L-Ribulose 5-Phosphate 4-Epimerase from *Aerobacter aerogenes*

EVIDENCE FOR A ROLE OF DIVALENT METAL IONS IN THE EPIMERIZATION REACTION*

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SUMMARY

L-Ribulose 5-phosphate 4-epimerase from *Aerobacter aerogenes* was inactivated by treatment with EDTA and was reactivated to varying extents by the addition of divalent metal ions in the order: Mn²⁺ > Co²⁺ > Ni²⁺ > Ca²⁺ > Zn²⁺ > Mg²⁺. When optimal Mn²⁺ was present, the homogeneous enzyme had a specific activity of 70 μmoles⁻¹ min⁻¹ mg⁻¹ of protein at 28° and pH 7.2. This value is about five times greater than that displayed by the crystalline enzyme as isolated and assayed in the absence of added metal ion.

In other mechanistic studies, L-ribulose 5-phosphate 4-epimerase was found to be stable to treatment with sodium sulfite and arsenite in the presence of a thiol compound. It was also stable to sodium borohydride in the presence or absence of substrate. Further, a reaction of tetranitromethane with the enzyme-substrate complex could not be detected. Possible mechanisms for L-ribulose 5-phosphate 4-epimerase are discussed.

L-Ribulose 5-phosphate 4-epimerase from *Aerobacter aerogenes*, which catalyzes the interconversion of L-ribulose-5-P and D-xylulose-5-P, is unique among 4-epimerases in that it neither contains nor requires NAD⁺ for catalysis (1). In addition, no evidence was found for the presence of chromophoric substances in the crystalline enzyme, nor did additional cofactors have an influence on the activity (1). In contrast, there is substantial evidence that epimerization by UDP-glucose 4-epimerase involves an oxidation-reduction mechanism utilizing NAD⁺ as the electron acceptor and donor (2-6). Thus, if L-ribulose-5-P 4-epimerase catalyzes a similar oxidation-reduction reaction, another as yet unrecognized electron acceptor must perform the function of NAD⁺.

It has also been observed that there is no kinetic isotope effect when 6-[4-T]ribose-5-phosphate is used as substrate (7). This is in contrast to UDP-glucose 4-epimerase where a normal isotope effect is observed (8). In this connection, it may be significant that the substrates, L-ribulose-5-P and D-xylulose-5-P, are open chain carbohydrates lacking a nucleotide moiety and possessing a carbonyl group two carbon atoms removed from the epimerization site. Undoubtedly, this confers chemical properties on the substrate which are considerably different from those of nucleotide sugars. For these reasons, mechanisms of 4-epimerization of L-ribulose-5-P other than oxidation-reduction have been considered.

The results presented in this paper indicate that L-ribulose-5-P 4-epimerase requires divalent metal ions for activity, and that different divalent metal ions activate to varying extents. In addition, an exploration of a number of mechanistic possibilities involving oxidation-reduction, or carbon-oxygen and carbonium ion formation, gave negative results.

MATERIALS

Chemicals—L-Ribulose-5-P was prepared according to the procedure of Anderson (9). Spectro-pure sulfate salts of Mn²⁺, Mg²⁺, Ni²⁺, Zn²⁺, and Co²⁺ were obtained from Johnson, Matthey and Co., Ltd. Chloride salts of the divalent metal ions were obtained from Mallinckrodt, Inc. Tris base was obtained from Sigma Chemical Co.

Enzymes—L-Ribulose-5-P 4-epimerase from *A. aerogenes* (constitutive for y-arabinose operon, uracil auxotroph designated "u-1-7") and D-xylulose-5-P phosphoketolase from *Leuconostoc mesenteroides* were purified by procedures previously reported (1). A triose phosphate isomerase-α-glycerol phosphate dehydrogenase mixture was obtained from Calbiochem.

