Epimerization via Carbon−Carbon Bond Cleavage. L-Ribulose-5-phosphate 4-Epimerase as a Masked Class II Aldolase†

Anne E. Johnson and Martin E. Tanner*

Department of Chemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z1, Canada

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ABSTRACT: Studies indicating that the *E. coli* L-ribulose-5-phosphate 4-epimerase employs an "aldolase-like" mechanism are reported. This NAD^+-independent enzyme epimerizes a stereocenter that does not bear an acidic proton and therefore it cannot utilize a simple deprotonation−reprotonation mechanism. Sequence similarities between the epimerase and the class II L-fuculose-1-phosphate aldolase suggest that the two may be evolutionarily related and that the epimerization may occur via carbon−carbon bond cleavage and re-formation. Conserved residues thought to provide the metal ion ligands of the epimerase have been modified using site-directed mutagenesis. The resulting mutants show low k_{cat} values in addition to a reduced affinity for Zn^{2+}. These observations serve to establish that there is a structural link between between the active site geometry of the epimerase and the aldolase. In addition, the H97N mutant was found to catalyze the condensation of dihydroxyacetone and glycolaldehyde phosphate to produce a mixture of L-ribulose-5-phosphate and D-xylulose-5-phosphate. This observation of aldolase activity establishes that the epimerase active site is capable of promoting carbon−carbon bond cleavage. Furthermore, glycolaldehyde phosphate was shown to be a competitive inhibitor of the mutant enzyme (K_I = 0.37 mM) but not of the wild-type enzyme. The mutation apparently causes the epimerase to become "leaky" and enables it to bind/generate the normal reaction intermediates from the unbound aldol cleavage products.

The bacterial enzyme L-ribulose-5-phosphate 4-epimerase (AraD, EC 5.1.3.4) catalyzes the interconversion of L-ribulose-5-phosphate (L-Ru5P)[1] and D-xylulose-5-phosphate (D-Xu5P) via a stereochemical inversion at C-4 (Figure 1). The epimerase serves to link the arabinose metabolic pathway to the pentose phosphate pathway and allows the bacteria to use arabinose as an energy source (*1*). The enzyme has a molecular mass of 102 kDa (*2*, *3*), and is composed of four identical 25.5 kDa subunits (*4*, *5*). The epimerase is also known to require one divalent metal cation per subunit for activity (*6*).

The mechanism employed by the enzyme is of interest because the center of inversion is not activated by an adjacent carbonyl or carboxylate functionality (*7*, *8*). Thus, the proton at the C-4 position of the epimeric sugars is not acidic, and a simple deprotonation/reprotonation mechanism can be ruled out. Furthermore, the enzyme has been shown not to use an NAD^+ cofactor during catalysis (*2*), and therefore does not employ an oxidation/reduction mechanism analogous to that of UDP-galactose 4-epimerase (*9*, *10*). Other studies have shown that the reaction proceeds without the incorporation of solvent-derived oxygen or hydrogen atoms and that there is no primary kinetic isotope effect in the epimerization of either D-[4-^{3}H]Xu5P or L-[4-^{2}H]Ru5P (*11*−*13*). Two mechanisms have been proposed to explain these observations (*6*). The first involves an initial retroaldol cleavage that produces the enolate of dihydroxyacetone and glycolaldehyde phosphate as enzyme-bound intermediates (Figure 2). Readdition of the same face of the enolate to the opposite face of the aldehyde generates the epimeric product. The metal ion acts as an electrophilic catalyst and stabilizes the enolate intermediate. The second is a dehydration/rehydration mechanism involving an enone intermediate (Figure 3). This mechanism demands that the eliminated hydroxide ion be able to add to either face of the planar intermediate for epimerization to occur. The metal ion could serve to bind the hydroxide ion, and prevent its exchange with bulk solvent. Other mechanisms involving multiple proton-transfer steps and carbonyl migration can be envisioned; however, they would have to take place without any solvent isotope incorporation.

Indirect evidence supporting the retroaldol/aldol mechanism (Figure 2) can be found by comparing characteristics of the epimerase with those of known bacterial (class II) aldolases (*14*−*16*). The reaction catalyzed by the aldolases differs in that the enolate may be protonated and released into solution, and that the carbon−carbon bond is generally formed with high stereospecificity. Nevertheless, the possibility of epimerization via metal-assisted aldol chemistry suggests that an evolutionary link may exist between the two types of enzymes. Indeed, reports describing the three-

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[1] Abbreviations: L-Ru5P, L-ribulose 5-phosphate; D-Xu5P, D-xylulose 5-phosphate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; LB, Luria broth; NAD^+/NADH, nicotinamide adenine dinucleotide/dihydronicotinamide adenine dinucleotide; TPP, thiamin pyrophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; CD, circular dichroism; NMR, nuclear magnetic resonance; ICPMS, inductively coupled plasma mass spectrometry; RCPCR, recombinant circle PCR; NMWL, nominal molecular weight limit.

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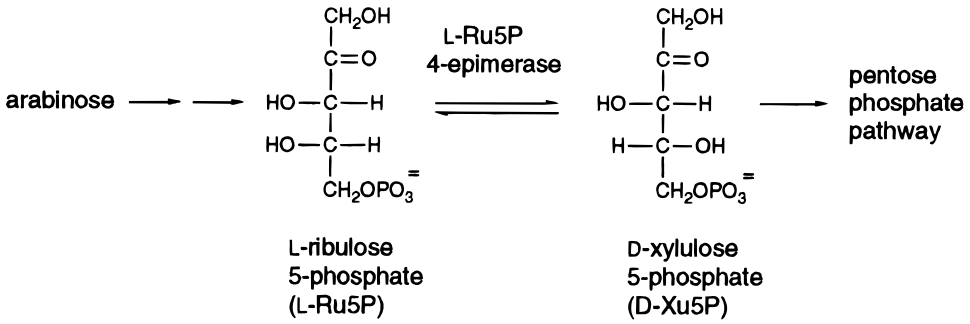

FIGURE 1: Reaction catalyzed by L-ribulose-5-phosphate 4-epimerase and its role in arabinose metabolism.

