Mechanism of enzymic isomerization and epimerization of D-erythrose 4-phosphate

Saburo HOSOMI,* Noriko NAKAI, Junko KOGITA, Tomoyuki TERADA and Tadashi MIZOGUCHI

Laboratory of Biochemistry, Faculty of Pharmaceutical Sciences, Osaka University, 1–6 Yamada-oka, Suita, Osaka 565, Japan

The mechanism of the enzymic isomerization and epimerization of D-erythrose 4-phosphate (Ery4P) by an enzyme preparation from bovine liver was investigated with the use of $^2\text{H}_2\text{O}$. The incorporation of ^2H was quantitatively determined by a procedure using gas chromatography–mass spectrometry. About one atom of ^2H was incorporated per molecule of the enzymic epimerization reaction product of Ery4P (D-threose 4-phosphate) or that of D-ribulose 5-phosphate. Computer simulation of the Ery4P isomerization reaction indicated that the ^2H of $^2\text{H}_2\text{O}$ was not directly incorporated into the enzymic reaction product (D-erythrulose 4-phosphate). Instead, intramolecular transfer of hydrogen atoms had occurred.

INTRODUCTION

D-Erythrose-4-phosphate isomerase is a bifunctional enzyme that catalyses the isomerization and epimerization of D-erythrose 4-phosphate (Ery4P) to D-erythrulose 4-phosphate (Eru4P) and D-threose 4-phosphate (Thr4P) in bovine liver (Ohashi *et al.*, 1984; Terada *et al.*, 1985). In the course of our studies we have found that Ery4P isomerase is similar to D-ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) (Wood, 1979). Ery4P isomerase might play an important role in the formation of urinary tetritols (especially D-threitol) (Pitkänen, 1972; Pfaffenberger *et al.*, 1975) in co-operation with D-erythrulose reductase (EC 1.1.1.162), which catalyses the reduction of D-erythrulose to D-threitol (Uehara & Hosomi, 1982). In addition, this enzyme can catalyse epimerization at C-2 of tetrose 4-phosphates. Though a number of enzymic epimerizations have been reported for monosaccharides, with the exception of cellobiose 2-epimerase (EC 5.1.3.11) from *Ruminococcus albus* (Amein & Leatherwood, 1969) no other enzyme catalysing such a reaction has been reported. Ery4P isomerase cannot catalyse the isomerization of D-ribulose 5-phosphate, and hence it was our interest to study the catalytic action of enzyme in the isomerization of Ery4P (or Thr4P) to Eru4P.

In the present investigation we have tried to determine whether the elements of water are incorporated into the isomerization and epimerization reaction products of Ery4P by g.c.–m.s. and computer simulation techniques.

MATERIALS AND METHODS

Materials

Homogeneous Ery4P isomerase was prepared from bovine liver by the method previously described (Ohashi *et al.*, 1984; Terada *et al.*, 1985). Preparations were stored at 4 °C in 10 mM-Bis-Tris/HCl buffer, pH 7.0. Under these conditions the enzyme may be kept for about 6 months without detectable loss of activity. Potato acid phosphatase (EC 3.1.3.2) was from Boehringer Mannheim. Trifluoroacetic anhydride and $^2\text{H}_2\text{O}$

were obtained from Nakarai Chemicals. Ery4P and Thr4P were prepared from D-glucose 6-phosphate and D-galactose 6-phosphate respectively by a slight modification of the method of Simpson *et al.* (1966). D-Ribulose 5-phosphate was purchased from Sigma Chemical Co. All other chemicals were of the highest grade commercially available.

Assay of Ery4P isomerase activity

The isomerase and epimerase activities of the Ery4P isomerase were measured by using g.l.c. as previously described (Ohashi *et al.*, 1984). The reaction mixture in water or in 97.5% $^2\text{H}_2\text{O}$ contained 2.85 mM-Ery4P, 87 mM-Bis-Tris/HCl buffer, pH 7.0, or pD 7.0, and 1.9 units of enzyme in a final volume of 1.43 ml. G.l.c. was performed on a Shimadzu GC-4CPTF gas chromatograph at 135 °C with a glass column (4 mm × 2000 mm) packed with 2% OV-105 on Gas-Chrom P.

Determination of the incorporation of ^2H by g.c.–m.s.