METHODS

L-Ribulose-5-P 4-Epimerase Assay The 4-epimerase was assayed by two methods designated as "continuous" and "two-step." The continuous assay involved the conversion of L-ribulose-5-P to α-glycerol phosphate with the concomitant oxidation of NADH utilizing phosphoketolase, triose phosphate isomerase, and α-glycerol phosphate dehydrogenase as coupling enzymes (1). The two-step assay involved the epimerization of L-ribulose-5-P to D-xylulose-5-P in Tris-Hepes' buffer, pH 8.0, in the absence of coupling enzymes. The 4-epimerase was then inactivated by the addition of acetic acid and heating in a boiling water bath for 1 min. The pH was readjusted to 7.0 by the addition of ammonium hydroxide, and an aliquot of the

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The abbreviation used is: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
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Results

Metal Ion Activation

Effect of Metal Chelators on \( L \)-Ribulose-5-P 4-Epimerase Activity

Although \( L \)-ribulose-5-P 4-epimerase does not require added organic or metal cofactors for activity, it is possible that tightly bound metal ions may participate in catalysis. As a first test of this hypothesis, the 4-epimerase was incubated with a series of metal chelators for various incubation times (Fig. 1).

A wide variety of responses was observed including both inhibition and stimulation of activity. Complete inhibition was obtained only with \( 10^{-2} \) M EDTA. Although \( 2 \times 10^{-3} \) M 8-hydroxyquinoline sulfonate did not alter the enzyme activity (not shown), \( 8 \times 10^{-2} \) M 8-hydroxyquinoline sulfonate inhibited 90% of the enzyme activity.

These results suggest that the enzyme, as isolated, may bind a variety of metals including those species which inhibit. Thus, EDTA may inactivate by removing all metal ions, whereas other metal chelators such as \( \sigma \)-phenanthroline may activate by preferentially complexing certain inhibitory metal ions. This possibility is supported by the fact that the stability constants for Mn-EDTA are in the same range as those for the heavy metals. However, the possibility that the metal chelators may nonspecifically activate or inactivate by means other than removal of a metal ion must be considered.

Activity of \( L \)-Ribulose-5-P 4-Epimerase after Removal of EDTA

In order to determine whether inactivation of the \( L \)-ribulose-5-P 4-epimerase by EDTA was due to chelation of metal ions or to binding of EDTA to the enzyme, it was necessary to determine the activity of the treated enzyme after removal of the EDTA. For this purpose, the enzyme was inactivated by incubation with \( 10^{-2} \) M EDTA for 1 hour at room temperature. The EDTA was then separated from the enzyme by passage through a Sephadex G-25 column as described under "Methods." All buffers used to elute the enzyme from the column and used in the enzyme assay were treated to remove trace contaminations of metal ions as described under "Methods."

The enzyme activity recovered from the Sephadex column varied from 0 to 10% of the initial activity. In the experiment cited in Table I no activity remained. In other cases where low activity remained, it was not ascertained how much was attributable to inaccuacies of the two-step assay, incomplete removal of metal, or recontamination by metal during passage.
through Sephadex. At any rate when metal ions were added to the first step of the two-step assay, the activity was greatly increased; the increase depended upon the metal ion species present as detailed below.

**Divalent Metal Ion Specificity**—To determine the activating capability of various metal ions, the 4-epimerase was dialyzed overnight against 0.05 M Tris-Heps buffer, pH 8.0, treated with EDTA, freed of EDTA on Sephadex G-25, and assayed in the presence of varying quantities of specific divalent metal ions as described under "Methods." The metal salts used were freely prepared solutions of spectrographically analyzed metal salts containing less than 5 ppm of most other metals. Under the conditions and concentrations used no precipitation of Mn was observed either in reagents or incubation mixtures. Since, in a preliminary test, the same activity was obtained when the enzyme was previously incubated with $10^{-3}$ M Co++, for 0, 10, or 30 min, the enzyme was not previously incubated with metal ions prior to assaying. Rather, metal ions and substrate were incubated to allow temperature equilibration of the assay mixture, and the reaction was started by the addition of a very small volume of the 4-epimerase.