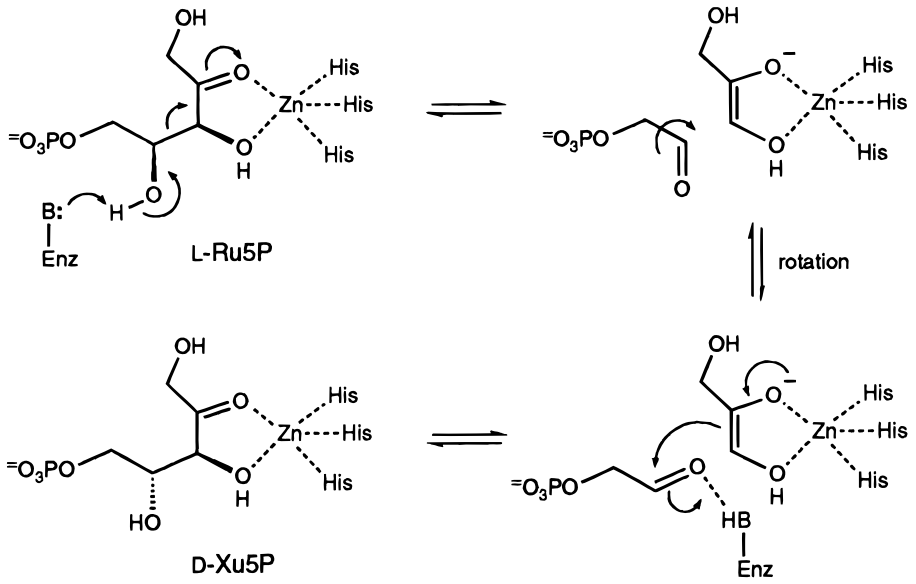

FIGURE 2: Potential retroaldol/aldol mechanism for the reaction catalyzed by L-ribulose-5-phosphate 4-epimerase.

dimensional structure of the class II L-fuculose-1-phosphate aldolase (FucA) from *E. coli* state that much of the N-terminal portion of L-Ru5P 4-epimerase is homologous to FucA and likely contains the amino acids that supply the metal ion ligands (*15*, *17*). Amino acid sequence alignments led the authors to suggest that Asp76, His95, and His97 are three of the four metal ligands in the uncomplexed epimerase active site (*17*).

In this paper, we describe the overexpression of L-ribulose-5-phosphate 4-epimerase at a very high level, its purification, and the preparation of site-directed mutants in which the putative metal ion ligands are modified. The mutants are isolated as stable, folded tetramers; however, they require exogenous metal ions for full activity. Their k_{cat} values are greatly reduced whereas the K_M values are only moderately affected. These observations are consistent with their postulated role as metal ligands and establish a structural link between this epimerase and the class II aldolases. In addition, low levels of aldolase activity are observed with the H97N mutant but not with the D76N or the H95N mutants. It appears that the epimerase active site is capable of promoting carbon−carbon bond cleavage and that the mutation caused the protein to become "leaky" and occasionally release or accept the products of this cleavage.

EXPERIMENTAL PROCEDURES

Materials and General Procedures. The following oligonucleotides (5′ to 3′) were used as primers for the cloning and mutagenesis (restriction sites underlined, mismatches in bold): primer 1 = GGCCCCATGGCCCATATGTTAGAAGATCTCAAACGCCAGG (*Nde*I); primer 2 = CCGCGCGGGGGATCCTGCAGTTACTGCCCGTAATATGCC (*Pst*I); primer 3 = GCGAGTGCGT**G**T**TA**ACAATGCCGCCAATGGA (*Hpa*I); primer 4 = CGTGGCGCGA**AT**T**C**GTATGCACAATGCCG (*Eco*RI); primer 5 = TGTGCATACG**A**A**T**TCGCGCCACGCGACCA (*Eco*RI); primer 6 = AAAAGCCCTCT**TC**GA**A**CACGCCAACTCACCGG (*Csp*45I); primer 7 = GTTGGCGTG**T**TC**GA**AGAGGGCTTTTTCGTACC (*Csp*45I); primer 8 = CGACGAGCGTGACACCACGATGCC; primer 9 = GCAGAGCGAGGTATGTAGGCGGTG.

The oligonucleotides were synthesized in the lab of Dr. Ivan Sadowski, Department of Biochemistry, University of British Columbia. Primers 1 and 2 are complementary to the 5′ and 3′ ends of the epimerase gene, respectively; primers 3−7 contain mismatches used to introduce mutations; and primers 8 and 9 are complementary to opposing strands of the pBS vector. Double-stranded sequencing of the wild-type and mutant plasmids was performed using a Sequenase Version 2.0 kit (U.S. Biochemical Corp.) and [^{35}S]dATPαS triethylammonium salt (400 Ci/mmol, Amersham). Restriction enzymes were purchased from either Promega or BRL.

The substrate L-Ru5P was prepared by treatment of L-ribulose with crude preparations of phosphoribulokinase and ATP (*18*). The kinase was obtained from strains of *E. coli* lacking a functional epimerase gene (either *E. coli* Y1090

