Purification, properties and assay of D-ribulose 5-phosphate 3-epimerase from human erythrocytes

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A direct assay procedure is described for D-ribulose 5-phosphate 3-epimerase (EC 5.1.3.1) which exploits differences in the c.d. spectra of substrate and product. The enzyme has been purified from human erythrocytes and was resolved by gel filtration and sucrose-density-gradient centrifugation into a major component of apparent M_r 45 000 and a minor component of M_r 23 000. Electrophoresis in sodium dodecyl sulphate gave a single component corresponding to M_r 23 000. Kinetic and sucrose-density-gradient centrifugation data indicate dissociation of the dimeric form of the enzyme into monomers of low specific activity; substrate favours the active dimeric form of the enzyme. At concentrations of the enzyme where both forms of the enzyme are present initial velocity data yielded a Hill plot with an interaction coefficient of approx. 2.0, indicating co-operative binding of substrate under these conditions.

D-Ribulose 5-phosphate 3-epimerase catalyses the interconversion of D-ribulose 5-phosphate and D-xylulose 5-phosphate and is implicated in the so-called non-oxidative part of the pentose phosphate pathway. In humans the enzyme has been studied in extracts from muscle and erythrocytes (Dickens & Williamson, 1956) and more recently has been detected in a wide range of tissues (Spencer & Hopkinson, 1980). The activity of the epimerase and of other enzymes of the pentose phosphate pathway have been measured in extracts of several tissues from rats (Novello & McLean, 1968) and in several areas of the central nervous system in the same species (Kauffman, 1972).

The enzyme has been isolated in a partially purified form from a variety of species, including *Lactobacillus pentosus* (Hurwitz & Horecker, 1956), yeast (Kiely *et al.*, 1973), rabbit muscle (Tabachnick *et al.*, 1958) and from both calf spleen (Ashwell & Hickman, 1957) and calf liver (Wood, 1979). On the basis of molecular weight determination by gel chromatography and electrophoresis in sodium dodecyl sulphate, Wood (1979) suggested that the calf liver enzyme contains two identical polypeptide chains.

Assay of the enzyme by chemical procedures is difficult because of the close similarity between substrate and product. Most studies published to date have relied on the linked spectrophotometric

assay devised by Wood (1970) that provides a simple and convenient procedure for assaying the enzyme but for detailed kinetic studies suffers from the disadvantages inherent in linked systems. Here we describe an assay which exploits differences in c.d. spectrum between ribulose 5-phosphate and xylulose 5-phosphate and present some preliminary data obtained through the application of this assay to the study of D-ribulose 5-phosphate 3-epimerase isolated from human erythrocytes.

Experimental

Materials

Sephacrose 4B-200, CNBr, dithiothreitol, D-xylulose 5-phosphate, D-ribose 5-phosphate, D-ribose 5-phosphate isomerase (EC 5.3.1.6), transketolase, D-ribulose 5-phosphate 3-epimerase, a mixture of triose phosphate isomerase and α -glycerophosphate dehydrogenase from rabbit muscle, thiamin pyrophosphate and NADH were all obtained from Sigma. Sephadex (DEAE A-50, G-100, G-50, G-25) were purchased from Pharmacia. Ampholytes were supplied by LKB. Acrylamide was obtained from BDH and bisacrylamide from Eastman Kodak. CM-cellulose was a product of Whatman, and agarose type HSIF was from Windsor Laboratories, Slough, Berks., U.K. D-Ribulose 5-phosphate was prepared enzymically from D-ribose 5-phosphate essentially as described by Dickens & Williamson (1956) except that the D-ribose 5-phosphate isomerase used here was from human

