Biochimica et Biophysica Acta, 293 (1973) 534–541

BBA 66825

PARTIAL PURIFICATION AND KINETIC PROPERTIES OF RIBOSE-5-PHOSPHATE KETOL-ISOMERASE AND RIBULOSE-5-PHOSPHATE 3-EPIMERASE FROM VARIOUS SOURCES

MAUREEN E. KIELY, ALLAN L. STUART AND TERRY WOOD
Department of Biochemistry, McGill University, Montreal, Quebec (Canada)

(Received September 8th, 1972)

SUMMARY

D-Ribose-5-phosphate ketol-isomerase (EC 5.3.1.6) was partially purified from calf spleen, calf liver and ox muscle. D-Ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) was prepared from calf liver. Mixtures of the isomerase and epimerase enzymes were separated by preparative electrophoresis in a 'Pevikon' block. Initial velocities of the isomerase and epimerase reactions were measured at 290 nm, at 340 nm, and by the cysteine–carbazole method. The K_m values for isomerase from spinach, spleen, muscle, and liver and for the epimerase from yeast and liver were determined. For spinach isomerase the K_m values and the maximal velocities of both the forward and the reverse directions were measured.

INTRODUCTION

Most of the K_m values for ribose 5-phosphate of D-ribose-5-phosphate ketol-isomerase (EC 5.3.1.6) have been measured on the enzyme from plant or microbial sources. In the case of the purified spinach enzyme, the two values that have been determined, 0.46 mM (ref. 1) and 4.6 mM (ref. 2), differ by a factor of 10. In *Escherichia coli*, two ribose 5-phosphate isomerase enzymes have been reported with K_m values of 0.19 mM and 2.2 mM, and a value as high as 5.4 mM was obtained after heat treatment to destroy the low K_m enzyme[3]. In the same microorganism, other workers have reported K_m values of 0.95 mM and 6.2 mM for the two isomerases[4]. The significance of these figures is enhanced by recent suggestions that the low K_m enzyme is involved in the catabolism of ribose-5-*P* while the other is concerned with its biosynthesis[3,4]. In the case of D-ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) there is a paucity of kinetic data and only the K_m values of the enzyme from *Lactobacillus pentosus* could be found in the literature[5]. Because enzyme activities in a tissue are usually determined at saturating concentrations of substrate, the Michaelis constant must be known before the rates can be calculated at which an enzyme is

likely to operate at *in vivo* substrate concentrations. In this paper we present K_m values that we have determined for these two enzymes prepared from mammalian tissues, and these may be used for the calculation of rates *in vivo*. In addition, measurements have been made on the enzymes from spinach and yeast.

MATERIALS

D-Ribose-5-phosphate, D-6-phosphogluconic acid sodium salt, D-ribose-5-phosphate ketol-isomerase (Type I, from spinach, 75 units/mg) and D-ribulose-5-phosphate 3-epimerase (Type III, from baker's yeast, 165 units/mg) were obtained from Sigma. D-Ribulose-5-phosphate was prepared from D-6-phosphogluconic acid[6,7]. 'Oxoid' cellulose acetate strips and 'Pevikon' (a vinyl chloride–vinyl acetate suspension copolymer) were from Shandon Ltd, (65, Pound Lane, London, N.W.10). Transketolase was prepared from *Candida utilis* as described previously[8]. Calf spleen, calf liver and ox-muscle were fresh from the abattoir by courtesy of Canada Packers Ltd.

METHODS

Analytical procedures

Ribulose-5-phosphate was measured either colorimetrically by the cysteine–carbazole method[9] or enzymically in the presence of an excess of epimerase and transketolase as described by Racker[10]. Both isomerase and epimerase activities were measured at 37 °C and 10 mM ribose-5-*P* by the spectrophotometric method at 290 nm described previously[11]. Protein was measured by the biuret method[12]. All enzyme activities are expressed in μmoles/min per mg protein.

