Biochimica et Biophysica Acta, 570 (1979) 352—362

BBA 68816

PURIFICATION AND PROPERTIES OF D-RIBULOSE-5-PHOSPHATE 3-EPIMERASE FROM CALF LIVER

TERRY WOOD

Department of Biochemistry, University of Rhodesia, P.O. Box MP 167, Mount Pleasant, Salisbury (Rhodesia)

(Received March 13th, 1979)

Key words: Ribulose-phosphate epimerase; (Calf liver)

Summary

D-Ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) was purified 760-fold from calf liver by adsorption on DEAE-cellulose, chromatography on DEAE-Sephadex, chromatography on D-ribose 5-phosphate-Sepharose and gel filtration on Biogel P200.

The purified enzyme of specific activity 617 units/mg was obtained in 28% yield and gave a single band on polyacrylamide gel electrophoresis. It had a molecular weight of 45 000 and appeared to contain two identical peptide chains of 22 900 daltons. The K_m for D-ribulose 5-phosphate was 0.19 ± 0.07 mM (S.E.). It was inhibited by reagents reacting with sulphydryl groups, by sulphate ion, and by D-deoxyribose 5-phosphate. The pH-stability and pH-activity curves were determined.

Introduction

Although D-ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) has been partially purified from rabbit muscle [1], calf spleen [2], *Lactobacillus pentosus* [3], and prepared in crystalline form from baker's yeast [4], there is relatively little information available about its properties. Ox muscle, calf spleen, and calf liver were found to contain 28, 37 and 112 units/g of tissue, respectively, measured at 37°C. The enzyme was purified from the latter source and its properties were investigated.

Materials and Methods

Candida utilis, protamine sulphate (Grade II from salmon), crystalline bovine serum albumin, ovalbumin, beef pancreas α-chymotrypsinogen A, D-ribose-

5-phosphate sodium salt, D-2-deoxyribose-5-phosphate, iodoacetamide, and phenylmethylsulphonyl fluoride were from Sigma. Activated thiol-Sepharose 4B, thiopropyl-Sepharose 6B, DEAE-Sephadex A-50, Sephadex G-100, epoxy-activated Sepharose 6B and the molecular weight marker kit were obtained from Pharmacia, Uppsala, Sweden. Whatman CM-52 carboxylmethyl cellulose and DE-52 diethylaminocellulose were from W. and R. Balston, Ltd., Maidstone, Kent, U.K. Biogel P200 (50—100 mesh) was from Bio-Rad laboratories, Richmond, CA. Diaflo UM-10 and PM-30 membranes were from Amicon Corporation, Lexington, MA. α-Glycerophosphate dehydrogenase (rabbit muscle 170 units/mg) and triose phosphate isomerase (rabbit muscle, 5000 units/mg) were obtained from Boehringer Mannheim.

Transketolase was isolated from *C. utilis* (Wood, T., unpublished results). An equilibrium mixture of D-ribulose-5-phosphate and D-ribose-5-phosphate was prepared as described previously [5]. D-Ribulose-5-phosphate was also prepared from 6-phosphogluconic acid [6]. D-5-phosphoribonic acid was prepared as described by Dickens and Williamson [7]. D-Ribose-5-phosphate-Sepharose 6B was prepared from D-ribose-5-phosphate and epoxy-activated Sepharose 6B as described previously [8].

Methods

Measurement of D-ribulose-5-phosphate 3-epimerase activity. The assay procedure measures NADH oxidation brought about by the following reactions:

D-ribulose-5-phosphate → D-xylulose-5-phosphate (1)

D-xylulose-5-phosphate + D-ribose-5-phosphate → D-sedoheptulose-7-phosphate + D-glyceraldehyde-3-phosphate (2)

D-glyceraldehyde-3-phosphate → Dihydroxyacetone phosphate (3)

Dihydroxyacetone phosphate + NADH + H^+ → α-glycerophosphate + NAD^+ (4)

Reaction 1 is catalysed by D-ribulose-5-phosphate 3-epimerase, and reactions 2—4, are catalysed by an excess of the auxiliary enzymes transketolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase, respectively.