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erythrocytes. It was found both convenient and economical to use isomerase covalently bound to Sepharose 4B-200 by using the procedure described by Lindberg (1974); the bound enzyme was easily recovered by filtration and could be re-used several times. The only other modification to the method of Dickens & Williamson was that ribulose 5-phosphate was eluted from Dowex-200 (formate) with 150 mM-ammonium formate containing 1 mM-sodium borate. The same column procedure was also used to remove ribulose 5-phosphate from commercial preparations of ribose 5-phosphate. Column fractions were analysed for aldo- and keto-pentoses by using the orcinol (Mejbaum, 1939) and cysteine carbazole reactions (Axelrod & Jang, 1954) respectively. Pooled fractions containing sugar phosphates were converted to the free acids by passage through Dowex-50 (H^+ form). After freeze-drying, residues were dissolved in a minimum volume of water and the pH adjusted to 6.3 by addition of saturated $Ba(OH)_2$ solution. Addition of 4 vol. of cold ethanol precipitated the barium salts which were collected by centrifugation. After washing three times with 80% (v/v) aqueous ethanol the product was dried *in vacuo* over $CaCl_2$. Ribulose 5-phosphate was identified by both chemical and enzymic methods as described by Pontremoli & Mangiarotti (1962).

All pH measurements were done at room temperature (20°C) and buffers were prepared as described by Savage & Spencer (1977).

Preparative agarose gel electrophoresis

Agarose gels (1% w/v) were prepared in 5 mM-citrate/Tris buffer, pH 6.0, and 100 mM-citrate/Tris of the same pH was used in the electrode compartments (Spencer & Hopkinson, 1980). Gels were poured to a depth of 0.5 cm in glass moulds (16 cm × 28 cm). The sample (approx. 0.5 ml) was soaked onto a strip of Whatman no. 17 paper (14 cm × 0.4 cm) which was then inserted into the gel about 4 cm from the cathode end. A potential of 10 V/cm was applied for 3 h with cooling provided by a metal plate maintained at 4°C. Marker strips (2 cm wide), cut from the long edges of the gel, were sliced and stained for D-ribulose 5-phosphate 3-epimerase and D-ribose 5-phosphate isomerase activity as described by Spencer & Hopkinson (1980). Both enzymes were eluted from the appropriate sections of the gel by using the following procedure: the excised portion of gel was placed in a 20 ml plastic syringe plugged with glass wool and left overnight at -20°C. After thawing at room temperature the pieces of agarose were compressed with the syringe plunger and the enzymes were eluted along with gel buffer through the glass wool plug. The eluted enzymes were concentrated to

approx. 1 ml by pressure dialysis by using a PM-1 membrane in an Amicon Diaflo ultrafiltration cell.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was carried out as described by Laemmli (1970) in a slab-gel apparatus identical to that of Harris & Hopkinson (1976).

Analytical isoelectric focusing

Agarose gels (1% w/v) containing 2% (v/v) ampholytes were poured on thin glass plates in a mould (0.1 cm × 16 cm × 20 cm). Focusing in the pH range 3–10 was carried out for 4 h at 800 V with cooling provided by a metal plate at 4°C. Duplicate portions of gel were stained for protein and for enzyme activity.

Determination of molecular weight

Relative molecular mass (M_r) was determined by gel filtration through columns of Sephadex G-100 as described by Andrews (1965) and by sucrose-gradient centrifugation (Martin & Ames, 1961). Experimental details for both techniques are described by Savage & Spencer (1977); in the present work 5 mM-triethanolamine/HCl buffer, pH 6.0, was used in both gel filtration and sucrose-gradient centrifugation experiments and the latter were carried out at 18°C. Fractions were analysed for both protein and epimerase activity.

D-Ribulose 5-phosphate 3-epimerase activity

This was monitored at all stages of the purification procedure by using the method of Wood (1970). Assays were carried out at 37°C in a double beam Unicam SP.1800 spectrophotometer connected to a Unicam AR 25 linear recorder by using quartz cuvettes of 1 cm path-length. The equilibrium mixture of D-ribose 5-phosphate and D-ribulose 5-phosphate that is used as substrate in this assay was freshly prepared and used within 50 min of preparation. Initial reaction rate was proportional to enzyme concentration provided the change in A_{290} fell within the range 0.002–0.01/min; activity is expressed as the change in A_{290} /min. Protein was measured as described by Brownson & Spencer (1972). In all experiments involving the purified enzyme the c.d. assay described in the following section was employed and all assays were done in duplicate.

