Purification and Properties of γ -Glutamylcyclotransferase from Human Erythrocytes

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1. γ -Glutamylcyclotransferase was purified 10000-fold from human erythrocytes. 2. The purification steps involved fractionation with $(\text{NH}_4)_2\text{SO}_4$ and chromatography on Sephadex G-75, DEAE-cellulose and hydroxyapatite. The purified enzyme was found to be homogeneous on density-gradient polyacrylamide-gel electrophoresis. 3. The maximum reaction rate was observed at pH 9.0 and the apparent K_m value for γ -glutamyl-L-alanine was 2.2 mM. 4. The molecular weight (25250) of the purified enzyme agreed well with the value (25500) in fresh haemolysates, indicating no apparent structural modification of the enzyme during purification. However, rapid processing of the blood through the initial $(\text{NH}_4)_2\text{SO}_4$ and Sephadex-chromatography steps was required to prevent formation of a high-molecular-weight aggregate with substantially lower specific activity. 5. γ -Glutamylcyclotransferase catalyses the formation of 5-oxoproline from γ -glutamyl dipeptides. The role of this enzyme in erythrocytes is of particular interest, because γ -glutamyl-L-cysteine serves as a substrate for both γ -glutamylcyclotransferase and glutathione synthetase. Thus the cyclotransferase could modulate glutathione synthesis.

γ -Glutamylcyclotransferase (EC 2.3.2.4), found in several animal tissues, catalyses the following general reaction: L- γ -glutamyl-L-amino acid $\rightarrow$ 5-oxoproline (pyroglutamate) + L-amino acid.

Recognition of this enzyme as an essential component of the proposed ' γ -glutamyl cycle' has increased interest in its metabolic role (Meister, 1973). γ -Glutamylcyclotransferase has previously been purified from human brain (Orlowski *et al.*, 1969) and rat liver (Orlowski & Meister, 1973). Technical difficulties in determining the enzyme activity have limited efforts to examine its properties in other tissues or species.

The role of γ -glutamylcyclotransferase in erythrocytes is of particular interest, because the γ -glutamyl cycle is not complete in them (Srivastava *et al.*, 1976; Board & Smith, 1977). In addition, the enzyme may play a significant role in regulating glutathione synthesis by interacting with γ -glutamylcysteine (Orlowski & Wilk, 1975; Board *et al.*, 1978).

We have described a new simplified method to determine γ -glutamylcyclotransferase activity (Board *et al.*, 1978). It has enabled us to rapidly purify the enzyme from human erythrocytes for further study.

Materials and Methods

γ -Glutamyl-L-alanine was obtained from Calbiochem, La Jolla, CA, U.S.A.; glutamate-pyruvate transaminase, lactate dehydrogenase, DEAE-

cellulose, and Sephadex G-75 and G-100 were from Sigma Chemical Co., St. Louis, MO, U.S.A. Hydroxyapatite was purchased from Bio-Rad, Richmond, CA, U.S.A. All other reagents were analytical grade.

γ -Glutamylcyclotransferase activity was determined, as described by Board *et al.* (1978), by linking alanine liberation with NADH oxidation enzymically as follows:

Protein was determined either by the method of Lowry *et al.* (1951) or by determining A_{280} .

Initial activation and preparation of the DEAE-cellulose was completed at room temperature. DEAE-cellulose was suspended (40 g/l) in 1 M-NaOH and stirred for 30 min. The suspension was allowed to settle and the fines were decanted. The DEAE-cellulose was washed, on a Buchner funnel, with 5 vol. of demineralized water.

This procedure was repeated with 0.5 M-HCl and 0.5 M-NaOH. After the final treatment, with NaOH, the DEAE-cellulose was washed repeatedly with demineralized water until the filtrate was neutral. Finally, the DEAE-cellulose was equilibrated (40 g/l)

with 0.005M-potassium phosphate buffer, pH 7.0, at 4°C.

γ -Glutamylcyclotransferase was purified from 1 litre of outdated bank blood. All procedures were performed at 4°C, unless otherwise stated.

Enzyme purification

Step 1: preparation of haemolysate. The blood was centrifuged for 10min at 6000g to facilitate removal of plasma and buffy coat. After the erythrocytes had been washed three times with 5vol. of 150mM-NaCl, they were haemolysed by adding 5vol. of 0.005M-potassium phosphate buffer (4°C), pH 7.0. The haemolysate was then centrifuged at 23000g for 15min to remove the stroma.

Step 2: $(\text{NH}_4)_2\text{SO}_4$ fractionation. The stroma-free haemolysate was added to the previously equilibrated DEAE-cellulose (1g of cellulose/2g of haemoglobin) and the mixture stirred for 30min. Haemoglobin was removed by collecting the DEAE-cellulose on a Buchner funnel, then washing it with 0.005M-potassium phosphate buffer, pH 7.0, until the filtrate was colourless. To elute γ -glutamylcyclotransferase, the DEAE-cellulose was suspended in a solution containing 0.4M-KCl and 0.005M-potassium phosphate buffer, pH 7.0 (2ml/ml of original packed cell volume). After the suspension had been stirred for 30min, the eluate containing the enzyme was collected by filtration.