D-Ribulose-5-phosphate is expensive and time consuming to prepare. At high concentrations, interference in the assay, from traces of D-xylulose-5-phosphate in the preparation and from traces of epimerase in the auxiliary enzymes becomes pronounced. Therefore routine assays of epimerase activity were conducted using 0.08 mM D-ribulose-5-phosphate and 1.24 mM D-ribose-5-phosphate. The activity of pure preparations of the enzyme was measured also at 0.5 mM D-ribulose-5-phosphate in the presence of 3 mM D-ribose-5-phosphate.

The assay mixture contained in 3.00 ml, 50 mM glycylglycine/chloride buffer, pH 7.4, 2.5 mM magnesium chloride, 1 mM dithiothreitol, 0.1 mM thiamine pyrophosphate, 0.13 mM NADH, 0.34 unit α-glycerophosphate dehydrogenase, 1 unit triose phosphate isomerase, 1.24 mM D-ribose-5-phosphate and 0.08 mM D-ribulose-5-phosphate. After incubating 3 min in the cell compartment of the spectrophotometer, 0.1 unit of transketolase was added

and, after any D-xylulose-5-phosphate present had reacted the epimerase was added. After an initial acceleration the rate of change of absorbance at 340 nm became constant and remained so until approximately 30% of the ribulose-5-phosphate had reacted. A correction was made for any epimerase contamination of the auxiliary enzymes by subtracting the rate observed in the absence of added epimerase. Rates were measured at 30°C in a Unicam SP 1800 spectrophotometer attached to a Vitatron 400 recorder.

Measurement of D-ribose-5-phosphate ketolisomerase (EC 5.3.1.6) activity. Activity was measured using the same procedure as above except that 1 mM D-ribose-5-phosphate was the sole substrate and 0.1 unit of D-ribulose-5-phosphate 3-epimerase was added as an additional auxiliary enzyme.

Effect of pH on the stability and activity of the enzyme. From pH 5.5 to 7.0, measurements were made in 50 mM imidazole/chloride buffer and from pH 7.0 to 9.0 in 50 mM triethanolamine/chloride. The activity in 50 mM glycylglycine/chloride buffer at pH 7.5 was also determined. For stability studies the enzyme was mixed with the appropriate buffer at 25°C in stoppered tubes. Aliquots were withdrawn after 20 min, 2 h and 24 h, and the activity assayed at 340 nm. In the activity studies, when a decrease in activity was observed, additional amounts of the three auxiliary enzymes were added to ensure that the effect of pH was on the epimerase itself.

Inhibition measurements. In these studies all solutions were adjusted to the pH used in the assay and the epimerase was dialysed to remove protective thiols.

The inhibiting effect of salts and phosphorylated compounds was studied at 37°C, pH 7.4, by the 290 nm procedure [9]. The inhibitor was included in the reaction mixture from the start and the absence of an inhibitory effect on the auxiliary enzyme D-ribose-5-phosphate ketolisomerase was deduced, after the addition of the isomerase, from the shape of the curve recording the increase in absorbance at 290 nm and the time taken to reach the ribose 5-phosphate-ribulose-5-phosphate equilibrium when compared with the control. The epimerase sample was then added and the initial rate of reaction was compared with that of a control devoid of inhibitor.

In the case of the sulphydryl reagents, cupric acetate, mercuric chloride and *p*-chloromercuribenzoate, the epimerase was incubated in assay buffer for 20 min at 25°C with the inhibitor, then diluted and assayed by the 290 nm method; at pH 7.4 in the case of *p*-mercurichlorobenzoate, and in the case of copper and mercury salts at pH 6.8 to avoid precipitation. The inhibitor was diluted fifteen hundred times in the assay cuvette. Inhibition of the auxiliary D-ribose-5-phosphate ketolisomerase was tested for by adding the same amount of inhibitor followed by non-incubated epimerase and comparing the rate with that of non-incubated epimerase alone. Significant inhibition of the isomerase was only observed by the addition of 2 mM *p*-chloromercuribenzoate which caused a 38% drop in the rate of epimerase at a final concentration of $1.3 \cdot 10^{-6}$ M (one fifteen-hundredth of 2 mM). This rate was used to calculate the degree of inhibition of the incubated epimerase. With the other concentrations used no such correction was necessary and the rate of the incubated enzyme was compared directly with the rate of the non-incubated enzyme assayed in the absence of inhibitor.