The reaction mixture in 91.1% $^2\text{H}_2\text{O}$ contained 14.3 mM-Ery4P, 87 mM-Bis-Tris/HCl buffer, pD 7.0, and 9.3 units of enzyme in a final volume of 1.5 ml. After incubation for appropriate times at 30 °C, the reaction was terminated by adding 0.7 ml of 10% (v/v) acetic acid. The pH was adjusted to 5 with 5 M-NaOH and dephosphorylation was accomplished by incubating the sample solution in the presence of 2 mg of potato acid phosphatase at 37 °C for 2 h. The sample was then passed over coupled ion-exchange columns of Amberlite CG 120 (H^+ form) (0.8 cm × 8 cm) and Amberlite IRA-47 (OH^- form) (0.8 cm × 8 cm). The resulting tetroses were applied on to a Dowex 1 X1 (borate form) column (0.6 cm × 16 cm) equilibrated with 16.7 mM-boric acid. D-Erythrulose was completely eluted with saturated boric acid solution, and then two aldotetroses (D-erythrose and D-threose) were eluted with the saturated boric acid solution containing 0.5 M-ammonium borate. After the removal of boric acid and ammonium borate, the samples were reduced by NaBH_4 . The incorporation of ^2H was analysed by g.c.–m.s. techniques after

Abbreviations used: Ery4P, D-erythrose 4-phosphate; Eru4P, D-erythrulose 4-phosphate; Thr4P, D-threose 4-phosphate; g.c.–m.s., gas chromatography–mass spectrometry.

* To whom correspondence should be addressed.

trifluoroacetylation and quantitatively determined by measuring the relative intensities of m/z 379 and 380 of sugar tetritol trifluoroacetate, as described previously (Ohashi *et al.*, 1984). G.c.-m.s. was performed on a Jasco JMA-2000 gas chromatography-mass spectrometer, coupled with a Jasco JMS-D-300 computer, at 20 eV with a 2 m glass column packed with 2% OV-105 on Gas-Chrom P (ionization current 300 μ A; source temperature 200 $^{\circ}$ C).

Simulation method

Analyses of the enzymic reactions of Ery4P by computer simulation were carried out on an NEC ACOS-1000 computer, located at the Computer Center of Osaka University. Differential equations representing the rate of change of reactant concentration were integrated by Gear's (1971*a,b*) method with the use of FORTRAN 66 with 18.9 decimal digit accuracy. Our initial analysis assumes that the enzymic reaction obeys classical Michaelis-Menten kinetics, and that the substrates combine reversibly with the enzyme.

RESULTS AND DISCUSSION

Quantitative determination of the incorporation of ^2H into the products of the Ery4P isomerase reaction by using g.c.-m.s.

In order to ascertain whether the elements of water participate in the reaction or remain stably bound to the substrate molecule, the enzymic reactions of Ery4P were performed in the presence of $^2\text{H}_2\text{O}$. If ^2H incorporation occurs during the enzymic reaction, ^2H should attach to C-1 of ketotetrose and C-2 of aldotetrose, as shown in Scheme 1. To determine the degree of ^2H incorporation into tetrose 4-phosphates, aldotetrose and ketotetrose were separated by using a column of Dowex 1 X1, after the dephosphorylation of the sugar phosphates, as described in the Materials and methods section. Then the sugars were reduced by NaBH_4 to the corresponding

Table 1. Incorporation of ^2H from $^2\text{H}_2\text{O}$ into tetrose 4-phosphates

Details of the enzymic reaction conditions and the procedure of tetrose 4-phosphate analysis by g.c.-m.s. are given in the Materials and methods section.

Substrate	Enzyme (units)	Time (min)	Incorporation of ^2H (%)
Ery4P	9.3	20	9.4
	9.3	60	21.3
	9.3	120	37.7
	0	0	0.0
	0	20	1.6
	0	120	2.3
Thr4P	9.3	20	86.7
	9.3	60	88.1
	9.3	120	93.2
	0	20	17.0
	0	120	4.9
	0	120	4.9
Eru4P	9.3	20	31.5
	9.3	60	54.0
	9.3	120	58.3
	0	20	4.5
	0	120	7.3
	0	120	7.3

tetritols (erythritol and threitol), and were analysed by g.c.-m.s. By noting the appropriate mass shifts upon g.c.-m.s., the relative percentages of deuterated aldotetroses and deuterated ketotetrose could be determined from the relative intensities of m/z 379 and 380 of each of the C_3 fragments (C-1-C-3 and C-2-C-4) of the corresponding tetritol derivatives (Scheme 1). The fundamental analytical theory was described previously (Ohashi *et al.*, 1984). The results for the incorporation of ^2H into aldotetroses and ketotetrose are shown in Table 1. The conversion of Ery4P into Thr4P is accompanied by 90% exchange of ^1H with ^2H of $^2\text{H}_2\text{O}$, and the Thr4P contains one ^2H atom per molecule of the sugar on C-2 at any time during the reaction. The degree of