Electrophoresis

Analytical electrophoresis was carried out on 'Oxoid' cellulose acetate strips in the cold room in Tris–glycine buffer, pH 8.5, ionic strength 0.22 (ref. 13). Preparative electrophoresis in a Pevikon block[14,15] was performed in the same buffer in the cold room and zones were removed and extracted with 100 mM triethanolamine–HCl buffer, pH 6.8.

Kinetic measurements

All determinations were made at pH 7.4 and either 25 or 37 °C. Measurements of epimerase rates at 290 nm (ref. 11) were carried out in 50 mM triethanolamine–HCl buffer, in the presence of equilibrium concentrations of ribulose-5-*P* generated by the addition of 2.2 units of isomerase to various amounts of ribose-5-*P*. It was confirmed in independent experiments using the 340 nm method[11] that concentrations of ribose-5-*P* up to 20 mM did not inhibit liver epimerase. Measurements of isomerase activity were carried out in the same manner without the prior addition of excess isomerase. Initial rates were linear over the first 6–10 min. In the case of spinach isomerase, small aliquots of the reaction mixture were withdrawn from the spectrophotometer cuvette at measured intervals, frozen immediately in tubes pre-cooled in solid CO_2–acetone, and later reacted with cysteine–carbazole to measure the ribulose-5-*P* formed. The reverse reaction of spinach isomerase was measured by incubating

mixtures containing 200 mM triethanolamine–HCl buffer and various amounts of ribulose-5-*P*. Suitable size aliquots were withdrawn before the addition of enzyme, and at intervals thereafter, and frozen immediately in solid CO_2–acetone. The samples were later reacted with cysteine–carbazole and the rate of ribulose-5-*P* disappearance was determined.

For measurements of epimerase rates at 340 nm, 2.0 ml of solution contained 50 mM glycylglycine buffer, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM thiamine pyrophosphate, 0.13 mM NADH, 0.3 units of glycerol-1-phosphate dehydrogenase (EC 1.1.1.8), 1.6 units triosephosphate isomerase (EC 5.3.1.1), 0.2 units of transketolase (EC 2.2.1.1) and a mixture of ribose-5-*P* and ribulose-5-*P* or ribose-5-*P* and a ribose-5-*P*/ribulose-5-*P* equilibrium mixture to give a fixed concentration of 15 mM ribose-5-*P* and variable concentrations of ribulose-5-*P*. The (small) initial rate due to traces of isomerase and epimerase in the auxiliary enzymes was measured before the epimerase was added. Initial velocities were calculated from the linear fall in absorbance at 340 nm minus the rate before the addition of epimerase. Measurements of isomerase activity were carried out in the same way, omitting ribulose-5-*P*, using different concentrations of ribose-5-*P* and a transketolase preparation containing 0.1 unit of epimerase.

Purification of isomerase from ox muscle

The muscle (75 g) was minced and extracted at 2 °C with 1 ml/g of 4 mM EDTA *plus* 30 mM KOH, pH 12. After squeezing through cheese-cloth, the residue was extracted a second time (0.5 ml/g). The combined extracts were adjusted to pH 7.5 and frozen. After 2 or 3 days, the extract was thawed, clarified by centrifugation at 11 000 × *g* for 10 min and $(NH_4)_2SO_4$ added to 60% saturation. After stirring for 30 min at 2 °C, the precipitate was brought down at 11 000 × *g* for 10 min. The precipitate was redissolved in 20 mM triethanolamine–HCl buffer, pH 7.4 and dialysed for 18 h at 2 °C against 100 volumes of 5 mM triethanolamine·HCl *plus* 1 mM EDTA, pH 7.4. Portions of 35 ml of the dialysed extract were heated rapidly at 80 °C and then held for 5 min in a bath at 60 °C before cooling in ice. The denatured protein was removed by centrifugation at 2700 × *g* for 10 min and the solution concentration to 5 mg/ml by ultrafiltration in the cold through a Diaflo PM-30 membrane. Further purification was obtained by electrophoresis in a 'Pevikon' block.

Purification of isomerase from calf spleen

An acetone powder of calf spleen was prepared, extracted, and the protein precipitated by $(NH_4)_2SO_4$ was fractionated with acetone as described by Ashwell and Hickman[16]. The 0–45% (v/v) acetone fraction contained the bulk of the isomerase and was free from epimerase.