Unlike the copper and mercury-containing inhibitors, *N*-ethylmaleimide and iodoacetamide had no effect on transketolase or other auxiliary enzymes used, so the 340 nm assay was employed to investigate their inhibitory effect on epimerase.

Inhibition by the specific sulphydryl group reagent 5,5'-dithiobis(2-nitrobenzoic acid) was tested by incubating epimerase with an excess of the reagent for 60 min at 30°C in the 40 mM triethanolamine/chloride/2 mM EDTA buffer, pH 8.0. As judged from the increase in absorbance at 412 nm, all available thiol groups had reacted after 30 min. The enzyme was then assayed and its activity compared with its original value.

Determination of molecular weights by gel filtration. The enzyme and molecular weight standards were chromatographed on a 1.5 × 40 cm column of Sephadex G-100 and K_{av} was plotted against the logarithm of the molecular weight [10]. A similar procedure was used with the 1.5 × 77 cm preparative Biogel P200 column employing bovine serum albumin, ovalbumin, and chymotrypsinogen as standards.

Protein determinations. Protein in the early stages of the purification procedure was determined by the biuret method [11] or from the absorbance at 280 nm in more purified preparations [11]. The protein content of solutions of the pure enzyme was measured by the Lowry procedure [11], after precipitation with deoxycholate [12], using bovine serum albumin as a protein standard.

Electrophoresis. Electrophoresis was carried out in a 7% polyacrylamide gel at pH 8.9 [13] and in a 7.5% gel at pH 7.5 [14]. Gels were stained with 0.5% amidoblack in 7% (v/v) acetic acid. SDS electrophoresis was performed as described by Weber and Osborn [15]. Gels were stained overnight in 0.25% Coomassie blue and destained for 24 h in 50% methanol/10% acetic acid followed by 20% methanol/10% acetic acid [16]. Epimerase and marker proteins (horse muscle myoglobin, bovine pancreatic chymotrypsinogen, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, ovalbumin, bovine serum albumin, bovine γ-globulin) were dissociated by procedure 1 of Weber and Osborn [17].

Results

Purification

Fresh calves' liver was brought from the local abattoir. A 200 g portion was used for the procedure described below and 100 g portions were placed in polythene bags and frozen at −20°C. Frozen liver stored for periods of up to six months can be used, although yields may be somewhat lower than with fresh material.

The tissue was homogenised in the cold room in a Waring blendor with 2 ml/g of ice-cold 50 mM triethanolamine/chloride/5 mM EDTA buffer, pH 7.4. The homogenate was centrifuged for 30 min at 25 000 × g_{av} in a Beckman J21B centrifuge at 5°C. The supernatant was filtered through cotton wool to remove fat and centrifuged a second time at 25 000 × g for 90 min. The supernatant was almost clear and free from ribosomes. Mercaptoethanol was added to 5 mM and the pH was adjusted to 7.7. A suspension of DE-52 DEAE-cellulose in 50 mM phosphate buffer was allowed to sediment under gravity and

washed with an equal volume of water. To the enzyme solution, cooled in ice, was added 40 ml of the packed adsorbent and the mixture was stirred for 5 min. A further 10 ml of DE-52 cellulose was added and the stirring was continued for a further 5 min. The adsorbent was allowed to settle and most of the clear supernatant was removed. The adsorbent was then packed into a 4 cm diameter column at room temperature, giving a bed approximately 4 cm high, using the supernatant as a suspending medium. The liquid in the column was drained down to the surface of the adsorbent and washed through with the void volume of buffer (50 mM sodium phosphate/1 mM EDTA/2 mM mercaptoethanol, pH 7.4). The enzyme was eluted with 0.3 M potassium chloride in the above buffer and 10-ml fractions collected until no more colour was eluted. Mercaptoethanol was added to the eluate (120 ml) to 5 mM and phenylmethylsulphonyl fluoride to 0.1 mM and the protein was precipitated by adding ammonium sulphate to the ice-cold solution to 90% saturation.