Scheme 1. Reactions of 'Ery4P isomerase' with Ery4P, and mass-spectral fragmentation pattern of trifluoroacetate derivatives of deuterated tetritols obtained by NaBH_4 reduction of aldotetrose (A) and ketotetrose (B)

R = COCF_3 . Abbreviation: TFAA, trifluoroacetic anhydride.

Table 2. Incorporation of ^2H from $^2\text{H}_2\text{O}$ into pentulose 5-phosphates

The reaction mixture in 99.2% $^2\text{H}_2\text{O}$ contained 2.67 mM-D-ribulose 5-phosphate, 99.3 mM-Bistris/HCl buffer, pH 7.0, and 0.93 unit of enzyme in a final volume of 1.5 ml. After incubation for 60 min at 30 °C, the reaction mixture was reduced with 1.5 ml of 1% NaBH_4 for 1 h. After dephosphorylation and trifluoroacetylation, the resulting pentitols were analysed by g.l.c. and g.c.-m.s. as described in the Materials and methods section.

Substrate	Enzyme (units)	Time (min)	Incorporation of ^2H (%)
D-Ribulose 5-phosphate	0.93	60	60.5
	0	0	0.9
	0	60	5.5
D-Xylulose 5-phosphate	0.93	60	95.1
	0	0	0.0
	0	60	0.0

incorporation of ^2H into another product (Eru4P) was very low at the beginning of the enzymic reaction, gradually increased and reached a maximum of 60% of the value calculated for the stoichiometric replacement of one ^1H atom of the original aldotetrose 4-phosphate by a ^2H atom of $^2\text{H}_2\text{O}$ after 120 min. Therefore, in the case of the isomerization reaction of Ery4P, the mode of the incorporation of ^2H might be different from that of epimerization.

On the other hand, when D-ribulose 5-phosphate was used as a substrate, ^2H was incorporated into approx. 95% of the enzymic epimerization product (D-xylulose 5-phosphate) (Table 2). These findings are in agreement with previous observations (McDonough & Wood, 1961; Davis *et al.*, 1972). Therefore it seems that the epimerization reactions of Ery4P and D-ribulose 5-phosphate by this enzyme proceed by the same reaction mechanism, and cause the exchange of ^1H at C-2 (tetrose phosphates) or C-3 (pentulose phosphates) with ^2H of $^2\text{H}_2\text{O}$.

Several studies have shown that $^2\text{H}_2\text{O}$ inhibits certain enzymes, and this could be related to isotope effects on allosteric proteins. However, in the present studies we found that the rate of the Ery4P isomerase activity in $^2\text{H}_2\text{O}$ was not different from that in $^1\text{H}_2\text{O}$ to any detectable degree (results not shown). Therefore the process of a ^2H exchange might not be the rate-limiting factor.

One of the plausible mechanisms for the isomerization reaction of Ery4P is the removal of the hydrogen (H-2) from C-2 of tetrose 4-phosphates and a transfer of the hydrogen to C-1 of the sugar phosphates to give Eru4P (intramolecular hydrogen transfer). Since this enzyme also catalyses an exchange of the hydrogen at C-2 of the substrate with the solvent, one also has to consider the possibility that only part of the hydrogen will exchange with each catalytic cycle and the remainder will be transferred as in the case of the phosphoglucose isomerase reaction (Rose & O'Connell, 1961).