Purification of epimerase from calf liver

Frozen calf liver (100 g) was chopped, thawed, and extracted at 2 °C with 200 ml ice-cold 50 mM triethanolamine buffer, pH 7.4. After centrifuging 30 min at 14 000 × *g*, the supernatant was stored frozen at −20 °C. After 2 days or longer, it was thawed, clarified by centrifuging and $(NH_4)_2SO_4$ added to 95% saturation at 0 °C. After 30 min stirring at 2 °C, the precipitate was centrifuged down for 10 min at 12 000 × *g* and it redissolved completely in 20 mM triethanolamine buffer, pH 7.4.

The solution was dialysed overnight at 2 °C against 2 l of 5 mM triethanolamine *plus* 1 mM EDTA, pH 7.4. The pH was adjusted to 7.4 and 10 ml portions of the dialysed solution were brought to 60 °C in a boiling water-bath and held for 5 min at 60 °C. The solution was cooled in ice-water and the heat-denatured protein centrifuged off. Samples were concentrated by ultrafiltration through a Diaflo PM-30 membrane prior to preparative electrophoresis on 'Pevikon'.

Purification of isomerase from calf liver

Following the dialysis step in the above procedure, the pH of the solution was adjusted to 6.0. The subsequent heat treatment resulted in complete destruction of the epimerase activity without affecting the isomerase activity. Further purification was achieved by preparative electrophoresis on 'Pevikon'.

TABLE I

PURIFICATION OF OX-MUSCLE ISOMERASE

The purification procedure is described under Methods.

Step	*Total units*	*units/mg*
Initial extract	895	0.7
0–60% $(NH_4)_2SO_4$ fraction	280	1.4
60% supernatant	195	0.18
Heat treated 0–60% fraction	238	17
Pevikon eluate	156	49*

* This fraction was free of epimerase activity.

TABLE II

PURIFICATION OF ISOMERASE FROM A CALF SPLEEN ACETONE POWDER

The purification procedure is described under Methods.

	Total units	*units/mg*
Initial extract	3920	1.3
0–50% $(NH_4)_2SO_4$ fraction	2680	4.0
50% supernatant	448	0.3
Heat treated 0–50% fraction	2860	11.6
0–45% acetone	1310	63
45–70% acetone	112	1.9

RESULTS

A preparation of isomerase was obtained from ox muscle which after electrophoresis was free of epimerase and had a specific activity of 49 units/mg protein (Table I). By following the procedure of Ashwell and Hickman[16] a spleen isomerase preparation of specific activity 63 units/mg was obtained by acetone fractionation (Table II). This preparation was free from epimerase, which only precipitated at higher concentrations of acetone. In the procedure for calf liver, the pH could be adjusted prior to the heat treatment step according to whether it was desired to destroy the epimerase or simply to remove extraneous protein (Table III). Subsequent

TABLE III

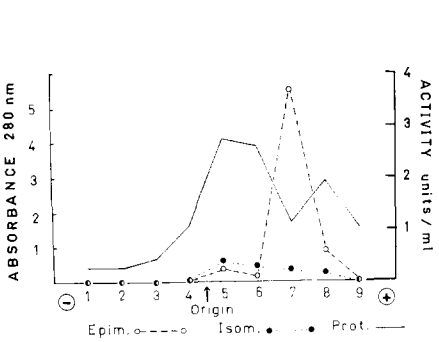
PURIFICATION OF ISOMERASE AND EPIMERASE FROM CALF LIVER

The purification procedure is described under Methods.