Assay of D-ribulose 5-phosphate 3-epimerase activity by measurements of circular dichroism

All measurements of c.d. were made at 20°C in a JASCO J40CS spectropolarimeter by using strain-free cylindrical silica cells of 1 ml capacity with 1 cm light path. Conversion of ribulose 5-phosphate to xylulose 5-phosphate was monitored by measuring

changes in c.d. at 272nm. The assay mixture contained ribulose 5-phosphate (sodium salt) at a final concentration of 5mM in 50mM-triethanolamine/HCl buffer, pH 7.4, and the reaction was started by the addition of a small volume (10–20 μ l) of purified enzyme to give a final volume of 1ml. Initial reaction velocity as measured by the change in $\Delta A/\text{min}$ was linear for at least 2min provided the differential absorption change did not exceed $1.5 \times 10^{-3}/\text{min}$.

Purification of D-ribulose 5-phosphate 3-epimerase

With the exception of the gel filtration step, which was carried out at 4°C, all purification procedures were carried out at room temperature (20°C). Erythrocytes were obtained as packed red cells from the Regional Blood Transfusion Service, Edgware, Middlesex, U.K. Erythrocytes were separated and washed as described by Brownson & Spencer (1972). Cells were lysed with 4 vol. of water and cell debris was removed by centrifuging at 18000 rev./min for 40 min. Haemoglobin was removed from the supernatant by using the procedure described by Osborne & Spencer (1973) which exploits the difference in pI between the epimerase and haemoglobin. The resulting filtrate, essentially free of haemoglobin, was concentrated by pressure dialysis in an Amicon Diaflo ultrafiltration cell with a PM-10 membrane. The concentrated solution (10ml) containing the enzyme was mixed with an equal volume of 50mM-triethanolamine/HCl buffer, pH 8.0, containing dithiothreitol (10mM) and D-ribose-5-phosphate (20mM). This solution was placed in a water bath at 65°C for 15 min. After cooling the solution to 0°C, denatured protein was removed by centrifuging at 17000 rev./min for 20 min at 4°C. The supernatant (20ml) was dialysed overnight against 5mM-triethanolamine/HCl buffer, pH 6, and then applied to a column (2cm $\times$ 40cm) packed with DEAE-Sephadex (A-50) equilibrated in the same buffer. After washing with starting buffer to remove unabsorbed protein the column was eluted with a linear gradient (0–0.5M) of NaCl in the same buffer at a flow rate of 30ml/h. Fractions (5ml) were collected and analysed for epimerase and protein. Cl^- concentration was estimated by titration with AgNO_3 . Fractions containing epimerase activity were combined, concentrated by pressure dialysis (8ml) and applied to a column (2.5cm $\times$ 80cm) of Sephadex G-100 equilibrated in 5mM-triethanolamine/HCl buffer, pH 8.0. The column was eluted with the same buffer at a flow rate of 35ml/h and fractions (5ml) were collected and analysed for epimerase activity and protein. Fractions containing the enzyme were combined and concentrated by pressure dialysis to about 1ml with a PM-1 membrane. The final stage in the purification procedure involves the separation of epimerase from

Fig. 1. C.d. spectra of D-ribulose 5-phosphate and D-xylulose 5-phosphate

C.d. spectra were measured as described in the text on (a) 5mM-ribulose 5-phosphate and (b) 5mM-xylulose 5-phosphate, both dissolved in 50mM-triethanolamine/HCl, pH 7.4.

D-ribose 5-phosphate isomerase, which comprises the major contaminant, by preparative agarose gel electrophoresis. The isomerase obtained after this procedure was used for the preparation of D-ribulose 5-phosphate as described above. Enzyme solutions obtained after preparative electrophoresis were contaminated with small particles of agarose which were removed by passing the solution through a column (2 cm $\times$ 20 cm) of Sephadex G-25 equilibrated in 5 mM-triethanolamine buffer, pH 7.4. Fractions containing the enzyme were pooled and concentrated by pressure dialysis with a PM-1 membrane and then either used directly or stored frozen (-10°C). After storage at -10°C for up to 2 weeks the epimerase retained 90% of its original activity.

