *Bacillus stearothermophilus* which is believed to proceed via a hydride transfer mechanism (Huskey, 1985).

Nonproductive Binding. If metal-assisted hydride transfer is the catalytic mechanism of D-xylose isomerase, pinpointing the interactions of the divalent metal cation with the sugar substrate becomes extremely important. Binding of magnesium cation to the hydroxyls of bound glucose is expected to produce a change in chemical shift of the attached carbons of 1.0 ppm for 5 mM enzyme. ^{13}C NMR experiments in which the enzyme–(U-C^{13})glucose complex is titrated with up to 0.01 M $MgCl_2$ show a change in chemical shift of ≤0.05 ppm for all six carbons of the glucose ring. In the case of $MnCl_2$, the binding of paramagnetic manganese to the enzyme-bound glucose hydroxyls is expected to produce line broadening of the peaks corresponding to the attached carbon atoms. Titration of the enzyme–(U-C^{13})glucose complex with up to 0.01 M $MnCl_2$ produced a broadening for all six carbons of the glucose ring. These results are consistent with the presence of incorrectly bound, inactive, glucose as well as correctly bound, reactive glucose. The nonproductive binding modes of glucose to the divalent metal cation along with the reactive binding mode lead to an equal amount of interaction of the metal cation with all six carbons of glucose. Although these results do not directly provide any information about the enzyme mechanism, they may provide an explanation as to why the enzyme has such a low rate of turnover (1 s^{-1}).

CONCLUSIONS

Our low-resolution studies of enzyme–substrate complexes of *Streptomyces olivochromogenes* D-xylose isomerase combined with the original data by Rose et al. (1969) showing an absence of solvent–isotope exchange for the enzyme catalyzed

reaction led us to suggest that mechanisms other than proton transfer should be considered for this enzyme (Farber et al., 1989). In particular, the prominent role of the metal ion led us to suggest the possibility of a hydride-transfer mechanism. This mechanism has been refined and elaborated upon by several other groups, on the basis of high-resolution crystallographic data (Collyer et al., 1990; Whitlow et al., 1991) and site-directed mutagenesis (Jenkins et al., 1992; Lambeir et al., 1992; van Tilbeurgh et al., 1992). However, some structural data has been interpreted in terms of a proton-transfer process (Carrol et al., 1989). Direct chemical and kinetic tests of the mechanism of this enzyme have not been reported. We have undertaken a series of such tests under a wide range of conditions, and our results are inconsistent with a proton-transfer mechanism for D-xylose isomerase with glucose as substrate. Our results are consistent with a mechanism in which cyclic α-D-glucose binds to the enzyme followed by enzymic base-catalyzed ring opening, and metal-assisted 1,2-hydride transfer, depicted in Scheme 1. Scheme 1 shows the ring-opening reaction as base catalyzed, not acid catalyzed, consistent with our results for D-glucal. The ring-opening base could be either a Mg^{2+} liganded water or hydroxide ion as seen in high-resolution crystallographic studies (Lavie et al., unpublished results; Collyer et al., 1990; Whitlow et al., 1991) and consistent with the pK_a of the enzyme–glucose complex (Allen et al., 1993), or possibly Lys182 as suggested by site-directed mutagenesis studies (Lambeir et al., 1992). In the ring-opened form, the substrate may be held in position by His53 (Gerhardt-Müller, unpublished results) and by the Mg^{2+} ligands. O2 may be deprotonated concomitant with hydride transfer as suggested (Whitlow et al., 1991; van Tilbeurgh et al., 1992). Current work in this laboratory with a potent inhibitor of xylose isomerase is consistent with such a deprotonation, with the resultant deprotonated O2 stabilized by the metals. The hydride transfer step is favored by the electron-deficient aldehydic carbonyl, C1, with Mg^{2+} acting as an electrophilic catalyst to stabilize the developing negative charge on O1 in the transition state. The hydride transfer as pictured has no protons in flight and has a pericyclic character. Once hydride transfer is complete, ring closing, to either the fructopyranose or fructofuranose form, may be catalyzed in the reverse of the ring-opening reaction. However, recent NMR experiments show that although the enzyme catalyzes the ring opening and closing of α-xylose it does not catalyze the ring closing of α-xylulose (Seeholzer, unpublished results).

Any mechanism proposed for this enzyme must account for the dependence of the reaction on divalent metal ion. The role of the metal is the subject of the accompanying paper.

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