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ERYTHROCYTE γ-GLUTAMYLCYSTEINE SYNTHETASE FROM NORMAL AND LOW-GLUTATHIONE SHEEP

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Summary

γ-Glutamylcysteine synthetase (L-glutamate:L-cysteine γ-ligase (ADP-forming), EC 6.3.2.2) was purified from the erythrocytes of normal and low-glutathione sheep. The molecular weight (78 000), pH optimum (pH 7), substrate specificity, inhibition constant for glutathione (0.44—0.50 mM), electrophoretic mobility, and heat stability were similar for the purified enzyme from both sources. Using immunological techniques, the specific activity of γ-glutamylcysteine synthetase from low-glutathione sheep was lower than that from normal sheep.

Introduction

Some sheep from the Merino and Corriedale breeds have genetically-determined low concentrations of erythrocyte glutathione [1—3]. We have previously reported that these diminished erythrocyte GSH concentrations are associated with decreased activity of γ-glutamylcysteine synthetase, the first enzyme required for glutathione synthesis in erythrocytes [4,5]. Since even in low-GSH erythrocytes with reduced γ-glutamylcysteine synthetase activity there is sufficient residual activity to allow the relatively rapid synthesis of GSH, it is not entirely clear whether diminished γ-glutamylcysteine synthetase activity is the cause of lowered GSH concentrations.

Previous studies of partially-purified γ-glutamylcysteine synthetase indicated that the diminished activity of γ-glutamylcysteine synthetase from low-GSH

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sheep did not result from a change in the affinity of enzyme for its substrates [5]. Data suggested that γ-glutamylcysteine synthetase from low-GSH sheep might be more susceptible to feedback inhibition by GSH than γ-glutamylcysteine synthetase from normal sheep; however, since the γ-glutamylcysteine synthetase from low-GSH sheep had low activity in hemolysates in which endogenous GSH had been diluted or removed by dialysis, further study of the role of feedback inhibition in the regulation of GSH concentrations in erythrocytes from low-GSH sheep appeared to be required.

To facilitate this study, we have prepared γ-glutamylcysteine synthetase from both normal and low-GSH sheep erythrocytes to a high degree of purity and determined some previously uninvestigated properties.

Materials and Methods

Purification. Blood from several Corriedale sheep, either normal (GSH > 2.4 μmol/g hemoglobin) or low-GSH type (GSH < 1.6 μmol/g hemoglobin) was pooled for batch purification of γ-glutamylcysteine synthetase. The purification procedures used were essentially those previously described by Majerus et al. [6] for human erythrocyte γ-glutamylcysteine synthetase.

Enzyme determinations. γ-Glutamylcysteine synthetase was determined initially in hemolysates by the method of Minnich et al. [7]. In hemoglobin-free preparations during the purification procedures, γ-glutamylcysteine synthetase was determined spectrophotometrically by linking the reaction to the oxidation of NADH and recording the change in optical density at 340 nm. The reaction mixture consisted of the following reagents: 100 μl 0.5 M imidazole buffer containing 0.05 M $MgCl_2$; 100 μl 15 mM phospho*enol*-pyruvate; 100 μl 5.5 mM glutamate; 100 μl 5.5 mM cysteine; 100 μl 2 mM NADH; 10 μl 250 U/ml lactate dehydrogenase; 10 μl 700 U/ml pyruvate kinase; 100 μl 3 mM ATP. The total volume was adjusted to 1 ml with water and the enzyme sample in 0.005 M potassium phosphate. Blanks were obtained by omitting cysteine. The assay is linear with time and enzyme activity.

Glutathione synthetase was determined by a similar procedure except that glutamate and cysteine were replaced by 5.5 mM glycine and 5.5 mM γ-glutamylcysteine. The γ-glutamylcysteine was prepared as previously described [4].

The pH for maximum activity was determined by using Tris/glycine/phosphate buffers [8]. The pH of the buffers was adjusted at room temperature and they were diluted 10-fold in the reaction mixture.

Molecular weight estimation. Molecular weight was estimated by gel filtration through a column (2.5 × 80 cm) of Sephadex G-100 equilibrated with 0.5 M Tris-HCl buffer, pH 7.5, containing 0.1 M KCl. The column was calibrated with the following proteins: aldolase (158 000), human hemoglobin (64 458), ovalbumin (45 000), chymotrypsinogen (25 000), and ribonuclease A (13 700).