Results and discussion

Assay procedure

The c.d. spectra of D-ribulose 5-phosphate (Fig. 1a) and D-xylulose 5-phosphate (Fig. 1b) are

virtually identical except that the sign of the differential absorption is reversed; the spectrum for D-ribulose 5-phosphate is similar to that reported by Wood (1975a). The reaction catalysed by D-ribulose 5-phosphate 3-epimerase is conveniently followed by measurements of differential absorption at 272 nm. The main advantage of the present procedure is that it allows the direct study of the effects of a variety of reagents on the activity of the enzyme provided that they do not exhibit appreciable c.d. absorption in the region 270–280 nm; unfortunately this restriction precludes a study of the effects of purine-containing metabolites. Possible interference through non-enzymic reactions of D-ribulose 5-phosphate with certain substances (Wood, 1975b) is avoided by inclusion of the appropriate controls.

Purification and properties

A recovery in excess of 100% at one stage of the purification procedure (Table 1) is probably due to the removal of a small molecular weight inhibitor. Apart from this the only feature of interest con-

Table 1. Purification of D-ribulose 5-phosphate 3-epimerase from human erythrocytes

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification factor	Recovery (%)
Stroma-free haemolysate	48.5	8153	0.0059	1	100
CM-cellulose filtrate	55.8	535.3	0.104	17.5	115
Concentrated filtrate	48.0	534.2	0.0898	15.1	98.9
Supernatant remaining after thermal denaturation	43.6	70.6	0.62	103.9	89.8
Fractions from DEAE-Sephadex column	25.5	12.1	2.11	354.8	52.5
Combined fractions from Sephadex G-100 column	19.2	4.1	4.68	788.4	39.5
Agarose gel extract	15.4	1.05	14.66	2468.0	31.7

Fig. 2. Chromatography on Sephadex G-100 of pooled and concentrated fractions from the DEAE-Sephadex column. Experimental conditions are described in the text. ●, D-ribulose 5-phosphate 3-epimerase activity, ○, protein (A_{280}).

Fig. 3. Relationship between D-ribose 5-phosphate 3-epimerase concentration and initial reaction velocity. Measurements of reaction velocity by using the c.d. assay were as described in the text. Enzyme concentration refers to final concentration in the reaction cuvette. Each assay was started by adding 100 μl of an appropriately diluted stock solution of epimerase (0.5 mg/ml in 50 mM-triethanolamine/HCl, pH 7.4); all dilutions were made in the same buffer immediately before assay.

Fig. 4. Relationship between enzyme concentration and duration of pre-steady-state lag.

D-Ribulose 5-phosphate 3-epimerase activity was measured by using the c.d. assay as described in the text. For different amounts of the purified enzyme the lag period was estimated by extrapolation from the linear portion of the progress curve. Each assay was started by adding 100 μl of an appropriately diluted stock solution of epimerase (0.5 mg/ml in 50 mM-triethanolamine/HCl, pH 7.4); all dilutions were made in the same buffer immediately before assay.

cerning the purification was the consistent observation of two zones of epimerase during gel filtration (Fig. 2).

The homogeneity of the purified enzyme was confirmed by electrophoresis in 1% agarose gels at pH 6 and by isoelectric focusing in the range of pH 3–10; in both systems the enzyme gave a single zone on staining for protein that was coincident with a zone of epimerase activity.

Electrophoresis of the purified enzyme in the presence of sodium dodecyl sulphate also revealed a single component, corresponding to an M_r of about 23 000. By contrast, gel filtration on Sephadex G-100 indicated a value of 45 000 for the M_r of the main zone of epimerase activity and a value of 23 000 for a minor zone. The enzyme would appear to be a dimer comprising two polypeptide chains that are either identical or of similar M_r . The calf liver enzyme studied by Wood (1979) also seems to be a dimer of M_r approx. 45 000. Previously, electrophoretic analysis of the epimerase components of extracts from man-mouse hybrid cells had provided an indication that the human (and also the mouse) enzyme is dimeric in structure (Spencer & Hopkinson, 1980).