Chromatography on DEAE-Sephadex

The ammonium sulphate suspension was centrifuged down for 10 min at 10 000 × *g*, resuspended in 30 ml of buffer (50 mM triethanolamine/chloride/5 mM EDTA/5 mM mercaptoethanol, pH 7.4) and dialysed at 5°C for 14 h against 1 l of 5 mM triethanolamine/chloride/1 mM EDTA/1 mM mercaptoethanol, pH 7.4. To the clear solution of enzyme was added a solution of 10 mg/ml protamine sulphate until there was no further precipitate. The mixture was stood for 30 min in ice, then centrifuged 10 min at 10 000 × *g*. The pH of the clear supernatant was adjusted to 6.0 with acetic acid and clarified if necessary. It was then passed at room temperature through a 4 × 1.7 cm diameter column of CM52 carboxymethyl cellulose packed in 10 mM sodium acetate/1 mM EDTA buffer, pH 6.0. The effluent was collected, one-twentieth its volume of 400 mM sodium phosphate buffer, pH 7.7, was added and the pH adjusted to 7.7. The solution was applied to a 10 × 1.6 cm diameter column of DEAE-Sephadex A-50 packed in buffer (50 mM sodium phosphate/1 mM EDTA/2 mM mercaptoethanol buffer, pH 7.7) and washed through with the

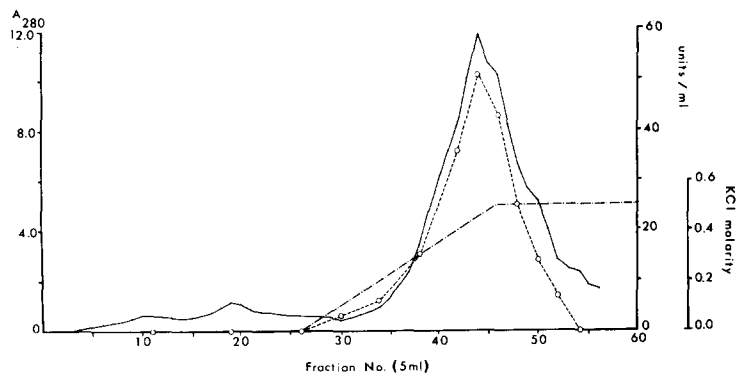

Fig. 1. Chromatography on DEAE-Sephadex at room temperature and pH 7.7. The sample was washed through with 50 mM sodium phosphate/1 mM EDTA/2 mM mercaptoethanol buffer, pH 7.7. A linear gradient of 50 ml of 0.5 M KCl in the above buffer running into 50 ml of buffer was begun at tube 26 and followed by 0.5 M KCl in buffer; ———, absorbance at 280 nm in a 1 cm cell (A_{280}); o- - - - -o, ribulose-5-phosphate 3-epimerase activity; · — · — · KCl concentration.

same buffer. When all unadsorbed protein had been removed, a linear gradient of 50 ml 0.5 M potassium chloride in buffer, pH 7.7, running into 50 ml of buffer, pH 7.7, was started. Fractions of 5 ml were collected and the distribution of enzyme and protein measured (Fig. 1).

The tubes containing enzyme were pooled and concentrated by ultrafiltration through a PM-30 membrane in a 50 ml cell. Phenylmethylsulphonyl fluoride was added to a concentration of 0.1 mM and the enzyme solution was dialysed against 1 l of 5 mM triethanolamine/chloride/1 mM EDTA/2 mM mercaptoethanol, pH 7.4.