Simulation of enzymic isomerization and epimerization of Ery4P

As previously stated, this enzyme can catalyse both the isomerization of Ery4P and Thr4P to a common product, Eru4P (scheme I), and we also found in this

experiment that the ^2H of $^2\text{H}_2\text{O}$ was incorporated at C-2 of Thr4P by the enzymic epimerization of Ery4P:

Scheme I

Therefore, when we consider the incorporation of ^2H into Eru4P with Ery4P as the initial substrate, we have to distinguish between the isomerization reaction of Ery4P and that of deuterated Thr4P. To study the time-dependent incorporation of ^2H into Eru4P we used computer simulation. By using a general FORTRAN program designed to estimate the solution of non-linear simultaneous differential equations (Gear, 1971a,b), the coefficients of complex rate equations could be determined. On the basis of our experimental evidence obtained previously (Terada *et al.*, 1985), the enzymic reaction of Ery4P can be described in terms of the mechanism illustrated in scheme II:

Scheme II

In this scheme E and EX represent enzyme molecule and enzyme-substrate reaction intermediate respectively. The reaction velocity constants k_{+2} and k_{+3} were almost the same because the initial rate of formation of Eru4P was equal to that of Thr4P, as described in a previous paper (Terada *et al.*, 1985). The velocity constant was calculated from the following equation:

$$k_{+3} \text{ (or } k_{+2}) = \frac{V_{\max.}(\text{Ery4P})}{[\text{E}]_0}$$

by using the Michaelis constant $[K_m(\text{Ery4P}) = 4.11 \times 10^{-3} \text{ M}]$ and $V_{\max.}$ [$V_{\max.}(\text{Ery4P}) = 2.18 \times 10^{-5} \text{ M} \cdot \text{min}^{-1}$] and the initial enzyme concentration $[\text{E}]_0 = 107.7 \text{ nM}$. k_{+4} and k_{-4} were determined by measuring the reaction rate of the non-enzymic isomerization of Thr4P. To test whether the scheme is compatible with our experimental results, we have used computer models to simulate the epimerization and isomerization of Ery4P. This is done by choosing a set of rate constants, calculating the concentration changes of metabolites with the aid of a computer, and then manipulating the rate constants until the computed concentrations match the data. Simulations of the enzymic reactions were performed with initial concentrations $[\text{E}]_0 = 107.7 \text{ nM}$, $[\text{Ery4P}]_0$ (or $[\text{Thr4P}]_0$) = 2.6667 mM and $[\text{Eru4P}]_0 = [\text{EX}]_0 = 0$ for each set of rate constants. The best-fit values for the rate constants are $k_{+1} = 3.416 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$, $k_{-1} = 5.22 \times 10^2 \text{ min}^{-1}$, $k_{+2} = 4.41 \times 10^2 \text{ min}^{-1}$, $k_{-2} = 3.416 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$, $k_{+3} = 4.41 \times 10^2 \text{ min}^{-1}$, $k_{-3} = 4.0 \times 10^{-3} \text{ M}^{-1} \cdot \text{min}^{-1}$, $k_{+4} = 4.066 \times 10^{-3} \text{ min}^{-1}$ and $k_{-4} = 2.0 \times 10^{-4} \text{ min}^{-1}$. The good correspondence between the experi-

Fig. 1. Computed time course of enzymic reactions for Scheme II

In the simulations, the initial concentrations of substrate, Ery4P (a) or Thr4P (b), and enzyme were set at 2.6667 mM and 107.7 nM respectively. The values of the rate constants, k_{+1} – k_{-4} , are provided in the text. The computed amounts, expressed in μmol of each tetrose 4-phosphate, are drawn in continuous lines as a function of reaction time. The experimental data obtained previously (Terada *et al.*, 1985) are also presented in the Figure as points: ●, Ery4P; ▲, Thr4P; ○, Eru4P. The broken line indicates the computed time course of the conversion of Thr4P, which is epimerized from Ery4P, into Eru4P.

mental data points of Fig. 1 and the simulated progress curves supports the validity of our theoretical model.

Alternative schemes were also examined. Schemes III and IV show that Ery4P is primarily epimerized to Thr4P by this enzyme and then isomerized enzymically (scheme III) or non-enzymically (scheme IV) to Eru4P:

Scheme III

Scheme IV

However, the models of schemes III and IV do not fit all the experimental results. Scheme II predicts the time course of the conversion of Thr4P into Eru4P, as shown in Fig. 1(a). The percentage of Eru4P formed from Thr4P compared with the total amounts of the Eru4P produced is initially very low, but increases to about 50% of the total at 120 min. The results of the simulation are in agreement with the mode of incorporation of ^2H from $^2\text{H}_2\text{O}$ into Eru4P as described above. Therefore we suggest that the ^2H of $^2\text{H}_2\text{O}$ does not participate in the isomerization reaction of tetrose 4-phosphates. The

reaction seems to proceed directly by the intramolecular transfer of the hydrogen (H-2 of Ery4P) or the deuterium (^2H -2 of Thr4P) to C-1 of Eru4P. In the case of non-enzymic conversion of Ery4P into Eru4P no incorporation of ^2H has occurred (Table 1). These observations are very unusual because other aldose-ketose isomerase systems exhibit exchange of carbon-bound hydrogen of the substrate with deuterium of $^2\text{H}_2\text{O}$.