The eluate was adjusted to 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ (313g/l) slowly while being stirred. After the $(\text{NH}_4)_2\text{SO}_4$ had dissolved completely, the solution was stirred for 30min, then centrifuged at 23000g for 20min. The resulting precipitate was discarded and the supernatant was adjusted to 80% saturation with $(\text{NH}_4)_2\text{SO}_4$ (a further 214g/l). The solution was again stirred for 30min and allowed to settle for 30min before being centrifuged at 23000g for 20min. The $(\text{NH}_4)_2\text{SO}_4$ precipitate was suspended in 0.005M-potassium phosphate buffer, pH 6.25, containing 0.01M-2-mercaptoethanol. The suspension was then dialysed against 16 litres of the same buffer overnight.

To prevent aggregation and substantial inactivation of the enzyme we found it necessary to complete steps 1 and 2 and to commence step 3 within 24h.

Step 3: Sephadex G-75 chromatography. The dialysed solution from step 2 was applied to a Sephadex G-75 column (2.5cm $\times$ 85cm) equilibrated with 0.005M-potassium phosphate buffer, pH 6.25, containing 0.01M-2-mercaptoethanol, and eluted with the same buffer; 6ml fractions were collected. γ -Glutamylcyclotransferase activity emerged from the column in a single peak after the appearance of a large peak composed of non-active high-molecular-weight protein. The fractions containing the major portion of enzyme activity were combined and further purified.

Step 4: DEAE-cellulose chromatography. The pooled active fractions from step 3 were applied to a DEAE-cellulose column (2.5cm $\times$ 20cm) that had been equilibrated with 0.005M-potassium phosphate buffer, pH 6.25, containing 0.01M-2-mercaptoethanol. The column, after being washed with 100ml of the equilibrating buffer, was eluted with a linear gradient formed from 1 litre of equilibrating buffer and 1 litre of the same buffer containing 0.4M-KCl. The flow rate was maintained at 60ml/h, and 5ml fractions were collected and assayed. The enzyme activity again emerged in one peak well behind the major peak of non-active protein. Fractions with γ -glutamylcyclotransferase activity were pooled and dialysed against 8 litres of 0.005M-potassium phosphate buffer, pH 6.25, containing 0.01M-2-mercaptoethanol.

Step 5: hydroxyapatite chromatography. The dialysed sample from step 4 was applied to a hydroxyapatite column (1.5cm $\times$ 8cm) previously equilibrated with the dialysis buffer. The column was eluted with a linear gradient formed from 250ml of 0.005M-potassium phosphate buffer, pH 6.25, containing 0.01M-2-mercaptoethanol and 250ml of 0.1M-potassium phosphate buffer, pH 6.25, containing 0.01M-2-mercaptoethanol. Fractions (5ml) were collected and assayed for γ -glutamylcyclotransferase activity, which was eluted as a single peak. The fractions containing enzyme activity were pooled and dialysed against 8 litres of 0.005M-potassium phosphate buffer, pH 6.25, containing 0.01M-2-mercaptoethanol.

Step 6: DEAE-cellulose chromatography. The dialysed sample from step 5 was rechromatographed on a small column of DEAE-cellulose (1cm $\times$ 10cm) equilibrated in the dialysis buffer. This column was eluted with a linear gradient formed from 200ml of 0.005M-potassium phosphate buffer, pH 6.25, containing 0.01M-2-mercaptoethanol and 200ml of 0.2M-potassium phosphate buffer, pH 6.25, containing 0.01M-2-mercaptoethanol. Fractions (3ml) were collected and assayed. The enzyme was again eluted from the column in a single peak (Fig. 1). Active fractions (32–54) were pooled and stored, for further study, at 4°C.

The pH for maximum activity of γ -glutamylcyclotransferase was determined by using the Tris/glycine/phosphate buffers described by Beutler *et al.* (1968). The pH of the buffers was adjusted at room temperature and they were diluted 10-fold in the reaction mixture.

The molecular weight was estimated by gel filtration through a column (2.5cm $\times$ 80cm) of Sephadex G-100 equilibrated with 0.5M-Tris/HCl/0.1M-MgCl₂ buffer, pH 7.5. The column was calibrated with aldolase (mol.wt. 158000), human haemoglobin (64500), ovalbumin (45000), chymotrypsinogen (25000) and ribonuclease A (13700).