The results in Table I show that dialysis against Tris-Heps buffer resulted in an activity loss of about 3-fold, presumably due to loss of metal ion. Following treatment with EDTA and passage through Sephadex G-25 no activity remained. After incubation with metals the highest 4-epimerase activity was obtained with Mn++, and this activation occurred at the lowest divalent cation concentration. A 17-fold stimulation over the activity present in the dialyzed preparation was observed. The MnSO$_4$ concentration was almost optimal at $10^{-3}$ M (17-fold versus 18-fold stimulation at $10^{-4}$ M), whereas $10^{-4}$ M NiSO$_4$ and $10^{-3}$ M or higher MgSO$_4$ were required for the maximum activation. Similar activating effects were obtained using Cl$^-$ salts of metal ions, thus indicating that a specific anion is not required.

To show the importance of EDTA treatment in obtaining full activation as described above, the 4-epimerase (90% pure) was dialyzed for 2 hours against 0.05 M barbital buffer, pH 8.0, then incubated for 1 hour with $10^{-3}$ M Co++, Mn++, Zn++, and MgCl$^+$ salts, and assayed in the two-step assay. Contaminating metals were not removed from the glassware or reagents. The results presented in Table II indicate that only Mn++ can stimulate 4-epimerase which had not been treated with EDTA. However, only a 2-fold stimulation was obtained, indicating that Mn++ was not able to activate completely without prior EDTA treatment. These results suggest that various nonactivator divalent cations are bound to the Mn$^{++}$ binding site of the 4-epimerase as isolated. These dissociate slowly even in the presence of Mn++, as reported for phosphoglucomutase by Ray (13).

**Specific Activity of Crystalline L-Ribulose-5-P 4-Epimerase in Presence of Mn$^{++}$**—Since the preceding results strongly indicated that L-ribulose-5-P 4-epimerase was activated by metal ions, Mn$^{++}$ being the most active, it was necessary to redetermine the specific activity of homogeneous Mn$^{++}$ 4-epimerase.

The L-ribulose-5-P 4-epimerase was twice crystallized as previously reported (1). The second crystals were at least 98% pure as determined by polyacrylamide gel electrophoresis. The enzyme solution was then incubated with $10^{-3}$ M EDTA, and the EDTA was removed by passage through a Sephadex G-25 column as before. The metal free enzyme was incubated with $10^{-4}$ M MnSO$_4$ (spectro-pure) and assayed with the two-step assay to which $10^{-3}$ M MnSO$_4$ had been added. A specific activity of 70 ± 7 units per mg of protein was obtained for the pure L-ribulose-5-P 4-epimerase, as compared with 12 units per mg of protein for the crystalline enzyme not so treated (1).

**Mechanistic Studies**

**Effect of Arsenite and Sulfite**—Since the 4-epimerase is devoid of NAD$^+$, it was considered possible that the epimerization process may involve an oxidation-reduction mechanism using enzyme-bound oxidized lipioic acid or cysteine as an electron acceptor. If this were true, either arsenite or sulfite should inhibit the 4-epimerase since dihydrolipoate and cysteine irreversibly react with sulfite.

Accordingly, L-ribulose-5-P 4-epimerase (85% pure) was incubated with $10^{-2}$ M, $10^{-3}$ M, $10^{-4}$ M, and $10^{-5}$ M sodium arsenite or sodium sulfite with and without prior incubation with either $10^{-2}$ M mercaptoethanol or $10^{-5}$ M dithiothreitol to reduce any

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**Table I**

| Conditions | Activity | Activation at metal ion concentration of |
|------------|----------|-----------------------------------------|
|            |          | $10^{-6}$ M | $10^{-5}$ M | $10^{-4}$ M | $10^{-3}$ M |
| Original enzyme | 10.0 | 15 | 17 | 18 |
| After dialysis | 3.4 | 0.4 | 11 | 15 | 18 |
| After EDTA treatment | 0.25 | 0.11 | 0.09 | 0.09 | 0.38 |
| After Sephadex G-25 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

*Expressed as 10-fold activation over the original activity.