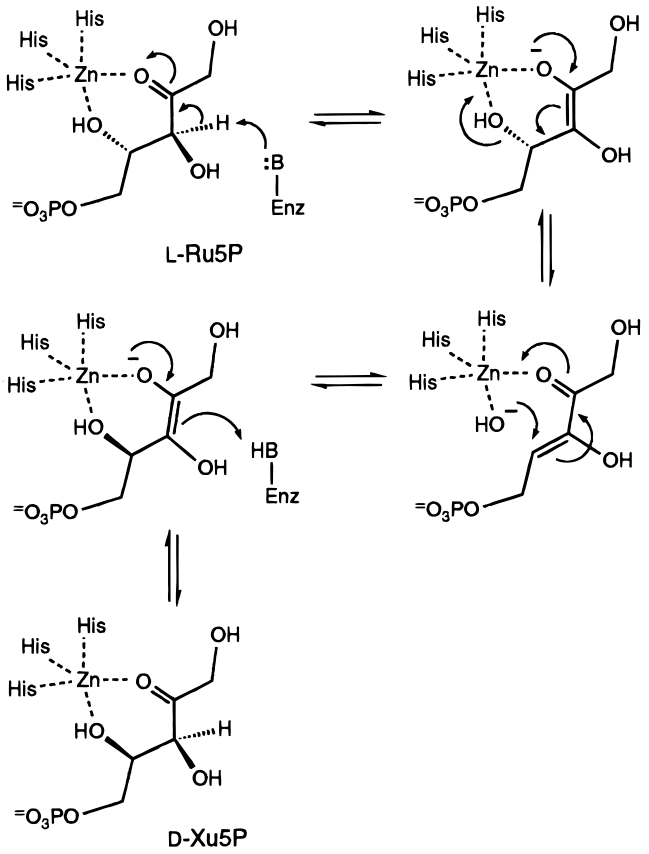

FIGURE 3: Potential dehydration/rehydration mechanism for the reaction catalyzed by L-ribulose-5-phosphate 4-epimerase.

(modified) [Δ(*lac*)*U169 lon-100 araD139 rpsL*(Str^r) *supF mcrA trpC22*::*Tn10* (cured of pMC9)] that was kindly provided by Dr. Neil Gilkes, Department of Microbiology, University of British Columbia, or *E. coli* MC4100 (ATCC 35695) [F$^-$ *araD139* Δ(*argF- lac*)*U169 rpsL150* (Str^r) *relA1 deoC1 ptsF25 rbsR flb5301* λ^-]. The substrate was purified by ion exchange chromatography (AG1-X8 resin, formate form, 100−200 mesh, eluted with a gradient of 0−3 N formic acid). It was found to be greater than 90% pure as judged by ^{1}H NMR spectroscopy.

Glycolaldehyde phosphate was prepared from allyl alcohol according to the method of Müller et al. (*19*). The calcium salt (1.0 g) was stirred with Amberlite DP-1 resin (0.5 mL, Na^+ form) in distilled water (10 mL). The resin was removed by filtration, and the filtrate was lyophilized to dryness. The solid (Na^+ form) was stored over desiccant at −20 °C.

The enzyme activity (37 °C, 25 mM glycylglycine buffer, pH 7.6) was routinely measured by monitoring the decrease in NADH using the coupled assay reported by Davis et al. (*11*). The coupling enzymes were purchased from Sigma Chemical Co. Protein concentrations were determined using A_{280} (1.73 mL mg^{-1} cm^{-1}). This value was obtained by amino acid analysis of a sample of the epimerase (in H_2O) to which norleucine (0.1 mM) had been added as an internal standard. The analysis was performed by Suzanne Perry of the Nucleic Acid Protein Service at The University of British Columbia.

Metal-free buffer was prepared by passing a solution of HEPES (50 mM, 2 L, containing 10% glycerol) through a column of Chelex-100 resin (70 mL, Na^+ form, 50−100 mesh, previously rinsed with 140 mL of 1 N HCl and 350 mL of deionized water) (*6*). The pH was adjusted to 7.6 using solid Tris base. All of the glassware used in this procedure had been previously soaked overnight in 4 N HCl and washed thoroughly with deionized water (17.8 MΩ/cm, 25 °C).

Cloning of L-*Ru5P 4-Epimerase*. To amplify the *araD* gene, aliquots (50 μL) of an overnight culture of *E. coli* DH1 (harvested and resuspended in an equal volume of water) were used directly in PCR with primers 1 and 2 [*Taq* DNA polymerase (Gibco BRL); 40 cycles; 95 °C, 1.5 min; 55 °C, 2 min; 72 °C, 2 min]. The products of the PCR amplification were purified by passage through an Ultrafree-MC 10 000 NMWL filter unit (Millipore), followed by extraction with phenol and SEVAG (25:1 $CHCl_3$−isoamyl alcohol), and then ethanol precipitated.

The overexpression vector employed was a slightly modified version of the pBSTIM vector (trc promoter) previously used (*20*) to express triosephosphate isomerase (the modifications were previously performed by Dr. S. Pollock in the laboratory of Dr. Jeremy Knowles, Department of Chemistry, Harvard University). The modifications included the removal of an *Nde*I restriction site at position 184 on the pBS vector (A for C replacement at position 186) and the introduction of the same site directly adjacent to the start codon (GCCATG was changed to CATATG). Both the vector and the PCR products (from six pooled reactions) were digested with *Nde*I and *Pst*I for 3 h at 37 °C. The vector was additionally treated with alkaline phosphatase for 2 h at 37 °C. These samples were isolated by electrophoresis on a 1% low melt Sea Plaque agarose gel (FMC BioProducts), and the appropriate bands (as visualized by ethidium bromide staining) were excised. An in-gel ligation was then performed using T4 DNA ligase for 30 h at 15 °C. The ligation mixture was melted and immediately used to transform *E. coli* XL1-Blue cells via electroporation. Colonies were picked from LB plates containing ampicillin (100 μg/mL), and 10 mL overnight cultures (LB containing 200 μg/mL ampicillin) were grown. Plasmid pRE1 was obtained using the Magic Minipreps DNA purification system (Promega), and the epimerase gene was fully sequenced to ensure that no errors had been introduced during PCR amplification.