Fig. 1. Rechromatography on DEAE-cellulose

The partially purified enzyme was rechromatographed to further purify and concentrate the enzyme. Fractions were assayed for activity and expressed in $\mu\text{mol/min per ml}$ (○). Protein was determined by A_{280} (●). The active fractions (32–54) were pooled and stored. See step 6 under 'Enzyme purification'.

Table 1. Summary of purification of γ -glutamylcyclotransferase from human erythrocytes

For full details see the Materials and Methods section. One unit is equivalent to $1 \mu\text{mol}$ of NADH converted into NAD^+/min at 37°C .

	Step	Volume (ml)	Total protein (mg)	Protein concn. (mg/ml)	Activity (units/ml)	Total activity (units)	Sp. activity (units/mg)	Yield (%)
1.	Haemolysate	1340	88000	65.7	0.224	300	0.0034	100
2.	$(\text{NH}_4)_2\text{SO}_4$ fractionation	45	534	11.9	3.71	167	0.313	56
3.	Sephadex G-75	55	61.9	1.12	2.63	145	2.34	48
4.	DEAE-cellulose	325	22.1	0.0679	0.362	118	5.33	39
5.	Hydroxyapatite	196	5.86	0.0299	0.518	101	17.3	34
6.	DEAE-cellulose	76	2.17	0.0285	0.965	84.9	33.8	24

Results

As summarized in Table 1, our purification procedures resulted in an approx. 10000-fold increase in the specific activity of γ -glutamylcyclotransferase. The product appeared to be homogeneous on density-gradient polyacrylamide-gel electrophoresis.

Storage of the $(\text{NH}_4)_2\text{SO}_4$ precipitate obtained at step 2 resulted in a high-molecular-weight form of the enzyme with decreased activity (Fig. 2). This high-molecular-weight aggregate was readily separated from the low-molecular-weight form by chromatography on Sephadex G-75, DEAE-cellulose or hydroxyapatite.

The molecular weight (25250) of the purified enzyme agreed well with the molecular weight (25500) of γ -glutamylcyclotransferase in freshly prepared haemolysate. The maximum reaction rate for the purified enzyme was observed at pH 9.0 (Fig. 3). The apparent K_m value from a Hanes–Woelf plot for

γ -glutamyl-L-alanine was 2.2 mM under the assay conditions (pH 7.52) previously described (Board *et al.*, 1978).

Discussion

The similarity of the pH optimum, K_m and molecular weight of purified γ -glutamylcyclotransferase to that obtained in fresh haemolysates (Board *et al.*, 1978) suggests that this purification procedure does not significantly modify the enzyme. Our findings suggest that under some conditions, i.e. storage of the $(\text{NH}_4)_2\text{SO}_4$ precipitate, the enzyme could be modified during purification. Orlowski & Meister (1973) found evidence that γ -glutamylcyclotransferase from rat liver was significantly modified during purification and storage.

The molecular weight of the purified enzyme (25250) was similar to the value obtained for that from rat liver (27500) by Orlowski & Meister (1973),

Fig. 2. Effect of storage on the $(\text{NH}_4)_2\text{SO}_4$ precipitate

The $(\text{NH}_4)_2\text{SO}_4$ precipitate was chromatographed on Sephadex G-75 equilibrated with 0.005 M-potassium phosphate buffer, pH 6.25 (see step 3 under 'Enzyme purification'). Fractions were assayed and activity was expressed in $\mu\text{mol/min per ml}$ (○). Protein was determined by A_{280} (●). (a) Elution pattern after 18 h dialysis of fresh $(\text{NH}_4)_2\text{SO}_4$ precipitate against 16 litres of equilibrating buffer; (b) chromatograph, showing substantial loss of enzyme activity, after the precipitate had been stored for 36 h and then dialysed for 18 h against 16 litres of equilibrating buffer.

Fig. 3. pH-dependence of γ -glutamylcyclotransferase. Under the assay conditions used the maximum rate was observed at pH 9.0. See under 'Enzyme purification'. pH-dependence is expressed as % of activity at pH 7.5.

monstrated the presence of multiple molecular forms of the enzyme in human and sheep brain. In contrast, we found only one form in human erythrocytes.

Wolfersburger & Tabachnick (1974) found that γ -glutamylcyclotransferase activity in guinea-pig epidermis, brain, liver and kidney was significantly stimulated by optimal concentrations of K^+ and Mg^{2+} . We examined the effects of K^+ , Mg^{2+} and Mn^{2+} on human erythrocyte γ -glutamylcyclotransferase activity, but failed to find any stimulation or inhibition by concentrations ranging up to 15 mM.

Further kinetic and substrate-specificity studies on the erythrocyte enzyme are necessary to determine its role in the erythrocytes' metabolic economy and to evaluate the contribution of γ -glutamylcyclotransferase in regulating glutathione synthesis.

No genetic variants or cases of inherited γ -glutamylcyclotransferase deficiency have been reported. Studying such cases might reveal the significance of the enzyme in the γ -glutamyl cycle.

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