Glutathione-Dependent Protection Against Oxidative Damage of the Human Red Cell Membrane

By S. Fujii, G. L. Dale, and E. Beutler

Glutathione (GSH) dependent protection against oxidative damage of human red cell membrane was examined. An artificial system was used in which chloroform/methanol-extracted red cell lipids, in the form of liposomes, were subjected to attack by a peroxidation system consisting of ascorbate-Fe^{3+}. Human erythrocytes contained a nondialyzable factor, completely inactivated by heating in a boiling water bath for 3 min, which showed GSH-dependent inhibition against lipid peroxidation and was devoid of GSH peroxidase activity. On the other hand, GSH-S transferase, highly purified by affinity chromatography, had no inhibitory activity. These findings strongly indicate that the GSH-dependent protection against lipid peroxidation of human red cell membrane is mediated by one or more proteins other than GSH peroxidase and GSH-S transferase.

REDUCED GLUTATHIONE (GSH) is an important constituent of erythrocytes. Its destruction precedes hemolysis of glucose-6-phosphate dehydrogenase deficient red cells, and hereditary deficiencies in the enzymes of GSH synthesis are associated with shortened erythrocyte lifespan. GSH serves as a substrate for the enzyme GSH peroxidase (E.C. 1.11.1.9), and it has been suggested that it is through the activity of this enzyme that GSH protects the red cell against oxidative damage. However, attempts to incriminate GSH peroxidase deficiency as the cause for oxidative hemolysis have been unconvincing. Patients with lowered red cell GSH peroxidase activity accompanied by hemolytic anemia manifested only a modest decrement of enzyme activity. The enzyme activity in such patients is no lower than that in healthy persons of Oriental or Mediterranean origin who inherit a gene for low GSH peroxidase activity or of healthy New Zealanders whose selenium intake is low.

GSH peroxidase utilizes many organic hydroperoxides as well as H$_2$O$_2$ as substrates. It has generally been believed that the protective effect of GSH against oxidative breakdown of lipids is mediated through GSH peroxidase by the reduction of endogenously formed hydroperoxides of unsaturated fatty acids to hydroxy derivatives. However, observations of McCay et al. on peroxidation of rat liver microsomes suggest that the GSH-dependent cytosolic factor, which inhibits lipid peroxidation, does so by preventing the peroxidation rather than by reducing lipid peroxides through another enzyme. Chromatographic studies by Burk et al. suggested that the protective effect of GSH against formation of malonyldialdehyde in rat liver preparations may be mediated by GSH-S transferase (E.C. 2.5.1.18), which cannot utilize H$_2$O$_2$. It has recently been reported that pig liver and heart contained a protein distinct from known GSH peroxidase and GSH-S transferase, which inhibits peroxidation in the presence of GSH.

In light of these observations, it seemed important to reevaluate factors that may protect against oxidative damage of the red cell membrane. We describe the presence in erythrocytes of a GSH-dependent enzyme that has an inhibitory effect against peroxidation of human erythrocyte membrane lipids. This factor differs from known GSH peroxidase and GSH-S transferase enzymes.

MATERIALS AND METHODS

Preparation of Liposomes

Two hundred milliliters of fresh human blood was drawn into heparin and freed of white cells, as reported previously. Red cells were washed with normal saline 3 times and lysed in 3 volumes of water containing 0.7 mM 2-mercaptoethanol. Red cell membranes were sedimented by centrifugation at 20,000 g for 45 min. The supernatant stroma-free hemolysate was used for enzyme purification, and the membranes were added to 2:1 chloroform:methanol and lipid extraction was performed as described by Folch et al. The extracted lipids were stored in chloroform-methanol solution. Before use, they were taken to dryness under nitrogen gas in a test tube. One milliliter of 0.15 M potassium phosphate buffer, pH 7.4, per 10 mg of lipids was added to the tube, followed by vigorous shaking. The liposomes formed were sonicated for 30 sec at 4°C.

Peroxidation of Liposomes

The incubation system for lipid peroxidation was composed of 0.15 M potassium phosphate buffer, pH 7.4, containing approximately 0.5 mg liposomes/ml buffer, 0.2 mM ascorbate, and 20 μM FeCl$_3$ in the absence and presence of 2.5 mM GSH. Incubation was carried out at 37°C in a shaking bath under room air.
dehydro formed was measured by the method described by Bieri and Anderson. In one experiment, the P02 was measured with an oxygen electrode during the reaction. Oxygen consumption was calculated from the known solubility of oxygen in water.

**GSH Peroxidase Assay**

GSH peroxidase activity was determined using t-butyl hydroperoxide as the substrate.15

**GSH-S Transferase Assay**

GSH-S transferase activity was assayed using 1-chloro-2,4-dinitrobenzene as the substrate, as reported previously.16

**Protein Measurements**

Protein concentration was measured by the method of Lowry et al.11

**GSH-S Transferase Purification Using Epoxy-Activated Sepharose 6B Coupled With GSH**

Fifty milliliters of stroma-free hemolysate was dialyzed overnight against 29 mM potassium phosphate buffer, pH 7.2, at 4°C, and GSH-S transferase was purified as described elsewhere.16,18 The purified enzyme contained no GSH peroxidase activity. It was stored at −20°C in 0.15 M potassium phosphate buffer, pH 7.4, containing 30% glycerol and 1.0 mM EDTA without significant loss of activity for several months. Before use, the enzyme was dialyzed extensively against 10 mM potassium phosphate buffer, pH 7.0, to remove completely EDTA, which interferes with malonyldialdehyde formation by the ascorbate-ferric ion peroxidation system.