Chromatography on D-ribose-5-phosphate-Sepharose

The solution was placed, at room temperature, on a 15 × 0.8 cm column of ribose-5-phosphate-Sepharose [8] and washed through with buffer (20 mM triethanolamine/chloride/1 mM EDTA/2 mM mercaptoethanol, pH 7.4). Elution was carried out with a linear gradient of 40 ml 0.1 M potassium chloride in the above buffer, running into 40 ml of buffer and 4-ml fractions were collected (Fig. 2). At the end of the gradient the column was washed with 0.5 M potassium chloride in buffer. Tubes from the enzyme peak were combined, concentrated by ultrafiltration through a UM-10 membrane in a 10 ml cell and phenylmethylsulphonyl fluoride was added to 0.1 mM.

Chromatography on Biogel P200

The sample was placed on a 77 × 1.4 cm column of Biogel P200 in the cold room and washed through with the 20 mM buffer, described above, at a flow rate of 3 ml/h and suitable size fractions were collected. Peak tubes were combined, concentrated by ultrafiltration through a UM-10 membrane, and applied to the column again. Four successive runs were carried out on the same column

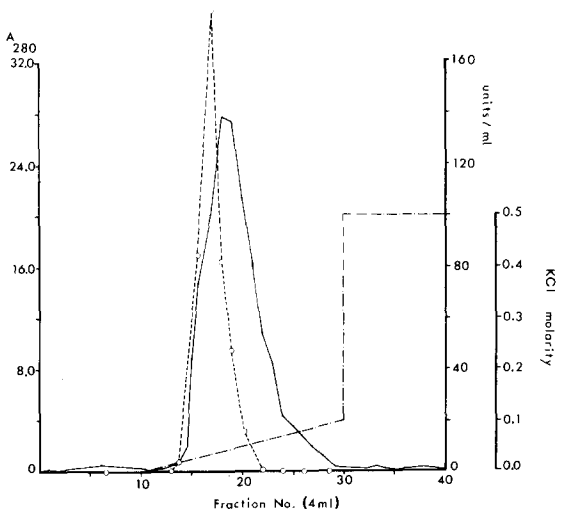

Fig. 2. Chromatography on ribose-5-phosphate-Sepharose at room temperature and pH 7.4. The sample was washed through with 20 mM triethanolamine/chloride/1 mM EDTA/2 mM mercaptoethanol buffer, pH 7.4. A linear gradient of 40 ml of 0.1 M KCl in buffer running into 40 ml of buffer was begun at tube 10 and followed by 0.5 M KCl in buffer at tube 30; ———, absorbance at 280 nm in a 1 cm cell (A_{280}); o- - - - -o, ribulose-5-phosphate 3-epimerase activity; · — · — ·, KCl concentration.

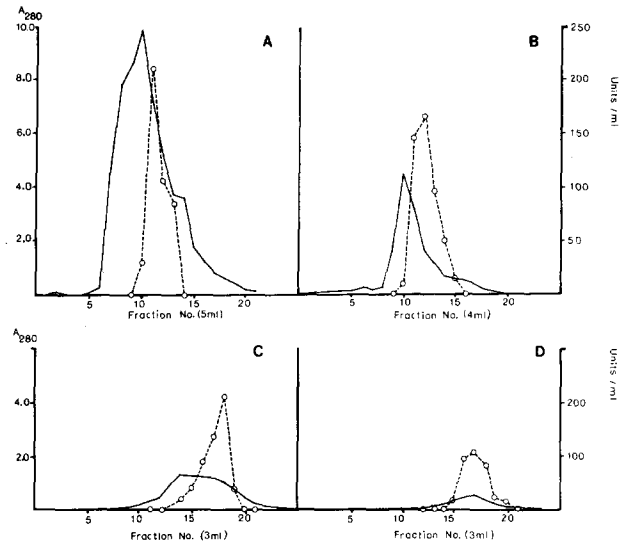

Fig. 3. Chromatography on Biogel P200 at 5° C and pH 7.4. The sample was washed through with 20 mM triethanolamine/chloride/1 mM EDTA/2 mM mercaptoethanol buffer, pH 7.4. After each run peak tubes were concentrated (see Table I) and run again. ———, absorbance at 280 nm in a 1 cm cell (A_{280}); ○· · · · ·○, ribulose-5-phosphate 3-epimerase activity.