	Epimerase		*Isomerase*	
	Total units	*units/mg*	*Total units*	*units/mg*
Initial extract	13850	1.8	1480	0.19
0–95% $(NH_4)_2SO_4$ fraction	15560	3.7	1670	0.40
Heat treated 0–95% fraction				
(a) at pH 6.0	0	0	825	0.64
(b) at pH 7.4	15000	10.3	825	0.57
Pevikon eluate after electrophoresis of (a)	—	—	—	2.1
Pevikon eluate after electrophoresis of (b)	—	30	—	—

electrophoresis of fractions enriched in either epimerase or isomerase was used to prepare each enzyme free from contamination by the other. The distribution of the isomerase and epimerase enzymes on a 'Pevikon' block (Fig. 1) was similar to their distribution in cellulose acetate strips when the same buffer was used.

When initial velocities were measured by the 290 nm method, it was not strictly necessary to have the epimerase free from isomerase, since isomerase was already present in excess in the reaction mixture. Similarly, provided initial velocities were measured, the presence of small amounts of epimerase in isomerase preparations should have had little effect on the apparent initial rate of the isomerase enzyme. In general, however, it was considered preferable to have each enzyme uncontaminated by the other. When kinetic measurements were made by the 340 nm method, epimerase contamination of isomerase was unimportant since epimerase was added as an auxiliary enzyme. However, even a small contamination of epimerase by isomerase



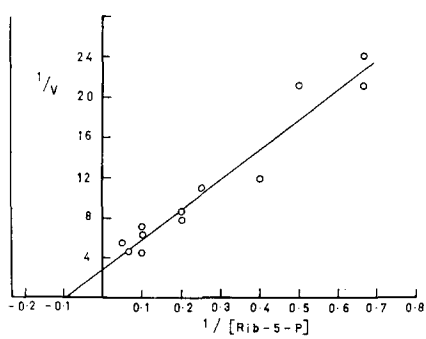
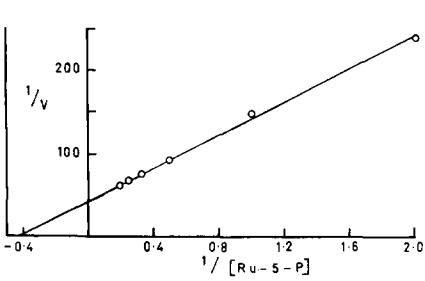


Fig. 1. A typical distribution of isomerase and epimerase from liver after electrophoresis on a 'Pevikon' block. Run for 12 h at 250 V and 0.8 mA. Abscissa represent zones 0.5 cm wide collected from either side of the origin; ordinates: —, protein concentration in eluates of each zone expressed as absorbance at 280 nm in a 1 cm cell; – – –, epimerase activity as units/ml of eluate; ·····, isomerase activity as units/ml of eluate. Units/ml determined by the 340 nm assay at a concentration of 0.1 mM ribulose-5-*P* and 2 mM ribose-5-*P* for epimerase and at 2 mM ribose-5-*P* for isomerase.

Fig. 2. Lineweaver–Burk plot of reciprocal rate against reciprocal ribose-5-*P* concentration for liver isomerase as determined by the 290 nm method at 25 °C. v is in μmoles/min; [ribose-5-*P*] in mM units.

made meaningful measurements of epimerase activity impossible in this assay because of the rapid changes in ribulose-5-*P* concentration brought about by the isomerase.

A Lineweaver–Burk plot was constructed from each set of data, a regression line was calculated, and the K_m value was obtained from the intercept on the $1/[S]$ axis. Plots of $[S]/v$ against $[S]$ gave similar values. Typical plots are shown for liver isomerase by the 290 nm method (Fig. 2), for yeast epimerase by the 340 nm method (Fig. 3), and for spinach isomerase with ribulose-5-*P* as a substrate using the cysteine–carbazole method (Fig. 4). The K_m values obtained are collected together in Table IV.



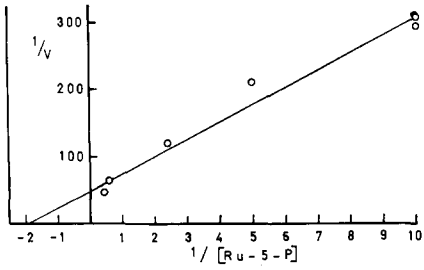


Fig. 3. Lineweaver–Burk plot of reciprocal rate against reciprocal ribulose-5-*P* concentration for yeast epimerase as determined by the 340 nm method at 25 °C v is in μmoles/min; [ribulose-5-*P*] in mM units.