+aCl$_3$ was Mallinckrodt analytical reagent grade.

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**Table II**

| Additions | Original activity |
|-----------|------------------|
| CoCl$_2$ | 89 |
| MnCl$_2$ | 205 |
| ZnCl$_2$ | 13 |
| MgCl$_2$ | 78 |

**Divalent metal ion activation of L-ribulose-5-P 4-epimerase**

The 4-epimerase (85% pure) was dialyzed overnight against 0.05 M Tris-Heps buffer, pH 8.0, incubated for 1 hour with $10^{-2}$ M EDTA, and passed through a Sephadex G-25 column (0.6 X 11 cm) which had been washed free of cations with EDTA and equilibrated with 0.05 M Tris-Heps buffer, pH 8.0. Activity was determined in the two-step assay containing spectro-pure metals at the levels indicated in the table. Precautions were taken to remove the contaminating metals from the glassware and the reagents as described under "Methods."

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**Effect of divalent metal ions on L-ribulose-5-P 4-epimerase**

The L-ribulose-5-P 4-epimerase (specific activity, 10.0) was dialyzed for 2 hours against 0.05 M barbital buffer, pH 8.0, and incubated for 1 hour with $10^{-3}$ M Co++, Mn++, Zn++, and Mg$^{++}$ as chloride salts and assayed in the two-step assay in the presence of glycerolglycine buffer, pH 8.0.
DISCUSSION

If L-ribulose-5-P 4-epimerase functions by a mechanism similar to that of UDP-galactose 4-epimerase, there must be a group on the enzyme capable of oxidizing the hydroxyl group on carbon atom 4 of the substrate. However, previous results have indicated that NAD\(^+\) is not present and is not required for enzyme activity (1). In addition, there were not chromophoric groups as are characteristic of many prosthetic groups and coenzymes. Since the 4-epimerase requires only the addition of metal ions for activity, the putative oxidation-reduction mechanism would have to involve only metal ions and the constituent amino acids.

Cystine and lipoic acid have reduction potentials comparable to that of NAD\(^+\) and, thus, could participate in the epimerization reaction. Presumably, the disulfide form would be required. Since sulfhydryl groups are often readily oxidized by air, the oxidized form could predominate in the active site. Although lipoic acid absorbs at 330 nm, its extinction coefficient is too low to have been readily detected in previous spectral studies (1). However, the evidence discussed below tends to eliminate the SH-disulfide oxidation-reduction mechanism. First, borohydride should reduce the disulfide bond with loss in activity. Concerning the possibility that the disulfide may have been quickly reoxidized prior to or during the assay, it has been observed that activity was not lost on incubation with 1 pH mercaptoethanol for 1 hour followed by assay in the presence of 0.05 pH mercaptoethanol; that is, under conditions which are usually sufficient to reduce and maintain the integrity of disulfide groups. Although 50% of the activity was lost on incubation with mercaptoethanol for an additional hour, the activity was not recovered on passage through the Sephadex column, suggesting that the activity loss was due to some phenomenon other than reduction of a disulfide bond at the active site. Second, arsencite should have reacted with the reduced disulfide and caused inactivation, and third, sulfite should have reacted with the disulfide group to form the stable sulfur-sulfonated derivative.

No data have been obtained to indicate that an indolenine intermediate derived from tryptophan functions as the electron acceptor in the 4-epimerization as reported by Schellenberg for alcohol dehydrogenase (17).

Consequently, the previous results (1) and those presented herein are not consistent with the electron-acceptor being NAD\(^+\), coenzyme A, lipoic acid, cystine, or an oxidized indolenine derivative of tryptophan.