and on the fourth run the specific activity of the enzyme was constant across the peak and equal to the highest value recorded (Fig. 3d). The peak tubes from the fourth run were combined, concentrated, and used for studies of the properties of the enzyme.

In order to protect the sulphydryl groups of the enzyme, dialysis was carried out in tubing that had been boiled for 20 min in 20 mM EDTA, and mercapto-

TABLE I

PURIFICATION OF D-RIBULOSE-5-PHOSPHATE 3-EPIMERASE FROM 200 g OF CALF LIVER

Rib-5-*P*, ribose 5-phosphate.

Sample	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Recovery (%)
Initial extract	310	10 300	2540	0.25	100
DEAE-cellulose supernatant	360	9 700	173	0.02	7
Ammonium sulphate suspension	164	1 380	2690	1.94	106
Dialysed suspension	60	1 380	1670	1.21	62
Protamine supernatant	72	970	1670	1.72	62
CM-cellulose effluent	81	770	1670	2.17	62
DEAE-Sephadex eluate	20	550	1290	2.34	51
Rib-5-*P*-Sepharose eluate	4.5	330 *	1730	5.25	68
Chromatography on Biogel P200					
Run A, conc. tubes 11—13, each 5 ml	2.5	70 *	1730	25	68
Run B, conc. tubes 11—14, each 4 ml	2.0	31 *	1880	61	75
Run C, conc. tubes 16—18, each 3 ml	1.0	9.7 *	1830	188	72
Run D, conc. tubes 16—18, each 3 ml	0.8	3.8 *	720	190	28

* Protein values based on absorbance at 280 nm assuming $A_{280}^{1\%} \approx 10.0$.

ethanol was added before ultrafiltration to a concentration of 5 mM. A summary of the purification is given in Table I.

Properties

The enzyme ran as a single band on polyacrylamide gel electrophoresis at pH 7.5 and 8.9. The molecular weight was determined as 45 000 by gel filtration on Sephadex G-100 and 44 700 from its elution volume on the Biogel P200 column. SDS electrophoresis gave a single band of molecular weight 22 900 indicating that the molecule contained two peptide chains of identical molecular weight.

The sample contained less than 0.004% of ribose-5-phosphate isomerase activity. This contaminating enzyme mostly remained unadsorbed by DEAE-cellulose under the conditions used and eluted before the epimerase during gradient elution on DEAE-Sephadex and ribose 5-phosphate-Sepharose. The enzyme protein had an $A_{280}^{1\%}$ value of 12.6. The specific activity was 190 units/A_{280} unit (Table I) and 240 units/mg at a ribulose-5-phosphate concentration of 0.08 mM when the protein content was measured by deoxycholate precipitation followed by the Lowry procedure. At 0.5 mM substrate (approximately 2.5 K_m) the measured specific activity was 617 units/mg.

The enzyme was stable to heat (2 min at 60°C) thus permitting many of the preparative steps to be carried out at room temperature. Outside the pH range 7.0—8.0 at 25°C some activity was lost (Fig. 4). The pH-activity curve closely resembled that published for the bacterial enzyme [3] with an optimum from pH 7.4 to 7.6. No activity was lost by exposure to the protease inhibitor phenylmethylsulphonyl fluoride at 1 mM for 1 h at 0°C, and after one month's exposure to a 0.1 mM concentration 80% of the activity remained.