Electrophoresis. Electrophoresis of γ-glutamylcysteine synthetase was carried out for 5—6 h at 100 V. in 90 mM Tris-HCl, 3 mM EDTA, 80 mM borate buffer (pH 8.3) on 7.5% polyacrylamide slab gels or on Gradipore density gradient (2.5—27%) polyacrylamide slab gels obtained from Isolab Inc., Akron, OH. After electrophoresis, γ-glutamylcysteine synthetase was stained

with Coomassie brilliant blue R or was histochemically localized by the following procedure. The slab gels were incubated for 12 h at 37°C in a solution containing 35 mM Tris-HCl, 20 mM $MgCl_2$, 20 mM $CaCl_2$, 10 mM glutamic acid, 10 mM aminobutyric acid, 5 mM ATP, and 10 mM dithiothreitol. The pH was adjusted to 8.0. The reaction catalyzed by γ-glutamylcysteine synthetase gives rise to products of γ-glutamylcysteine, ADP, and P_i. The P_i then combines with calcium to form an insoluble white precipitate. The zone of γ-glutamylcysteine synthetase activity thus becomes visible as a white band when the gel is viewed against a black background.

Inhibition kinetics. The inhibitory effects of GSH on the activity of γ-glutamylcysteine synthetase from normal and low-GSH sheep were evaluated, assuming competitive inhibition, with a computer program developed by Cleland [9]. γ-Aminobutyrate (1 mM) was used to replace cysteine in these experiments, to avoid changes in the concentration of cysteine due to oxidation [10]. Glutamate concentration was 0.2, 0.4 or 0.8 mM and GSH varied from 0 to 3 mM.

Immunochemistry. Antibodies to γ-glutamylcysteine synthetase from both normal and low-GSH sheep were raised in White New Zealand rabbits. Purified γ-glutamylcysteine synthetase (0.2 ml containing 20 μg protein) was emulsified in 0.5 ml of Freund's complete adjuvant. The emulsion was then injected subcutaneously at four positions on the rabbits' backs. This procedure was repeated at weekly intervals. After the third week, serum containing antibodies to γ-glutamylcysteine synthetase was obtained.

Ouchterlony double-diffusion was carried out in 1% agar plates containing 0.15 M potassium phosphate buffer, pH 7.0, and 2 μM sodium azide.

Titration of γ-glutamylcysteine synthetase with undiluted antiserum was carried out by adjusting samples of γ-glutamylcysteine synthetase from normal and low-GSH sheep to an activity of 0.356 units/ml. Aliquots of these enzyme samples (100 μl) were then mixed with varying quantities (0—100 μl) of antiserum. The total volume of this reaction mixture was adjusted to 200 μl with 200 mM potassium phosphate buffer, pH 7.25. After 30 min at room temperature the antibody-enzyme complexes were removed by centrifugation 1500 × *g* for 10 min) and the residual γ-glutamylcysteine synthetase activity in the supernatant was determined.

Results

The purification procedure resulted in a 935-fold increase in specific activity of γ-glutamylcysteine synthetase from the group of normal GSH sheep and a 747-fold increase in that from the group of low-GSH sheep. At all stages during the purification procedure, the enzyme from normal sheep preparations and that from low-GSH sheep preparations behaved identically.

Majerus et al. [6] reported difficulty in separating human erythrocyte γ-glutamylcysteine synthetase from glutathione synthetase. In contrast, sheep erythrocyte γ-glutamylcysteine synthetase and glutathione synthetase were clearly separated by the DEAE-cellulose column (Fig. 1).

Electrophoresis on density gradient polyacrylamide revealed that γ-glutamylcysteine synthetase from normal sheep possibly was purified to homogeneity,