Fig. 4. Lineweaver–Burk plot of reciprocal rate against reciprocal ribulose-5-*P* concentration for spinach isomerase at 37 °C by the cysteine–carbazole method. v is in μmoles/min; [ribulose-5-*P*] in mM units.

A K_m of 0.53 mM was calculated from the Lineweaver–Burk plot (Fig. 4) for spinach isomerase acting in the reverse direction on ribulose-5-*P*. The K_m was also calculated from the Haldane relationship

$$K_{eq} = \frac{V_1 K_m \text{ (ribulose-5-}P\text{)}}{V_2 K_m \text{ (ribose-5-}P\text{)}},$$

(where K_{eq} is the equilibrium constant of the reaction and V_1 and V_2 are the maximal velocities in the forward and reverse directions respectively.) The maximal velocity

TABLE IV

MICHAELIS CONSTANTS OF D-RIBOSE-5-PHOSPHATE KETOL-ISOMERASE AND D-RIBULOSE-5-PHOSPHATE 3-EPIMERASE FROM VARIOUS SOURCES

The method of measuring initial velocities and the temperature used are shown in parentheses. 290 = 290 nm method; 340 = 340 nm method; c–c = cysteine–carbazole method. All values in mM.

	Spinach	*Yeast*	*Calf spleen*	*Ox muscle*	*Calf liver*
Isomerase					
K_m (ribose-5-*P*)	5.3 (290, 37 °C)	—	2.6 (290, 37 °C)	6.5 (290, 37 °C)	10.2 (290, 25 °C)
	5.4 (c–c, 37 °C)	—	—	—	9.1 (340, 25 °C)
K_m (ribulose-5-*P*)	0.78 (c–c, 37 °C)	—	—	—	—
Epimerase					
K_m (ribulose-5-*P*)	—	2.4 (340, 25 °C)	—	—	2.9 (340, 25 °C)
	—	—	—	—	2.9 (290, 25 °C)

V_1 was calculated with considerable precision (± 2%) from duplicate measurements of initial velocities in the 290 nm assay at 10 mM and 20 mM ribose-5-*P* concentrations on the same enzyme preparation that was used for measurements in the reverse direction. V_2 was obtained from the Lineweaver–Burk plot of the reverse reaction (Fig. 4) and was very close to the measured velocity at 2.09 mM ribulose-5-*P*, which was the highest substrate concentration used. The ratio V_1/V_2 was 2.30, K_{eq} of 0.333 at 37 °C reported by Tabachnik *et al.*[17] and the value of 5.4 mM for K_m (ribose-5-*P*) (Table IV) were substituted in the expression, and a value of 0.78 mM was calculated for K_m (ribulose-5-*P*). This is the value listed in Table IV.

DISCUSSION

The close agreement between the K_m values determined by the 340 nm and the 290 nm procedures and between the cysteine–carbazole method and the 290 nm procedure gave confidence that the latter, which is a relatively new procedure for observing these reactions, was a valid method for following the rate of reaction in kinetic studies and that what was being observed did not include changes in absorbance due to extraneous reactions.

The K_m values for the epimerases from yeast and calf liver were close to each other, but nearly three times the only value found in the literature of 1 mM for the epimerase of *L. pentosus*[5]. Of the two K_m (ribose-5-*P*) values for spinach isomerase in the literature, our value was close to the figure of 4.6 mM measured by Knowles *et al.*[2], thus confirming that this enzyme has a high K_m. For the mammalian enzyme our values range from 2.6 mM for the isomerase from calf spleen to 9–10 mM for the calf liver enzyme. The high K_m value of calf liver isomerase did not appear to be an artefact of the heating procedure since measurements on a fresh extract of liver showed that when the ribose-5-*P* concentration was raised from 10 mM to 20 mM the rate of this enzyme increased by 30% compared to an increase of 32% calculated from the mean K_m value of 9.6 mM. The only figures that could be found in the literature for a mammalian isomerase were 2.1 mM (ref. 18) and 2.2 mM (ref. 19) for the enzyme in red blood cells, values close to our figure of 2.6 mM for the spleen enzyme. It appears that the isomerase in each of the three bovine tissues studied has a characteristic Michaelis constant which presumably reflects the characteristic conditions under which it operates in each tissue.