K_m for D-ribulose-5-phosphate

Initial velocities were determined at 30°C, pH 7.5, over the range of substrate concentrations 0.1—1.5 mM in the presence of 3 mM ribose-5-phosphate. When the data were plotted as a direct linear plot [18] a value of 0.16 mM was obtained for K_m. The data were also treated as described by Cornish-Bowden

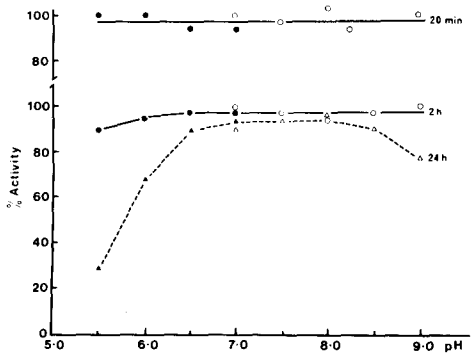

Fig. 4. pH-stability curves. Enzyme activity was measured after incubation at 25°C for the times shown; filled symbols, 50 mM imidazole/chloride buffer; open symbols, 50 mM triethanolamine/chloride buffer.

TABLE II

INHIBITION OF LIVER EPIMERASE AFTER INCUBATION WITH REAGENTS REACTING WITH SULPHYDRYL GROUPS

Rates measured by the 290 nm method at 37°C and by the 340 nm method at 30°C after incubation at 25°C for the times shown. See Materials and Methods for details.

Inhibitor	Incubation time and assay conditions	Concentration (mM)	Inhibition (%)
Cupric acetate	20 min, pH 6.8, 290 nm	0.5	0
		1.0	28
		2.0	90
Mercuric chloride	20 min, pH 6.8, 290 nm	0.1	20
		0.5	43
		1.0	70
p-Chloromercuribenzoate	20 min, pH 7.4, 290 nm	0.1	0
		0.5	27
		2.0	55
Iodoacetamide	30 min, pH 7.4, 340 nm	5.0	0
		10.0	10
		15.0	15
N-Ethylmaleimide	30 min, pH 7.4, 340 nm	5.0	20
		10.0	35
		15.0	65
Excess 5,5′-dithiobis-(2-nitrobenzoic acid)	60 min, pH 7.4, 340 nm	0.05	0

[19] and the best value of K_m and the standard error were calculated to be $K_m = 0.19 \pm 0.07$ mM.

Effect of inhibitors

Because of the large number of compounds that cause inhibition of trans-

TABLE III

INHIBITION OF LIVER EPIMERASE BY SALTS AND OTHER COMPOUNDS

Inhibitor included in the assay mixture. Rates measured by the 290 nm method at 37°C, pH 7.4. See Materials and Methods for full details.

Inhibitor	Concentration (mM)	Inhibition (%)
Ammonium sulphate	20	27
	50	46
	100	57
Sodium sulphate	20	28
	50	40
	100	52
Potassium phosphate	10	0
	50	5
	100	10
D-Glucose 6-phosphate	10	0
D-6-Phosphogluconate	20	0
D-5-Phosphoribonate	2	0
	5	0
D-Ribose 5-phosphate	20	0
D-2-Deoxyribose 5-phosphate	5	45

ketolase most substances were studied using the 290 nm assay. However, the 340 nm procedure could be used with iodoacetamide and *N*-ethylmaleimide. Cupric acetate, mercuric chloride and *p*-chloromercuribenzoate all inhibited at concentrations of 1—2 mM, but iodoacetate and *N*-ethylmaleimide only inhibited after incubation with relatively high concentrations of reagent, and the specific reagent for reactive thiol groups, 5,5′-dithiobis(2-nitrobenzoic acid) had no effect (Table II). Sulphate was a moderately powerful inhibitor, accounting for the low recoveries of activity when undialysed ammonium sulphate fractions were assayed. Phosphate only inhibited slightly and substrate analogs such as glucose 6-phosphate, 6-phosphogluconate, 5-phosphoribonate, and ribose-5-phosphate not at all. However, D-2-deoxyribose-5-phosphate produced inhibition at concentrations similar to that of the substrate (Table III).