Overexpression and Purification of L-*Ru5P 4-Epimerase*. The purification of the recombinant epimerase was achieved using a modification of the procedure reported by Gielow and Lee (*21*). Plasmid pRE1 was transformed into *E. coli* Y1090 (modified, lacking a functional epimerase gene as described above), and individual colonies (grown on LB agar containing 50 μg/mL ampicillin) were cultured overnight in LB broth (500 mL, containing 50 μg/mL ampicillin). The cells were harvested and resuspended in buffer A [10 mM potassium phosphate, pH 7.6, containing glycerol (10%), pepstatin (1 mg/L), aprotinin (1 mg/L), and phenylmethanesulfonyl fluoride (1.5 mM)]. Cells were lysed by two passages through a French pressure cell at 20 000 psi. Following ultracentrifugation at 30 000 rpm for 1 h, the supernatant was brought to 43% saturation with solid ammonium sulfate. The precipitated protein was collected as described in the original procedure (*21*), resuspended, and dialyzed against buffer A. This was applied to a column (15 mL) of (diethylamino)ethylcellulose (DE-52, from Whatman) that had been preequilibrated with buffer A. The

column was washed with buffer A (25 mL), and the protein was eluted with buffer A containing 0.4 M NaCl (50 mL). The eluted protein was concentrated, exchanged into buffer B (10 mM potassium phosphate, pH 7.6, containing 10% glycerol), and frozen rapidly with liquid nitrogen. Portions of this sample were thawed, applied to a Protein-Pak Q 8HR column (Waters), and eluted with a linear gradient of buffer B containing NaCl (0−40 mM, 1 mL/min). Fractions containing homogeneous epimerase as analyzed by SDS−PAGE were pooled and frozen as above. A typical preparation yielded 100 mg of epimerase per liter of culture with a specific activity of 34 μmol min^{-1} mg^{-1}.

Preparation of Mutant Plasmids and Enzymes. The mutants H97N and D76N were prepared using the recombinant circle PCR (RCPCR) method (*22*). To obtain mutant H97N, pRE1 (previously linearized with *Pst*I) was amplified by PCR using primers 4 and 8, and pRE1 (previously linearized with *Eco*RI) was amplified by PCR using primers 5 and 9. To obtain mutant D76N, pRE1 (previously linearized with *Pst*I) was amplified by PCR using primers 7 and 9, and pRE1 (previously linearized with *Eco*RI) was amplified by PCR using primers 6 and 8. In both cases, the resulting PCR products were cotransformed according to the RCPCR technique. Silent mutations that introduced a unique restriction site were incorporated in the mutagenesis primers (in addition to the desired mutation) in order to facilitate rapid screening of the resultant colonies (*Eco*RI in the case of H97N and *Csp*45I in the case of D76N). Plasmids pAJ2 (containing H97N) and pAJ3 (containing D76N) were obtained, and the inserts were fully sequenced, confirming that only the desired mutation was present.

The mutant H95N was obtained by first preparing a double-stranded megaprimer (*23*) (containing the desired mutation and a silent mutation that introduced the *Hpa*I restriction site) using PCR with primers 3 and 1 on pRE1. The megaprimer was purified using a Wizard Magic PCR Preps DNA purification kit (Promega) and was used in two subsequent rounds of PCR. Reactions were run with the megaprimer and primer 8 on pRE1 (previously linearized with *Pst*I) and with the megaprimer and primer 9 on pRE1 (previously linearized with *Eco*RI). Plasmids from colonies obtained by cotransformation (RCPCR method) of these two PCR products were analyzed by restriction digestion analysis using *Hpa*I. The construct pAJ1 was found to contain only the desired H95N mutation by sequencing of the epimerase gene.

The three mutant enzymes were expressed and purified as described for the wild-type enzyme.

Preparation of Zinc-Substituted Enzymes and Analysis of Metal Content. Apoenzyme was prepared by incubating protein samples (5 mg) in 20 mM EDTA (2.5 mL) for 3 h at 25 °C (*6*). The samples were then dialyzed against metal-free HEPES−Tris buffer (50 mM, pH 7.6, 2 × 500 mL, containing 10% glycerol) at 4 °C. An end-point assay for L-Ru5P was employed to ensure that the enzyme samples were inactive. The samples were reconstituted by the addition of 99.99% pure $ZnCl_2$ (10 equiv, 250 μL of a 3.9 mM solution) and incubated for 2 h at 25 °C. Routine samples were then dialyzed against metal-free HEPES−Tris buffer (50 mM, pH 7.6, 2 × 500 mL, containing 10% glycerol) at 4 °C and flash-frozen in liquid N_2. Samples to be analyzed by ICPMS were passed through a size-exclusion column (Waters Protein Pak 125), eluted with the same HEPES−Tris buffer, diluted 1/400 in 10% nitric acid (ultrapure grade, twice distilled on quartz, from Seastar, Sidney, B. C., containing 20 ppb Sc as an internal standard), and digested at 50 °C for 12 h prior to analysis. The column had previously been treated with 200 mM EDTA and then equilibrated with the HEPES−Tris buffer. These samples were analyzed on a VG Elemental Plasma Quad mass spectrometer that had been calibrated with a standard curve of Zn (0, 5, 15, 40 ppb, prepared in 10% nitric acid containing a 1/400 dilution of the elution buffer and 20 ppb Sc as an internal standard). The analyses were performed by Dr. Bert Mueller in the Department of Oceanography at The University of British Columbia.

Circular Dichroism and Thermal Stability. Solutions of the zinc-substituted enzymes were exchanged into potassium phosphate buffer (10 mM, pH 7.6) using Amicon Centricon-10 concentrators. These solutions (0.17 mg/mL) were scanned in the far-UV (300−190 nm) on a Jasco J-720 spectropolarimeter.

The thermal stabilities of the wild-type and mutant epimerases (0.17 mg/mL) were determined by observing the change in CD signal at 220 nm as a function of temperature in HEPES−Tris buffer (50 mM, pH 7.6). The temperature of the sample was raised from 30 to 75 °C at a rate of 50 °C/h using a NESLAB RTE-111 waterbath, a NESLAB M-RS-232 bath/computer interface, and a NESLAB RS-2 remote sensor.

Native Molecular Mass Determinations. Solutions (1 mg/mL) of the epimerase, epimerase mutants, and protein standards were prepared in potassium phosphate buffer (100 mM, pH 7.0). Aliquots (50 μL) were injected onto a Protein-Pak 300 SW column (Waters) and eluted at a flow rate of 1 mL/min with the same buffer. Blue dextran (2000 kDa) was used to mark the void elution time, and pyruvate kinase (237 kDa), aldolase (158 kD), rabbit muscle lactate dehydrogenase (140 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa) were used as molecular mass standards.