In the absence of any substantial evidence for participation of an oxidizing group on the enzyme, it is necessary to consider other mechanisms for epimerization such as: (a) a \(\text{S}_{\text{N}}\text{Z} \) (Walden) inversion at C-4; (b) carbon-carbon bond cleavage and re-forma-

![Fig. 2. Proposed dealdolization-aldolization mechanism for L-ribulose-5-P 4-epimerase. \(M\) depicts a divalent metal ion in the active site and \(R\) indicates a base function in the active site.](image)

![Fig. 3. Proposed dehydration-rehydration mechanism for L-ribulose-5-P 4-epimerase.](image)
tion between C-3 and C-4; and (c) dehydration-rehydration at the same location. An Sn2 inversion is not considered probable since McDonough and Wood (18) previously reported no isotope incorporation into L-ribulose-5-P and D-xylulose-5-P when the epimerization was conducted in H218O.

The mechanism proposed in Fig. 2 depicts a metal ion-assisted aldololytic cleavage in a manner strictly analogous to the Schiff base mechanism (19, 20). The metal ion chelates with the carbonyl group (and possibly a hydroxyl group) and serves as an electrophile. A base on the enzyme surface acting as a nucleophile impinges on the C-4 hydroxyl group. In the ensuing rearrangement of electrons, C-3–C-4 cleavage occurs and C-3 takes on carbanion character. In completion of the rearrangement, a metal-oxygen bond is formed at C-2 along with a double bond at C-2–C-3. These intermediates would be analogous to the enamine and ketamine intermediates in the Schiff base mechanism. In this scheme, it is not intended to favor a discrete as opposed to a concerted mechanism.

If the characteristics of the epimerase are such that (a) the carbanion of dihydroxyacetone from carbon atoms 1, 2, and 3 cannot be discharged by a proton as in the case of transaldolase (21, 22) and (b) the glycolaldehyde phosphate moiety does not readily dissociate, it would follow that the carbon-carbon bond would immediately re-form. If there were a high probability that the bond between C-4 and the hydroxyl group would reform cis or trans in this process, epimerization would be observed. In such a mechanism, L-ribulose 5-phosphate 4-epimerase would, in fact, be a special kind of transaldolase to the extent that the enol of dihydroxyacetone does not dissociate. It would differ in that the other fragment, glycolaldehyde phosphate, is bound and precludes other aldehydes functioning in dihydroxyacetone transfer reactions.

If this hypothesis is correct, the carbanion intermediate would be very short lived because the proximity of glycolaldehyde phosphate would favor condensation. In this connection, the reaction of tetranitromethane in fructose diphosphate aldolase and transaldolase-catalyzed reactions may be observable because dissociation of glycoaldehyde 3-phosphate allows access to the carbanion intermediates.

An alternative mechanism would be dehydration-rehydration by acid-base catalysis as shown in Fig. 3. The first step would involve a base-catalyzed removal of the proton on C-3 leaving either a carbanion at C-3 or a double bond between C-2 and C-3. The presence of the metal ion in the active site would facilitate removal of the C-4 hydroxyl group in a manner proposed for enolase (23) and aconitase (24). In the reverse reaction, the return of the hydroxyl group would have high probabilities of occupying either bonding position.

Since McDonough and Wood (18) were unable to find an incorporation of T or 18O from the medium into the substrate, the limitation on this mechanism is that the same proton and hydroxyl groups removed must be involved in the reverse reaction. In this connection, Rose (25) has produced evidence with phosphoglucoisomerase that the intramolecular transfer of a proton can be faster than equilibration with the surrounding medium. Thus, it is conceivable that the proton removed (from C-3) becomes bound to the enzyme and is not free to diffuse into the medium. The hydroxyl group would probably be chelated by the metal ion in a position where it would be readily accessible to both sides of C-4 but not to the medium.

Neither dealdolization-aldolization nor dehydration-redehydration can participate in the mechanism of the other carbohydrate 4-epimerases since the substrates of all other 4-epimerases do not possess a free carbanion group which could participate in the mechanism.

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