Discussion

Calf liver was a rich source of epimerase. The data in Table I indicate the extraction of about 25 units/g liver assayed at 0.08 mM substrate corresponding to approximately 50 units/g at 0.5 mM substrate (approaching saturation) at 30°C. This value is in accordance with the content of 112 units/g measured at 2.5 mM ribulose-5-phosphate and 37°C by the 290 nm method described in Introduction.

A 760-fold purification of the enzyme was achieved. The recovery of activity at each step was high and tended to increase beyond 100% during the gel filtration steps despite the discarding of some enzyme at the edges of the peaks. This was interpreted as the result of the removal of proteins interfering with the assay. Although, in the particular preparation described in Fig. 1, the distribution of enzyme on DEAE-Sephadex followed closely that of the protein and no great purification resulted (Table I), in many other preparations a substantial increase in specific activity was obtained at this stage. The drop in recovery from 72% to 28% at the end of run D (Table I) was due to the considerable amount of concentrated enzyme left in the 10 ml ultrafiltration cell after the 0.8 ml of concentrated epimerase had been removed. These recoveries, of 72% and 28% may be compared with an overall yield of 4% for the enzyme from yeast [4].

The specific activity of the enzyme of 617 units/mg (measured at approximately 2.5 K_m at 30°C) was greater than the value of 258 units/mg (measured at 5 mM D-ribulose-5-phosphate and room temperature) for the yeast enzyme [4], and much greater than the values of 29, 30 and 69 units/mg for the enzymes purified from rabbit muscle [1], calf spleen [2] and *L. pentosus* [3], respectively. The molecular weight of the enzyme, estimated at 45 000, was close to the value of 46 000 reported for the yeast enzyme [4].

The K_m value for D-ribulose-5-phosphate of 0.19 mM was about one-tenth of that reported previously [20]; the reason for this is not known.

The only inhibition studies to be found in the literature were those of Tabachnik et al. [1] of the action of sulphydryl reagents on the rabbit muscle enzyme. In agreement with these authors, the enzyme was inhibited by *p*-chloromercuribenzoate at concentrations around 1 mM and by high concentrations of *N*-ethylmaleimide after a lengthy preincubation. These observations

are now extended to include the effects of cupric and mercuric ions, iodoacetamide, sulphate and phosphate ions, and 5- and 6-carbon sugar phosphates and their derivatives. Apart from compounds reacting with sulphydryl groups, the only inhibitor effective at a concentration comparable to that of the substrate was D-2-deoxyribose-5-phosphate, which probably acts by virtue of the structural resemblance of its straight chain form to D-ribulose-5-phosphate.

The enzyme lost activity during the purification if mercaptoethanol was omitted from the buffers used. Dialysis in tubing that had not been boiled with EDTA also led to a massive loss of activity, probably due to the presence of copper and other heavy metals. These observations together with the inhibition studies indicated that intact sulphydryl groups are required for enzyme activity, although the failure of 5,5′-dithiobis(2-nitrobenzoic acid) to inhibit the enzyme discounted the idea of a reactive sulphydryl group at the active site, unless such a group was present but inaccessible to the reagent. The enzyme bound readily to activated thiol-Sepharose 4B and to thiopropyl-Sepharose 6B but attempts to apply this as a purification step were hindered by an inability to recover all the enzyme from the adsorbent.

All workers with this enzyme are agreed that it is extremely stable when stored in solution at −10—−20°C [1,21]. Some of my preparations have retained their activity for 3 years at −15°C. Repeated freezing and thawing, however, can be harmful. Preparations stored at 2°C as suspensions in 70% saturated ammonium sulphate showed no loss of activity after 18 months.

Acknowledgements

I am indebted to Mrs. P. Appelt, Mrs. C. Watts, and Miss S. Fletcher for devoted technical assistance and to Mr. A.L. Stuart for participation in the preliminary stages of this investigation. Part of this work was supported by the Medical Research Council of Canada and part by the University of Rhodesia Research Board.

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