Studies on γ-Glutamyl Transpeptidase in Human and Rabbit Erythrocytes
By Satish K. Srivastava, Yogesh C. Awasthi, Steven P. Miller, A. Yoshida, and E. Beutler

γ-Glutamyl transpeptidase transfers the γ-glutamyl moiety of glutathione to a variety of acceptor amino acids. Through the operation of the γ-glutamyl–cyclotransferase cycle, this enzyme has been implicated in the transport of amino acids into cells, especially the cells of the proximal tubules of kidney. It has been reported to be present in rabbit erythrocytes. However, using white cell-free preparations, we have not been able to demonstrate the presence of γ-glutamyl transpeptidase in human or rabbit erythrocytes either by measuring the utilization of GSH or by following the formation of the product. Using similar conditions, we have been able to demonstrate the presence of γ-glutamyl transpeptidase in human and rabbit leukocytes and in human kidney. In contrast to a previous report, we were unable to find the accumulation of 5-oxoproline, an intermediate of the γ-glutamyl–cyclotransferase pathway in human red cells incubated in Krebs-Ringer solution. Immunologic studies demonstrated that human red cell membranes contained no protein antigenically similar to kidney γ-glutamyl transpeptidase. Thus our studies indicated that in human and rabbit erythrocytes, the γ-glutamyl transpeptidase–cyclotransferase pathway was not operative.

Glutathione is synthesized in the erythrocytes with a half-life of 2–4 days. However, the fate of glutathione is not clear. This tripeptide is not cleaved by red cell peptidases because of the presence of the γ-peptide bond; most of the peptidases are specific for α-peptide bonds. It has been suggested that the transport of oxidized glutathione from the red cells may account for at least some of the turnover rate of glutathione in erythrocytes.

γ-Glutamyl transpeptidase transfers the γ-glutamyl moiety from GSH to a variety of acceptor amino acids. This enzyme has been found to be present in various human tissues such as kidney, liver, lens epithelium, cornea, iris, and retina. Minimal activity of γ-glutamyl transpeptidase in human erythrocytes was reported to be present by Jackson, and Azzopardi and Jayle. Recently, Palekar, Tate, and Meister reported a considerably higher level of γ-glutamyl transpeptidase activity in rabbit erythrocytes. They demonstrated that rabbit erythrocytes lacked 5-oxoprolinase. Therefore, when erythrocytes were incubated for several hours, 5-oxoproline, a product of the γ-glutamyl peptide formed in the γ-glutamyl transferase reaction accumulated. In the present...
studies, we showed that \(\gamma\)-glutamyl transpeptidase was, in fact, absent from rabbit or human red cells and that 5-oxoproline did not accumulate. An extremely small amount of \(\gamma\)-glutamyl transpeptidase activity in the erythrocyte preparations was due to enzyme in the leukocytes which contaminated the washed red cells.

**MATERIAL AND METHODS**

\(\gamma\)-Glutamyl p-nitroanilide, GSH, GSSG, glutathione reductase (yeast), NADPH, and L-pyroglutamic acid were purchased from Sigma Chemical Co. \(^{14}\)\(^{C}\)-L-pyroglutamic acid was purchased from New England Nuclear Corp. All the other reagents used were of analytical grade. The samples were analyzed for radioactivity by using a Beckman liquid scintillation spectrometer Model L230. \(\gamma\)-Glutamyl transpeptidase activity was determined also by using \(\gamma\)-glutamyl p-nitroanilide as substrate.\(^{12}\)