Assays for Aldol Activity. The aldol reaction was followed spectrophotometrically using an end-point assay for total D-Xu5P and L-Ru5P that was modified from the continuous D-Xu5P assay of Davis et al. (1972). Solutions (1 mL, pH 7.6) containing glycolaldehyde phosphate (5 mM), dihydroxyacetone (50 mM), $ZnCl_2$ (0.1 mM), and the epimerase/mutant (0.5 mg, zinc form) were incubated at 37 °C for 48 h. At timed intervals, aliquots (50 μL) were removed and added to cuvettes containing glycylglycine buffer (25 mM, pH 7.6, 0.5 mL), NADH (0.15 mM), TPP (0.1 mM), D-ribose 5-phosphate (5 mM), α-glycerol-phosphate dehydrogenase (2.5 units), triosephosphate isomerase (25 units) and transketolase (0.125 unit) at 37 °C. With the mutant enzymes, an additional 0.03 unit of wild-type epimerase was added to the assay mixture. The amount of total pentose phosphates present was determined by the rapid decrease in absorbance at 340 nm due to NADH consumption.

To prepare the ^{1}H NMR samples, the contents of two incubation mixtures (48 h, one with the H97N mutant and one without) were filtered through Amicon Centricon-10 concentrators, frozen in liquid N_2, and lyophilized to dryness. The samples were redissolved in D_2O and analyzed by ^{1}H NMR spectroscopy (500 MHz).

RESULTS

Expression, Purification, and Characterization of L*-Ribulose-5-phosphate* 4*-Epimerase from E. coli.* The *araD* gene encoding for the epimerase was amplified directly from *E. coli* DH 1 cells using PCR. The PCR product was then cloned into a modified pBS vector (trc promoter) to give the new construct pRE1. To avoid contamination by endogenous enzyme, this plasmid (and subsequent mutant plasmids) was transformed into a strain of *E. coli* lacking the gene for a functional L-Ru5P epimerase. Extremely high levels of expression were observed, and we estimate that the recombinant epimerase accounted for >60% of the total protein present in a crude cell extract. The epimerase was purified by ion-exchange chromatography, and its mass was shown, using electrospray mass spectrometry, to be consistent with that expected from the gene sequence: calcd 25 520 Da; found 25 522 Da. Enzyme prepared in this fashion was assayed in the L-Ru5P → D-Xu5P direction and gave kinetic constants of $k_{cat} = 13.5\ s^{-1}$ and $K_M = 0.026$ mM. Treatment of a commercial sample of D-Xu5P with the epimerase and analysis by ^{1}H NMR spectroscopy indicated that the equilibrium constant was 1.2, in favor of the D-Xu5P epimer. This value agrees with a previous report by Burma and Horecker (*24*).

The epimerase can accept a variety of divalent metal ions for activity, and it is not entirely clear which ion is present in the enzyme isolated from natural sources (*6*). The recombinant enzyme described above was therefore submitted for metal ion analysis by ICPMS. The results indicated that the enzyme preparation contained a mixture of the following metals as listed in order of decreasing abundance: $Zn^{2+} > Mn^{2+} > Cu^{2+}$. It is possible that the very high levels of overexpression resulted in the sequestration of a variety of metal ions and that this distribution does not reflect that obtained under normal growth conditions. Since zinc is commonly used by enzymes (such as the class II aldolases) to promote electrophilic catalysis and since it was the most abundant metal present in our preparation, we decided to prepare a homogeneous sample of the zinc form for further studies. The apoenzyme was prepared by treatment with EDTA and dialyzed against metal-free buffer. It was subsequently reconstituted with excess zinc and isolated by size-exclusion chromatography. Enzyme prepared in this fashion was analyzed for zinc content by ICPMS, and the results showed that there was 1.05 equiv of zinc present per subunit of enzyme as expected (*6*). The zinc enzyme was assayed in the L-Ru5P → D-Xu5P direction and gave kinetic constants of $k_{cat} = 20.4\ s^{-1}$ and $K_M = 0.087$ mM.

Preparation and Characterization of Mutant Enzymes. Sequence alignment studies have shown that the N-terminal portion of the L-fuculose-1-phosphate aldolase (class II) from *E. coli* (residues 1−125) shares 34% identity with that of L-Ru5P epimerase and suggest that an evolutionary link exists between these two enzymes (*17*). In addition, X-ray crystallographic studies on the aldolase have unambiguously identified the ligands for the active site zinc to be three histidine residues and one glutamate residue (*15*, *17*, *25*). Three of these residues are conserved among all known L-Ru5P epimerase sequences, and in the case of the *E. coli* enzyme are provided by His95, His97, and Asp76. We therefore decided to prepare mutants of the epimerase in which each of these residues was independently converted to an asparagine residue via site-directed mutagenesis. These mutations should directly perturb the coordination sphere of the bound metal and affect both the enzyme's affinity for the metal and its catalytic constants.

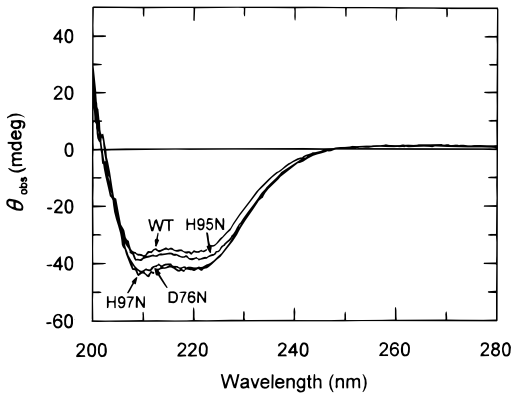

FIGURE 4: Circular dichroism spectra of the wild-type and mutant L-ribulose-5-phosphate 4-epimerases. The samples contained 0.17 mg/mL protein in 10 mM potassium phosphate buffer, pH 7.6, at 25 °C.