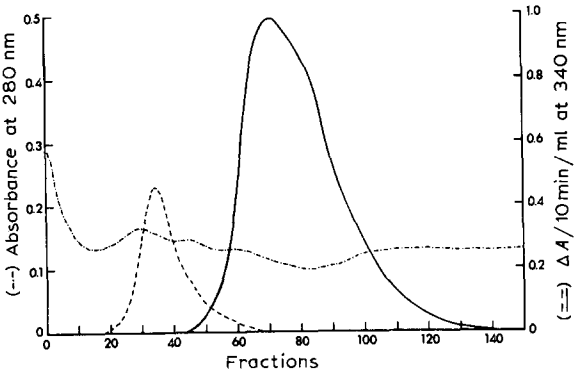
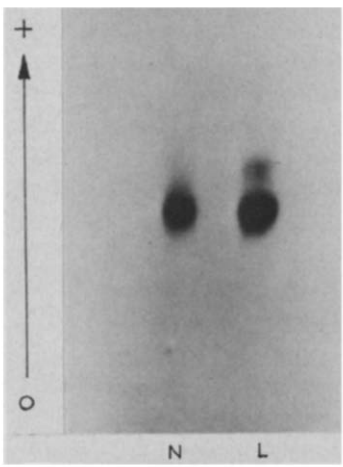


Fig. 1. Separation of γ-glutamylcysteine synthetase (———) and glutathione synthetase (- - - - -) from normal sheep erythrocytes on DEAE-cellulose. The enzymes were eluted with a linear gradient established between 0.05 and 0.2 M KCl.

because it migrated as a single band (Fig. 2). In contrast, at least two proteins were present in the sample prepared from low-GSH sheep erythrocytes. The most intense of these two protein bands corresponded exactly to the band seen in the normal enzyme preparation. Specific histochemical localization of γ-glutamylcysteine synthetase indicated that the second, lower molecular weight protein in the low-GSH γ-glutamylcysteine synthetase sample had no γ-glutamylcysteine synthetase activity and was therefore probably only a contaminant. The trapping of both normal and low-GSH γ-glutamylcysteine synthetase samples is at the same position with the same molecular weight.



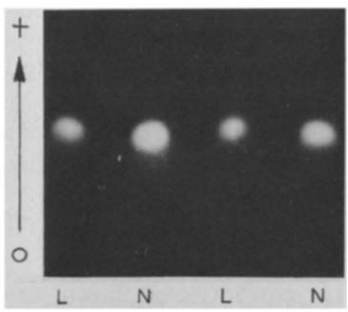


Fig. 2. Electrophoretic separation of γ-glutamylcysteine synthetase purified from normal (N) and low-glutathione (L) sheep on a polyacrylamide density gradient (2.5—27%). The gel was stained with Coomassie brilliant blue.

Fig. 3. Electrophoretic separation of γ-glutamylcysteine synthetase purified from normal (N) and low-glutathione (L) sheep on 7.5% polyacrylamide. The enzyme was localized by the specific staining procedure.

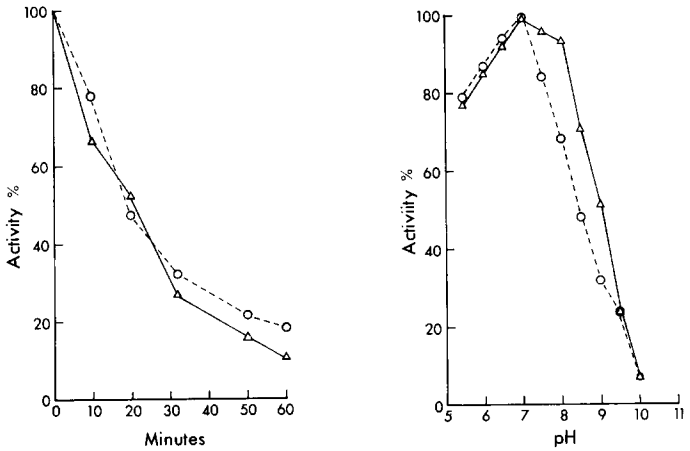


Fig. 4. Relative stability of γ-glutamylcysteine synthetase from normal (△) and low (○) GSH sheep erythrocytes at 48°C. Purified enzyme preparations were incubated at 48°C. Samples were taken and assayed at 10 min intervals. Results are expressed as percent of initial activity.

Fig. 5. Variation of γ-glutamylcysteine synthetase activity with pH. The pH was varied using Tris/glycine/phosphate buffers, as described previously [8].

Electrophoresis on standard 7.5% polyacrylamide (Fig. 3) indicated that both samples have similar mobilities at pH 8.3.

The molecular weight of normal and low-GSH γ-glutamylcysteine synthetase was determined by gel filtration and was found to be 78 000 in each case.

Investigation of stability at 48°C indicated that γ-glutamylcysteine synthetase from low-GSH sheep has the same stability as the normal enzyme (Fig. 4).