Venous blood was drawn into heparin from the marginal ear vein of rabbits and from the arm of human subjects. The blood was centrifuged at 2,500 g for 20 min at 4°C, and the plasma and buffy coat were removed by aspiration. The red cells were washed three times with 5-7 volumes of buffered sodium chloride solution (potassium phosphate buffer pH 7.0, 0.1 M, 1 part + NaCl 0.145, M 9 parts) each time. Wherever specified, washed red cells were filtered through cotton wool to remove leukocytes.\(^{13}\) Red cells were suspended in an equal volume of 10 mM Tris-HCl buffer, pH 8.0, containing 80 mM MgCl\(_2\) and hemolyzed by sonication for 15 sec exactly as described by Palekar et al.\(^{11}\) The hemolyzate was centrifuged at 17,000 g for 20 min, and the membrane fraction was solubilized by sodium deoxycholate (1%) to make up the original volume of the hemolyzate. In about 10 min most of the membranes dissolved. This preparation was centrifuged either after 20 min, or after 4 hr, and the supernatant was used for the determination of \(\gamma\)-glutamyl transpeptidase activity. The reaction mixture, final volume 0.2 ml, contained 0.1 M Tris-HCl buffer, pH 8.5, 5 mM glutathione (adjusted to pH 8.5 by addition of Tris), 40 mM methionine and deoxycholate extract. Controls from which the enzyme or amino acid was separately omitted were included. The reaction mixture was incubated at 37°C for 0-60 min. At different time intervals 0.1 ml samples were withdrawn and added to 0.9 ml of 2% sulfoalicylic acid. The samples were centrifuged, and 0.5 ml of protein free supernatant solution was used for the determination of glutathione.\(^{14,15}\) In some cases, 0.1 ml samples were added to 1.5 ml of 5% trichloroacetic acid (TCA) for the determination of total acid soluble glutathione by glutathione reductase and NADPH\(^{16}\) after oxidation of acid soluble glutathione by bubbling oxygen through the solution in the presence of 1 mM CuSO\(_4\).\(^{12}\)

For the analysis of the reaction product, 5 mM L-\(^{14}\)C-methionine (10,000 counts/min per \(\mu\)mole) and 50 mM Tris-HCl buffer, pH 8.5, were used. Either acceptor amino acid or the enzyme was omitted from the controls. One milliliter of the reaction mixture was added to 1 ml of 10% TCA and centrifuged. TCA was extracted from the supernatant solution three times with three volumes of peroxide free ether. It was determined in separate experiments that methionine and \(\gamma\)-glutamyl-L-methionine were not extracted by ether and remained in the water phase. The samples were lyophilized and analyzed for methionine and \(\gamma\)-glutamyl-L-methionine using the amino acid analyzer. One-half of each fraction from the automatic amino acid analysis column was used for the determination of radioactivity and the other half was used for the amino acid analysis. To confirm the validity of the assay procedures, \(\gamma\)-glutamyl transpeptidase activity was also determined in a 5% homogenate of human kidney cortex.\(^*\)

\(^*\)Substantial decreases of GSH and methionine, accompanied by the formation of \(\gamma\)-glutamyl methionine, were observed in the case of kidney homogenate. Beside \(\gamma\)-glutamyl methionine, a larger quantity (several times that of \(\gamma\)-glutamyl methionine) of a peptide containing methionine was eluted from the column slightly faster than \(\gamma\)-glutamyl methionine. Acid hydrolysis of the peptide provided methionine sulfoxide and glutamic acid, suggesting that \(\gamma\)-glutamyl methionine produced might be oxidized during the incubation. However, incubation of an authentic \(\gamma\)-glutamyl methionine with the tissue extract did not induce any secondary modification of the peptide. The origin of the modified glutamyl methionine remains to be elucidated.
Formation of 5-oxoproline was followed as described by Palekar et al. A 50% suspension of three times washed red cells in Krebs-Ringer solution containing 5 mM glucose was incubated at 37°C for 7 hr in a Dubnoff shaker with about 50 oscillations per min. Aliquots were removed after 2, 4, and 7 hr of incubation for the determination of GSH and 5-oxoproline. In all the supernatants, 5 µl of 14C-5-oxoproline solution containing about 3 nmoles (100,000 cpm) were added as carrier, and the solution was passed through a Dowex-50 (hydrogen) column prepared in a Pasteur pipette. The elution was carried out with water and the eluate was lyophilized, dissolved in 0.2 ml of water, and subjected to descending paper chromatography (Whatman 3 MM thoroughly washed with distilled water) in a solvent system consisting of N-butanol-acetic acid-water, 4:1:1 (v/v). 14C-5-oxoproline was used as standard. The areas corresponding to 5-oxoproline were cut out and eluted with water. The eluate was passed through a Dowex-50 (chloride) column prepared in a Pasteur pipette. The elution of 5-oxoproline was carried out with 10 ml of 100 mM NaCl. The elution was followed by counting the radioactivity of 14C-5-oxoproline. The eluates were evaporated to 5 ml and an equal volume of 12 N HCl was added. The mixture was placed in a boiling water bath for 4 hr. The hydrolysates were lyophilized and analyzed for glutamic acid using a Beckman amino acid analyzer.