The mutagenesis was carried out using PCR techniques, and in each case the mutant enzymes were overexpressed at levels comparable to wild type. The mutant enzymes were purified in the same fashion as the wild-type enzyme, and their molecular weights were shown by electrospray mass spectrometry to be consistent with that expected from their gene sequences. The mutants were found to be indistinguishable from the wild-type enzyme when analyzed by circular dichroism spectroscopy (Figure 4). In addition, the loss of ellipticity at 220 nm was followed as a function of temperature, and all of the proteins were found to have thermal denaturation temperatures in the range of 49.5−54.5 °C. These observations support the notion that the mutant proteins are fully folded with a tertiary structure and stability comparable to those of the wild type. To investigate the quaternary structure of the proteins, size exclusion chromatography was employed. The wild-type and mutant enzymes all eluted with similar retention times, and a comparison to molecular weight standards indicated that they had masses of approximately 127 kDa. This shows that the mutants retained the ability to form tetrameric structures.

Kinetic Analysis of the Mutant Enzymes. To prepare protein samples with homogeneous metal ion content, the purified mutants were converted to their apoenzyme forms by dialysis against EDTA, and then reconstituted with excess zinc. The excess zinc was removed by dialysis against metal-free buffer prior to kinetic analysis. The kinetic constants for these samples were compared with those of a similarly treated sample of wild-type enzyme both in the presence and in the absence of 0.1 mM added Zn^{2+} (Table 1). The value of k_{cat} for the wild-type Zn^{2+} enzyme was relatively unaffected by the presence of added zinc. This is consistent with previous reports that the metal is bound tightly enough to survive dialysis against metal-free buffer (*6*). It also shows that excess zinc does not greatly affect the enzyme activity. The data obtained with the mutant enzymes were somewhat different in that notable increases in the k_{cat} values were observed in the presence of added zinc (particularly in the

Table 1: Catalytic Constants for Wild-Type and Mutant L-Ru5P 4-Epimerases[a]

	Zn^{2+} form after dialysis			Zn^{2+} form + 0.1 mM Zn^{2+}		
	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
wild type	20.4	0.087	2.3×10^5	22.4	0.060	3.7×10^5
H95N	0.047	0.14	3.4×10^2	0.10	0.096	1.0×10^3
H97N	0.048	0.10	4.8×10^2	7.3	0.14	5.2×10^4
D76N	0.073	0.11	6.6×10^2	0.16	0.14	1.2×10^3

[a] Assays were performed in 25 mM glycylglycine buffer, pH 7.6, at 37 °C.

case of H97N). However, the K_M values remained relatively unaffected. It would appear that the mutant enzymes lost varying amounts of the bound metal during dialysis against metal-free, buffer resulting in mixtures of the apoenzyme and holoenzyme. Indeed, passage of the mutant enzymes through a size exclusion column produced proteins with low metal ion content (as analyzed by ICPMS) and low activity, particularly with the H95N and H97N mutants (<0.1 and 0.1 equiv of zinc per subunit, respectively). These observations are consistent with the notion that the mutations have modified a residue involved in metal ion complexation. Measurements made in the presence of 0.2 mM Zn^{2+} caused no further changes in the k_{cat} values, indicating that 0.1 mM Zn^{2+} was sufficient to fully activate these mutants.

In each case, the k_{cat} values obtained with the mutants in the presence of Zn^{2+} were lower than that of the wild-type enzyme. This indicates that the modified residues play a role in catalysis such as providing a ligand for metal ion complexation or acting as general acid/base catalysts. Furthermore, the values of K_M were relatively unaffected by the mutations, as might be expected for modifications of residues that are not involved in substrate binding.

Tests for Aldolase Activity. The structural similarities between the epimerase and the class II aldolases suggest that the enzyme may be capable of promoting carbon−carbon bond cleavage and indirectly support the retroaldol/aldol mechanism (Figure 2). More direct evidence could be obtained by observing low levels of aldolase activity with the purified epimerase. The wild-type enzyme was therefore assayed for aldolase activity (C−C bond formation) by extensive incubation with dihydroxyacteone (50 mM) and glycolaldehyde phosphate (5 mM). At timed intervals, aliquots were removed, and the total amount of L-Ru5P and D-Xu5P was determined using an end-point assay for D-Xu5P (the large amounts of epimerase present ensured rapid equilibration between the epimers). This assay could not be run continuously since very long incubation times were employed and a small background rate was observed when glycolaldehyde phosphate itself was incubated in the presence of the coupling enzymes and NADH. The results showed that low levels of the pentose phosphate epimers were produced under these assay conditions; however, it was difficult to demonstrate unambiguously that they were not a result of a background, buffer-catalyzed reaction. The aldol substrates did not appear to bind to the epimerase since the epimerization of L-Ru5P (0.095 mM) was not significantly inhibited by the presence of either dihyroxyacetone (50 mM) or glycolaldehyde phosphate (5 mM).

The observation that the epimerase mutants still retained significant activity despite an altered metal coordination

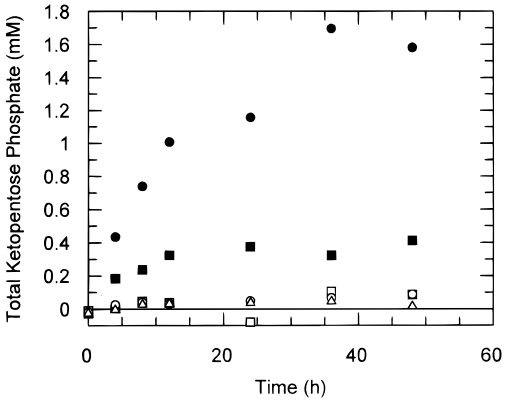

FIGURE 5: Reaction progress curves for the formation of L-ribulose 5-phosphate and D-xylulose 5-phosphate by the wild-type and mutant epimerases. The reactions contained 5 mM glycolaldehyde phosphate (pH 7.6), 50 mM dihydroxyacetone, 0.1 mM $ZnCl_2$, and 0.5 mg/mL protein: wild type (■), D76N (□), H95N (△), H97N (●), and equal volume of supernatant from heat-inactivated H97N (○).