The possibility that the enzyme can dissociate into its corresponding monomeric subunits is suggested by the gel filtration data referred to previously and also by the data shown in Fig. 3: a marked decline in

specific activity on dilution is indicative of dissociation into inactive or less active subunits (Wang & Weissmann, 1971). At low concentrations of enzyme a change in kinetic behaviour was also noted, with either ribulose 5-phosphate or xylulose 5-phosphate as substrate, progress curves showed a lag before steady state kinetics were observed. The duration of the lag is inversely proportional to enzyme concentration over a limited range (Fig. 4) and is not detectable at concentrations of 10 μg/ml or greater. The subject of lag kinetics has been reviewed by Kurganov *et al.* (1976a,b) who also provided a detailed theoretical treatment of the phenomenon. Association of enzyme subunits to higher oligomers, and/or the isomerization of an allosteric enzyme, are cited as the main causes of a pre-steady state lag; both processes may be induced by substrate and may be relatively slow by comparison with the reaction velocity of the enzyme. According to Kurganov *et al.* (1976b), if on raising the enzyme concentration the lag eventually disappears, as is the case here, then this implies that the lag is due solely to an effect of substrate on the association-dissociation equilibrium involving different polymeric forms of the enzyme. However, since the kinetics have not been studied here by using for example,

Fig. 5. Sucrose-density-gradient centrifugation profiles of purified D-ribulose 5-phosphate 3-epimerase

Experimental conditions are indicated in the Experimental section. The profile shown in (a) was obtained by centrifuging 200 μl of a solution containing 0.5 mg of epimerase/ml in 50 mM-triethanolamine buffer, pH 7.4. An aliquot (200 μl) from fraction 8 of this profile was dialysed for 16 h against 50 mM-triethanolamine buffer, pH 6; the profile obtained after re-centrifuging this sample is shown in (b). Conditions for (c) were identical to those used for (a) except that all solutions contained 5 mM-ribulose 5-phosphate.

stopped-flow procedures, the possibility that an allosteric transition might be a component of the observed lag cannot be discounted.

Additional evidence to suggest that the enzyme dissociates on dilution and that substrate affects the dissociation equilibrium is provided by the results of

Fig. 6. Hill plot of initial velocity data for purified D-ribulose 5-phosphate 3-epimerase at various concentrations of ribulose 5-phosphate

Initial velocity data were obtained over the range, 0.1–5 mM-ribulose 5-phosphate in 50 mM-triethanolamine/HCl buffer, pH 7.4, by using the c.d. assay. A constant amount of enzyme was used in each assay by adding 100 μl of a stock solution containing 20 μg of epimerase/ml in 50 mM-triethanolamine/HCl buffer, pH 7.4.

sucrose-gradient-centrifugation experiments. At concentrations of enzyme where both monomer and dimer are present, gradient profiles showed two peaks of epimerase activity corresponding to a dimer of M_r 45 000 and a monomer of M_r 23 000 (Fig. 5a). On re-centrifuging a portion of the fraction containing highest activity of the dimer a similar profile was obtained with two zones of activity (Fig. 5b); this is consistent with further dissociation of the enzyme on dilution. The absence of a peak corresponding to monomer when the enzyme is centrifuged in the presence of its substrate indicates that substrate induces the association of monomers to form dimers (Fig. 5c). Similar effects where substrate induces polymerization of enzyme subunits have been reported for several enzymes, including α -acetylgalactosaminidase (Wang & Weissmann, 1971), threonine dehydratase (Vanquickenborne & Phillips, 1968) and lactate dehydrogenase (Hathaway & Criddle, 1966).

Preliminary data from initial velocity measurements are also consonant with an effect of substrate on the dissociation of the enzyme. Initial velocities measured over a range of ribulose-5-phosphate concentrations gave a Hill plot that indicated co-operative binding of substrate (Fig. 6); this is to be expected whenever a ligand binds preferentially to one polymeric form of an enzyme and thereby induces polymerization (Frieden & Colman, 1967).

Whether such properties are of significance in the context of metabolic control (Frieden, 1971) remains to be seen, but D-ribulose 5-phosphate 3-epimerase

would seem to be strategically placed to control the rate of the non-oxidative part of the pentose shunt pathway.

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