RESULTS

Using either γ-glutamyl p-nitroanilide or GSH as substrate and 14C-L-methionine as acceptor amino acid, a very small amount of γ-glutamyl transpeptidase activity was observed in three times washed erythrocytes (Table 1). However, after erythrocytes were filtered through cotton wool, practically no γ-glutamyl transpeptidase activity was observed in either human or rabbit erythrocytes. Rabbit and human leukocyte preparations were found to contain significant amounts of γ-glutamyl transpeptidase activity (Table 1). Under identical conditions, human kidney cortex was found to contain about 75 units of enzyme per gram wet weight using γ-glutamyl p-nitroanilide as substrate and about 58 units/g wet weight using GSH as substrate (Table 1). The results when GSH was used as substrate were confirmed by following the formation of γ-glutamyl-14C-L-methionine. No increase in the level of 5-oxoproline was observed during the 7-hr incubation period (Table 2). A minute amount of 5-oxoproline, however, was present even in the 0-hr sample. This finding may have been due to contamination of red cells with leukocytes or due to an arti-

<table>
<thead>
<tr>
<th>Table 1. γ-Glutamyl Transpeptidase Activity in Human and Rabbit Tissues</th>
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<tr>
<td><strong>γ-Glutamyl Transpeptidase Activity (Units)</strong></td>
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<tr>
<td><strong>γ-Glutamyl p-Nitroanilide as Substrate</strong></td>
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<tr>
<td><strong>GSH as Substrate</strong></td>
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<tr>
<td><strong>Sample</strong></td>
</tr>
<tr>
<td>1. Filtered RBC</td>
</tr>
<tr>
<td>2. RBC without buffy coat</td>
</tr>
<tr>
<td>3. RBC with buffy coat</td>
</tr>
<tr>
<td>4. Leukocytes</td>
</tr>
<tr>
<td>5. Kidney</td>
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</table>

*A unit of enzyme activity catalyzes the formation of 1 µmole of product per minute under the assay conditions. The results presented above were calculated by the determination of GSH. Almost identical results were obtained when total glutathione was determined after oxidation as GSSG by the enzymatic method.15,16
Table 2. Formation of 5-Oxoproline in Human Red Cells

<table>
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<tr>
<th>Incubation Time (hr)</th>
<th>nmoles/ml Red Cells</th>
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<tbody>
<tr>
<td></td>
<td>GSH</td>
</tr>
<tr>
<td>0</td>
<td>1902</td>
</tr>
<tr>
<td>2</td>
<td>1972</td>
</tr>
<tr>
<td>4</td>
<td>2016</td>
</tr>
<tr>
<td>7</td>
<td>2068</td>
</tr>
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Five milliliters three times washed red cells suspended in equal volume of Krebs–Ringer solution with 5 mM glucose. The samples were incubated at 37°C in a Dubnoff shaker. Aliquots were removed at various time intervals for the determination of GSH, GSSG, and 5-oxoproline as described in the text. To monitor recoveries, 200 nmoles of 14C-5-oxoproline were added just before the addition of trichloroacetic acid. About 90% of the added 5-oxoproline was recovered.

A difference of 5–10 nmoles 5-oxoproline per milliliter red cells between samples drawn at different time intervals represented experimental deviation. Under identical conditions Palekar et al. reported the formation of about 160 nmoles of 5-oxoproline per milliliter red cells in 8 hr.