The K_m values for ribose-5-*P* of the various isomerases are rather high by comparison with the 25–300 μM concentrations reported in mammalian tissues[20–22]. As the main physiological function of the enzyme is, presumably, to form ribose-5-*P* this may merely be a reflection of a low K_m value for the substrate of the reverse reaction. This supposition was confirmed with spinach isomerase by a direct measurement of the K_m for ribulose-5-*P* and by a calculation of its value from measurements of the maximal velocities of the forward and reverse directions. We believe that this is the first time that the K_m of the isomerase for ribulose-5-*P* and the relative rates of the maximal velocities in the forward and reverse directions have been reported. The latter ratio is of considerable significance since estimations of ribose-5-*P* isomerase activity in tissues are usually based upon measurements of near-maximal velocities in the forward direction, whereas, its ability to function in the formation of ribose-5-*P* for nucleotide synthesis depends upon its activity in the reverse direction.

ACKNOWLEDGMENTS

We wish to thank Mrs Pamela Appelt for skilled technical assistance. This research was supported by grant MA 2205 from the Medical Research Council of Canada.

REFERENCES

1 Rutner, A. C. (1969) *Biochemistry* 9, 178–184
2 Knowles, F. C., Pon, M. R., and Pon, N. G. (1969) *Anal. Biochem.* 29, 40–47
3 David, J. and Wiesmeyer, H. (1970) *Biochim. Biophys. Acta* 208, 56–67
4 Skinner, A. J., and Cooper, R. A. (1971) *FEBS Lett.* 12, 293–296
5 Hurwitz, J. W. and Horecker, B. L. (1956) *J. Biol. Chem.* 223, 993–1008
6 Horecker, B. L., Smyrniotis, P. Z. and Seegmiller, J. E. (1951) *J. Biol. Chem.* 193, 383–396
7 Simpson, F. J. and Bhuyan, B. K. (1962) *Can. J. Microbiol.* 8, 663–672
8 Kiely, M. E., Tan, E. L. and Wood, T. (1969) *Can. J. Biochem.* 47, 455–460
9 Dische, Z. and Borenfreund, E. (1951) *J. Biol. Chem.* 192, 583–587
10 Racker, E. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 186–187, Academic Press, New York and London
11 Wood, T. (1970) *Anal. Biochem.* 33, 297–306
12 Gornall, A. G., Bardawill, C. S., and David, M. M. (1949) *J. Biol. Chem.* 177, 751–766
13 Duesburg, D. H. and Rueckert, R. R. (1965) *Anal. Biochem.* 11, 342–361
14 Muller-Eberhard, H. J. (1960) *Scand. J. Clin. Lab. Invest.* 12, 33–36
15 Foxwell, C. J., Buck, G. M. and Baron, D. N. (1968) *Biochem. J.* 110, 721–724
16 Ashwell, G. and Hickman, J. (1957) *J. Biol. Chem.* 226, 65–76
17 Tabachnik, M., Srere, P. A., Cooper, J. and Racker, E. (1958) *Arch. Biochem. Biophys.* 74, 315–325
18 Urivetzky, M. and Tsuboi, K. K. (1963) *Arch. Biochem. Biophys.* 103, 1–8
19 Bruns, F. H., Noltmann, E. and Valhaus, E. (1958) *Biochem. Z.* 330, 483–496
20 Carl, J. L. and King, L. J. (1970) *J. Neurochem.* 17, 293–295
21 Kauffman, F. C., Brown, J. G., Passoneau, J. V. and Lowry, O. H. (1969) *J. Biol. Chem.* 244, 3647–3653
22 Gumaa, K. A. and McLean, P. (1969) *Biochem. J.* 115, 1009–1029