sphere led us to test them for aldolase activity as well. We reasoned that a crippled enzyme may permit protonation of the bound enolate (or conversely deprotonation of dihydroxyacetone) and result in the release (or conversely consumption) of dihydroxyacetone and glycolaldehyde phosphate. Indeed, we found that the H97N mutant clearly did catalyze the formation of L-Ru5P and D-Xu5P from dihydroxyacetone and glycolaldehyde phosphate in the presence of 0.1 mM Zn^{2+} (Figure 5). The aldolase activity exhibited by this mutant was quite low (approximately 0.004 unit/mg), but the reaction was catalytic and was followed until 30% of the glycolaldehyde phosphate was consumed. Both the wild-type and H97N enzymes were found to lose their activity with a half-life of about 25 h under these conditions, preventing the aldol process from reaching equilibrium (product inhibition is also a factor here). To identify the products unambiguously as the epimeric pentose phosphates, the reaction mixture was lyophilized, dissolved in D_2O, and analyzed by ^1H NMR spectroscopy. The resulting spectrum showed signals identical to those of an authentic sample of the epimeric mixture that had been independently prepared using the wild-type epimerase and D-Xu5P. Control reactions run with the supernatant obtained from heat-denatured enzyme samples showed no detectable aldol products above background, indicating the reaction was not a result of a nonprotein impurity in the enzyme preparation. In addition, neither the wild-type enzyme nor the other two mutants showed activity comparable to that of the H97N mutant (all were isolated in an identical manner and assayed in the presence of 0.1 mM Zn^{2+}).

Aldol activity was also detected in the cleavage reaction direction by extensively incubating the enzymes with the epimeric sugar phosphates. At timed intervals, aliquots were removed and assayed for the formation of dihydroxyacetone using glycerol dehydrogenase and NADH. The results were similar to those seen in the coupling experiments, with the H97N mutant clearly catalyzing the reaction at rates greater than the wild-type enzyme; however, these rates were very low and difficult to quantitate (data not shown).

The observation of aldolase activity indicates that the mutant enzyme is able to bind the cleaved products from solution. In fact, glycolaldehyde phosphate was found to

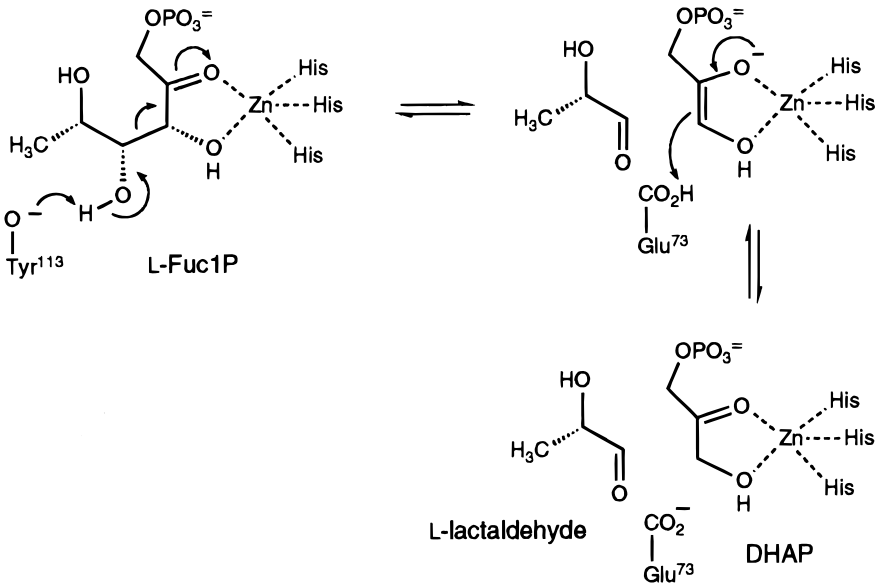

FIGURE 6: Proposed mechanism for the reaction catalyzed by L-fuculose-1-phosphate aldolase (*14*, *15*).

act as a competitive inhibitor (K_I = 0.37 mM) of the H97N enzyme in an assay for L-Ru5P epimerization. Dihydroxyacetone did not significantly inhibit the epimerization.

DISCUSSION

This work provides evidence that there is both a structural and a mechanistic link between the L-ribulose-5-phosphate 4-epimerase (AraD) from *E. coli* and the class II L-fuculose-1-phosphate aldolase (FucA) from *E. coli*. The aldolases have traditionally been characterized as either class I in which a lysine residue forms a Schiff base with a carbonyl of the nucleophilic substrate or as class II in which a metal ion serves to stabilize the enediolate intermediate (*16*). Class II aldolases are primarily found in bacteria, and the best characterized example is the *E. coli* FucA. The structure of FucA has been determined with (*14*, *15*) and without (*17*) the bound inhibitor phosphoglycolohydroxamate at 2.4 and 1.92 Å, respectively, and a proposed mechanism for the reaction has recently been published (Figure 6) (*14*, *15*). In the uncomplexed form of the enzyme, the zinc ion is coordinated by three histidine residues and one glutamate residue. Dihydroxyacetone phosphate is thought to displace the glutamate residue and bind to the zinc atom in a bidentate fashion via its carbonyl oxygen and hydroxyl group. Deprotonation of the bound substrate (presumably by the displaced glutamate) generates a metal-bound enediolate. This species attacks the carbonyl of lactaldehyde to form the carbon−carbon bond. A tyrosine is thought to help stabilize the developing charge and to control the stereochemistry of the attack by hydrogen bonding to the aldehyde. A very similar mechanism could be employed by the epimerase provided that the attack on the aldehyde is nonstereospecific and the bound enediolate is protected from any proton source (Figure 2). The exposure of either face of the carbonyl could be achieved by a simple bond rotation (as implied in Figure 2) or by a rotation of the intermediate as a whole (retaining its original conformation).

The report on the high-resolution structure of FucA indicated that the N-terminal portion of the aldolase was homologous to that of the Ru5P epimerase and suggested that Asp76, His95, and His 97 supply three of the expected four metal ligands in the epimerase active site (*17*). We have mutated each of these residues to asparagine and found that the resulting enzymes had significantly reduced epimerase activity. The reduction was largely expressed in lower k_{cat} values as might be expected for the alteration of residues that are not directly involved in substrate binding. In the case of the histidine mutants, this is presumably due to changes in the ligand sphere about the metal ion that alter its ability to serve as an electrophilic catalyst. In the case of the aspartate residue (that is likely displaced from the metal upon substrate binding), it may be due to the modification of a catalytic base. Furthermore, the observation that only the mutant enzymes lose the divalent metal upon dialysis against metal-free buffer or size exclusion chromatography provides evidence that the modified residues serve as metal ligands. Further sequence alignments (Figure 7) carried out in our laboratory suggest that the fourth metal ligand is supplied by His171; however, we have not yet prepared the corresponding mutant.