Antiserum raised in rabbits against an apparently homogeneous preparation of human kidney γ-glutamyl transpeptidase was used to determine the presence of γ-glutamyl transpeptidase in human red cells. This antiserum precipitated γ-glutamyl transpeptidase activity from the kidney, lens, iris, and retina. Red cell membrane γ-glutamyl transpeptidase” was extracted with sodium deoxycholate in 0.1 M Tris-HCl buffer, pH 8.0 as described in Materials and Methods. Using the reported levels of γ-glutamyl transpeptidase of about 300 mU/ml packed red cells, the equivalent of 18 mU of enzyme activity was mixed with an equal amount of the enzyme purified from the

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Fig. 1. The experimental tubes contained 18 mU of red cell γ-glutamyl transpeptidase’ (calculated according to Palekar et al.11), 18 mU of kidney γ-glutamyl transpeptidase, and various amounts of antiserum in 20 mM potassium phosphate buffer, pH 7.0, in a total volume of 0.4 ml. The control contained no red cell extract. The samples were incubated overnight at 4°C and centrifuged at 20,000 g at 4°C. γ-Glutamyl transpeptidase activity was determined in the supernatant by using γ-glutamyl p-nitroanilide. The parallel slopes of enzyme activity (without a shift in the curve) indicate that no red cell membrane protein cross-reacted immunologically with the kidney γ-glutamyl transpeptidase antiserum.
GLUTAMYL TRANSPEPTIDASE

The control samples contained only kidney enzyme. The samples were incubated overnight at 4°C with various amounts of antisera in a total volume of 0.4 ml. The samples were subsequently centrifuged for 1 hr at 20,000 g at 4°C, and the enzyme activity was determined by using γ-glutamyl p-nitroanilide as described by Szasz. The enzyme activity precipitation curve presented in Fig. 1 for the kidney enzyme was almost the same after the addition of red cell ghost extract. This finding would indicate that there was no protein in the red cells which was immunologically similar to kidney γ-glutamyl transpeptidase.

DISCUSSION

Recently, Palekar et al. reported the presence of a substantial amount of γ-glutamyl transpeptidase activity in rabbit erythrocytes. They also observed the accumulation of 5-oxoproline in the incubation system containing washed erythrocytes and Krebs-Ringer solution. This finding was attributed to the lack of 5-oxoprolinase in erythrocytes. Using exactly the same methods as described by Palekar et al., we were not able to demonstrate γ-glutamyl transpeptidase activity in either human or rabbit erythrocytes or the accumulation of 5-oxoproline. We were, however, able to find a considerable amount of γ-glutamyl transpeptidase in human leukocytes and kidney and in human cultured lens epithelium cells, iris and retina.

Washing of red cells only removes approximately 60%–90% of the leukocytes. It is likely that some of the activity which Palekar et al. observed was, in fact, leukocyte enzyme. However, the activity they have reported, i.e., 300 mU/ml erythrocytes, cannot be accounted for entirely by the contamination of washed erythrocytes with leukocytes, and the reason for this discrepancy is unclear.

No accumulation of 5-oxoproline was observed when human erythrocytes were incubated for 8 hr in Krebs–Ringer solution. These data were further supported by the maintenance of GSH levels in the red cells for 7 hr in Krebs–Ringer solution at 37°C. If γ-glutamyl transpeptidase activity were present in the red cells, one would expect a decrease in the GSH level during 7 hr of incubation at 37°C with a simultaneous increase in the 5-oxoproline content. The immunologic studies also demonstrated that red cells do not have an antigen similar to the kidney γ-glutamyl transpeptidase.

Since there is no detectable γ-glutamyl transpeptidase activity in human or rabbit erythrocytes, the γ-glutamyl transpeptidase–cyclotransferase pathway proposed by Meister for the transport of amino acids into the cells cannot be operative in the erythrocytes. Thus, the turnover rate of glutathione in erythrocytes must be explained by some other mechanism, possibly by the transport of oxidized glutathione from red cells as proposed earlier.

REFERENCES

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cyte glutathione flux to the oxidized glutathione transport. J Lab Clin Med 83:444, 1974
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