On the basis of our experimental results, it is clear that both H-3 of D-ribulose 5-phosphate and H-2 of tetrose 4-phosphate are the same distance away from the phosphate group. We have also found that the phosphate group of the substrate may play an important role in the binding of the substrate to the enzyme (Terada *et al.*, 1985). From the above-mentioned results, we predict that at the enzyme's active site a basic group is positioned in a definite geometry with respect to the phosphate-binding site, and the initial step apparently is the attack by a basic group of the enzyme on the hydrogen at C-2 of tetrose 4-phosphate, as shown in Scheme 2. This would result in the formation of a carbanion-type intermediate having a near-planar configuration at C-2 of tetrose 4-phosphate (Scheme 2b). In the isomerization reaction of Ery4P, we were not able to detect incorporation of deuterium from the medium into the substrate. Therefore, it is conceivable that the proton removed from C-2 becomes bound to the enzyme and is not free to diffuse into the medium, and can be transferred to C-1 of Eru4P. On the other hand, if at the same time a deuterium atom from either the medium or an exchangeable site on the enzyme could enter at the remote face from the leaving hydrogen atom, the result would be an inverted-exchange product (the enzymic epimerization reaction of tetrose 4-phosphate).

Scheme 2. Possible mechanism of the enzymic isomerization and epimerization of Ery4P

These data also can be interpreted in terms of the 'enediol' mechanism usually proposed for aldose-ketose isomerism, as well as a mechanism involving such

a carbanion-type intermediate that has been proposed for a unique cellobiose 2-epimerase (Amein & Leatherwood, 1969). However, we have previously observed that 3-phosphoglycerate, which is assumed to be the transition-state analogue of the cis-1,2-enediolate intermediate in the Ery4P isomerase reaction, was not an inhibitor for Ery4P isomerase (Terada *et al.*, 1985). In addition, we know that the vicinal hydroxy group of the enediolate intermediate has a hydrogen atom that is easily exchangeable with the deuterium of $^2\text{H}_2\text{O}$. As a result of the enzymic isomerization reaction of tetrose 4-phosphate, the deuterium might not be incorporated directly into the Eru4P. From these considerations, we suppose that the carbanion-type intermediate model is more appropriate than the enediolate-type intermediate model for the mechanism of the enzymic isomerization and epimerization of Ery4P.

REFERENCES

- Amein, M. & Leatherwood, J. M. (1969) *Biochem. Biophys. Res. Commun.* **36**, 223-227
- Davis, L., Lee, N. & Glaser, L. (1972) *J. Biol. Chem.* **247**, 5862-5866
- Gear, C. W. (1971a) *Commun. ACM* **14**, 176-179
- Gear, C. W. (1971b) *Numerical Initial Value Programs in Ordinary Differential Equations*, Prentice-Hall, Englewood Cliffs
- McDonough, M. W. & Wood, W. A. (1961) *J. Biol. Chem.* **236**, 1220-1224
- Ohashi, K., Terada, T., Kohno, T., Hosomi, S., Mizoguchi, T. & Uehara, K. (1984) *Eur. J. Biochem.* **142**, 347-353
- Pfaffenberger, C. D., Sazfranek, J., Horning, M. G. & Horning, E. C. (1975) *Anal. Biochem.* **63**, 501-512
- Pitkänen, E. (1972) *Clin. Chim. Acta* **38**, 221-230
- Rose, I. A. & O'Connell, E. L. (1961) *J. Biol. Chem.* **236**, 3086-3092
- Simpson, F. J., Perlin, A. S. & Sieben, A. S. (1966) *Methods Enzymol.* **9**, 35-38
- Terada, T., Mukae, H., Ohashi, K., Hosomi, S., Mizoguchi, T. & Uehara, K. (1985) *Eur. J. Biochem.* **148**, 345-351
- Uehara, K. & Hosomi, S. (1982) *Methods Enzymol.* **89**, 232-237
- Wood, T. (1979) *Biochim. Biophys. Acta* **570**, 352-362

Received 3 February 1986/2 June 1986; accepted 14 July 1986