The similarities between the epimerase and FucA indicate that the enzymes share a common metal binding motif but this does not necessarily mean that they employ related mechanisms. The observation of any aldolase activity with the epimerase would strongly support the notion that the enzyme promotes carbon−carbon bond-breaking/making reactions during catalysis. Studies with the wild-type enzyme did show this activity, but at levels barely above the background rates. If the enediolate and aldehyde are intermediates in this reaction, the enzyme has gone to great lengths to sequester them effectively in its active site. Greater insight into the role of the metal in the epimerization reaction is found in the observation that the H97N mutant does show significant aldolase activity, yet retains substantial epimerase activity. This indicates that the active site geometry has not been dramatically altered and is capable of promoting C−C bond cleavage. The mutation has presumably lowered the rate of this cleavage as well as damaged the fidelity of the enzyme with regards to intermediate sequestration. The fact that glycolaldehyde phos-

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AraD:  MLEDLKRQVLEANLALPKHNLVTLTWGNVSAVDRERGVFVIKPSGVDYS  49
FucA: MERNKLARQIIDTCLEMTRLGLNQGTAGNVSV  RYQDGMLITPTGIPYE  48

AraD: VMTADDMVVVSIETGEVVEGTKKPSSDTPTHRLLYQAFPSIGGIVHIHSR  99
FucA: KLTESHIVFIDGN GKHEEG KLPSSEWRFHMAAYQSRPDANAVVHNHAV  96

AraD: HATIWAQAGQSIPATGTTHADYFYGTIPCTRKMTDAEINGEYEWETGNVI 149
FucA: HCTAVSILNRSIPAIHYMIAAAGGNSIPCAPYAT         FGTR    134

AraD: VETFEKQGIDAAQMPGVLVHSHGPFAWGKNAEDAVHNAIVLEEVA  YMG 197
FucA:  ELSEHVALALKNRKATLLQHHGLIACEVNLEKALWLAHEVEVLAQLYLT 183

AraD: IFC RQLAPQLPDMQQTLLDKHYLRKHGAKAYYGQ               231
FucA: TLAITDPVPVLSDEEIAVVLEKF KTYGLRIEE                 215
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FIGURE 7: Sequence alignments of the *E. coli* L-Ru5P 4-epimerase (AraD) and the *E. coli* L-fuculose-1-phosphate aldolase (FucA) using the program SEQSEE (*35*). Vertical lines denote identical residues. Asterisks denote similar residues. Boxed residues denote metal ligands.

phate is a competitive inhibitor of the mutant enzyme, but not of the wild-type enzyme, could be a result of extremely slow binding in which the form of the enzyme that normally binds the intermediates is kinetically inaccessible. Alternatively, the mutation may simply have altered the active site structure and coordination properties of the metal in a manner that causes the mutant enzyme to bind the aldehyde more tightly. It should be noted that the free aldehyde exists primarily in a hydrated form (*19*) and this may be the species causing the inhibition.

The observed aldol reaction could result from an infrequent protonation of the bound enolate (or deprotonation of dihydroxyacetone in the reverse direction) by an enzymic acid/base residue. A residue that may play this role is the displaced ligand, Asp76, with analogy to the glutamate of FucA. Alternatively, the mutant may simply be releasing the enolate or the zinc−enolate complex into solution. This would demand, however, that the enzyme binds the enolate (or zinc−enolate complex) directly from solution in the reverse reaction.

The "aldol-like" mechanism requires that the enolate be able to add to either face of the bound aldehyde to promote stereochemical inversion. Although the aldolases are usually thought to exhibit high stereoselectivity, this is not always the case. The class II tagatose-1,6-bisphosphate aldolase is able to cleave the C-4 epimer (D-fructose 1,6-bisphosphate) of its natural substrate, endowing it with what is effectively an epimerase activity (*26*). In certain cases, the use of unnatural aldehyde substrates results in modest or even reversed stereoselectivity with the class II aldolases (*27*, *28*). Class I aldolases that lack stereospecificity are also known and include serine hydroxymethylase (*29*−*31*), D-ketohexose-1,6-diphosphate aldolase (*32*), and 4-hydroxy-2-ketoglutarate aldolase (*33*).

The aldolase activity of the mutant supports the notion that the L-Ru5P 4-epimerase reaction involves carbon−carbon bond cleavage and that it is evolutionarily linked to the class II aldolases. One noticeable difference is that FucA and the related aldolases are highly specific for dihydroxyacetone phosphate as a donor substrate (*27*). In the case of the epimerase, dihydroxyacetone must play this role. The three-dimensional structure of FucA shows that the phosphate of the intermediate analogue lies in a pocket lined with neutral residues that participate in hydrogen bonds. These residues are conserved in the epimerase sequence even though the phosphate is lacking. One possible explanation for this is that the residues have been conserved in order to bind to the displaced Asp76 residue and prevent it from protonating the bound enolate. Another difference is that sequence alignments place a threonine residue of the epimerase at the position of the FucA tyrosine 113. This tyrosine is thought to to act as a general base during the C−C bond cleavage step (Figure 6). In the case of the epimerase, it is likely that two spatially distinct bases would be required in order to promote the cleavage of both epimers. The changes required to permit this may have caused significant restructuring in this portion of the active site. A possible candidate that may serve as a catalytic base is the displaced Asp76.

Future studies on the epimerase will include attempts to increase both the aldolase activity and the stereospecificity of the enzyme through mutagenesis. This work will be greatly aided by knowledge of the structure of the epimerase; preliminary crystallographic studies have been reported (*34*). Eventually it may be possible to design an aldolase that will employ dihydroxyacetone as a donor substrate.

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