The variation of γ-glutamylcysteine synthetase activity with pH is shown in Fig. 5. Although the optimum for γ-glutamylcysteine synthetase from both normal and low-GSH sheep was found to be pH 7, γ-glutamylcysteine synthetase from normal sheep appears to have a slightly broader range.

In the reaction catalyzed by γ-glutamylcysteine synthetase, cysteine can potentially be replaced by other amino acids. γ-Aminobutyrate (at 0.55 mM) gave a substantial reaction rate, but there was no evidence to suggest any difference in the substrate specificities of γ-glutamylcysteine synthetase from normal (20.5% of the reaction with cysteine) and low-GSH sheep (23.2%). Asp, Gly, Leu, Lys, Thr, Ser, Val, and Ala did not serve as substrate (less than 1% of the cysteine activity) for either normal or low γ-glutamylcysteine synthetase when tested at 0.55 mM.

GSH was found to competitively inhibit γ-glutamylcysteine synthetase (Fig. 6) from both normal and low-GSH sheep. The K_i values of 0.44 ± 0.055 mM for normal γ-glutamylcysteine synthetase and 0.50 ± 0.077 mM for low-GSH γ-glutamylcysteine synthetase, were not significantly different.

Antibodies raised against either normal or low-GSH enzyme were found to cross-react with the alternate enzyme. Fig. 7 shows a reaction of identity between both enzyme variants and antiserum to γ-glutamylcysteine synthetase from low-GSH sheep. A similar result was obtained using antiserum to γ-glutamylcysteine synthetase from normal sheep. Both antibodies formed

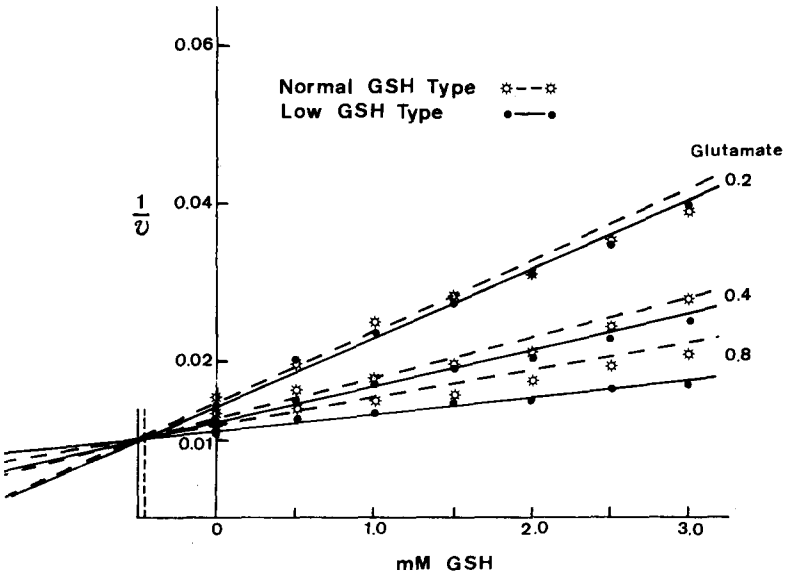
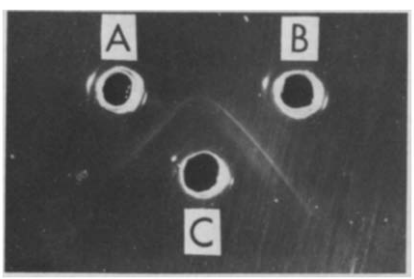


Fig. 6. $1/v$ vs. inhibitor concentration. 5.5 mM γ-Aminobutyrate and 1 mM ATP were kept constant with increasing concentrations of glutamate and varying concentrations of GSH as inhibitor. The vertical lines intersecting on the (—) x axis correspond to the K_i values; normal, 0.44 ± 0.055 mM; low, 0.50 ± 0.077 mM.

complexes with either enzyme variant that retained their enzyme activity. This indicated that the antigenic site differed from the active site. The antibody-enzyme complexes could be rapidly removed from solution by centrifugation.

Because of the cross-reactivity of both antibodies, both enzyme variants could



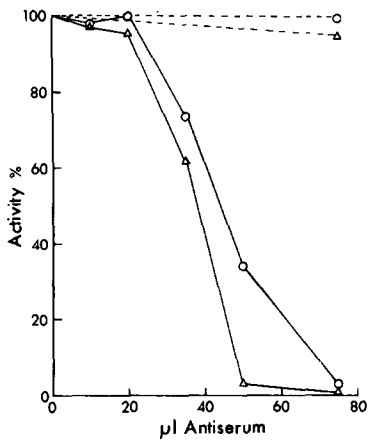


Fig. 7. Ouchterlony double-diffusion of γ-glutamylcysteine synthetase from normal (A) and low-GSH (B) sheep, against rabbit antiserum (C) raised to γ-glutamylcysteine synthetase purified from low-GSH sheep erythrocytes.

Fig. 8. Inactivation of γ-glutamylcysteine synthetase from normal (△) and low (○) GSH sheep erythrocytes by rabbit antiserum raised against normal γ-glutamylcysteine synthetase. Each point is the mean of three separate determinations. The broken lines indicate the effect of control rabbit serum obtained before immunization.

be titrated against either antibody. This property allowed us to determine if there is a difference in the specific activity of the two forms of enzyme. When equivalent activities of either normal or low-GSH γ-glutamylcysteine synthetase were combined with increasing quantities of antiserum, γ-glutamylcysteine synthetase activity was increasingly precipitated. Fig. 8 shows that a greater quantity of antiserum was required to remove γ-glutamylcysteine synthetase from low-GSH sheep. These data indicate that γ-glutamylcysteine synthetase from low-GSH sheep has a lower specific activity than γ-glutamylcysteine synthetase from normal sheep, assuming that equivalent quantities of antibody are required to complex either enzyme molecule. Similar data were obtained using either normal or low antiserum and enzyme purified separately from different individual sheep of each type.

Discussion

The most striking feature of this study was the similarity of the two forms of enzyme. Many properties frequently modified in enzyme variants were found to be unchanged when comparison was made between γ-glutamylcysteine synthetase from normal sheep and γ-glutamylcysteine synthetase from low-GHS sheep. Board et al. [5] previously reported no difference in the Michaelis-Menten constants (K_m), but their data, obtained from partially purified extracts, suggested a difference in the feedback inhibition of γ-glutamylcysteine synthetase by GSH. The present investigation of highly purified γ-glutamylcysteine synthetase failed to find any significant difference in the K_i for GSH.

The molecular weights of the γ-glutamylcysteine synthetase samples from both groups of sheep were found to be the same, suggesting that the difference between the two enzyme variants is probably restricted to a minor amino acid substitution. Wendel [11] reported the molecular weight of bovine erythrocyte γ-glutamylcysteine synthetase as 78 000, which is identical to our finding for the sheep enzyme.

The titration of both γ-glutamylcysteine synthetase samples against the same antiserum clearly demonstrates that γ-glutamylcysteine synthetase from low-GSH sheep has lower activity per molecule than γ-glutamylcysteine synthetase from normal sheep. Unfortunately, our data do not allow an accurate quantitative estimate of the difference in specific activity of the two enzyme variants. However, preliminary estimates suggest that the specific activity of γ-glutamylcysteine synthetase from low-GSH sheep is probably reduced by only approx. 25%. The observed differences in mean GSH concentration between the two groups of sheep is much greater, the GSH concentration in the low-GSH sheep being reduced by 60—70% [4]. This discrepancy suggests that some factor other than the difference in specific activity of γ-glutamylcysteine synthetase variants may contribute to the difference in the final equilibrium GSH concentration in vivo.

Of additional interest is the clear separation of γ-glutamylcysteine synthetase from glutathione synthetase on DEAE-cellulose (Fig. 1). Majerus et al. [6] reported some difficulty in separating these two enzymes from human erythrocytes and suggested that they may be closely associated in vivo, thereby

providing a unified site for glutathione synthesis. It has been postulated that a physical association between γ-glutamylcysteine synthetase and glutathione synthetase may allow glutathione synthesis to take place without the direct interaction of γ-glutamyl cyclotransferase, which can break down γ-glutamylcysteine to pyroglutamate and cysteine [12,13]. Our data indicate that, in sheep erythrocytes, γ-glutamylcysteine synthetase and glutathione synthetase are not firmly associated. Thus, in this case at least, some other mechanism must protect γ-glutamylcysteine from the action of γ-glutamyl cyclotransferase.

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