**RESULTS**

**Lipid Peroxidation and Its Inhibition**

Figure 1 shows that the time course of malonyldialdehyde formation was linear during the first 10 min. Accordingly, the incubations were carried out for 10 min in subsequent experiments.

Figure 2 demonstrates the inhibition of malonyldialdehyde formation by the crude enzyme. Inhibition was observed only in the presence of both GSH and the crude enzyme. The effect of heating this crude enzyme preparation on its ability to protect against lipid perox-

<table>
<thead>
<tr>
<th>Addition to Peroxidation System</th>
<th>O2 Consumed (nmol/ml/10 min)</th>
<th>MDA Formed (nmol/ml/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>51.4</td>
<td>0</td>
</tr>
<tr>
<td>Liposomes</td>
<td>47.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Liposomes + GSH</td>
<td>100.7</td>
<td>3.8</td>
</tr>
<tr>
<td>Liposomes + GSH + E</td>
<td>52.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Liposomes + GSH + heated E</td>
<td>93.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

E: Crude enzyme prepared in Fig. 2 (100 µl/ml of reaction mixture). Heated E: Crude enzyme was heated in a boiling water bath for 3 min.

GSH, reduced glutathione; MDA, malonyldialdehyde.
dialyzable, heat-labile factor(s) that protect against lipid peroxidation.

Partial Purification of the GSH-Dependent Inhibitory Factor

Two hundred milliliters of stroma-free hemolysate was dialyzed against 3 changes of 10 volumes of 10 mM phosphate buffer, pH 7.0, containing 0.7 mM 2-mercaptoethanol. After dialysis, the pH of the hemolysate was adjusted to 7.0. Ethylene glycol and Triton X-100 were added to the hemolysate to final concentrations of 5% (v/v) and 0.1% (w/v), respectively. The hemolysate was applied to a (1.5 x 17 cm) DE 52 column, equilibrated with 10 mM phosphate buffer, pH 7.0, containing 0.7 mM 2-mercaptoethanol, 5% (v/v) ethylene glycol, and 0.1% Triton X-100, and washed extensively with the same buffer. No hemoglobin remained on the column. Elution was performed with a 600-ml linear gradient of 0–0.3 M KCl in the same buffer; 4-ml fractions were collected, and GSH peroxidase activity and protein content of each fraction was measured. To assay inhibitory activity against lipid peroxidation, sets of five fractions were pooled and reabsorbed on small columns (bed volume: 2 ml) of DE 52 after the conductivity was adjusted by the addition of H2O to the same as that of 10 mM phosphate buffer. The columns were washed with 10 mM phosphate buffer, containing 0.7 mM 2-mercaptoethanol, in order to remove Triton X-100, which interferes with malonyldialdehyde formation by ascorbate-ferric iron peroxidation system. Each column was eluted with 10 mM phosphate buffer, pH 7.0, containing 0.3 M KCl. Solid ammonium sulfate was added to each eluate to 90% saturation. Each pellet was dissolved in the same volume of 10 mM phosphate buffer, pH 7.0, dialyzed against the same buffer, and tested for GSH-dependent inhibitory factor against lipid peroxidation. As shown in Fig. 3, the fractions containing inhibitory activity were separate from the major peak of GSH peroxidase activity. The active fractions were pooled, and ammonium sulfate was added to give a saturation of 45%. Most of the GSH peroxidase activity was precipitated. Ammonium sulfate was added to provide 90% saturation. The pellet, which contained the inhibitory activity, was dissolved in 10 mM phosphate buffer, pH 7.0, dialyzed against the same buffer, and applied to Sephadex G75 (1.5 x 30 cm). The elution profile is depicted in Fig. 4. A small peak of GSH peroxidase activity was followed by a high broad peak of GSH-dependent inhibitory activity, with a partial overlap. The inhibitory activity was clearly distinct from GSH peroxidase activity.

However, the active fractions after G75 gel filtration still contained a considerable amount of GSH-S transferase activity. Therefore, the effect of highly purified GSH-S transferase on peroxidation of liposomes was examined (Table 2). Even large amounts of GSH-S transferase had little effect on malonyldialdehyde formation in the presence of GSH, whereas the active fractions after G75 filtration showed a remarkable inhibition in the presence of GSH in spite of its low GSH-S transferase activity. The further addition of purified GSH-S transferase did not enhance the inhibition induced by the active fractions alone. Increasing the amount of the active fractions, on the other hand, brought about further inhibition of malonyldialdehyde formation.

![Fig. 3. Chromatographic profile of GSH peroxidase activity and GSH-dependent inhibitory activity against malonyldialdehyde formation obtained by fractionation of hemolysate on a DE 52 column. The volume of each fraction was 4 ml. (O—O) GSH peroxidase activity; (●—●) protein concentration as measured by the method of Lowry et al. The percent inhibition of malonyldialdehyde (MDA) formation (□) in the presence of the fractions and 2.5 mM GSH is shown.](image)

![Fig. 4. The elution pattern of GSH-dependent inhibitory activity from a Sephadex G75 column. The inhibitory activity is graphed as shown in Fig. 3. (O—O) GSH peroxidase activity; elution of the proteins (●—●) was determined by measuring absorbance at 280 nm.](image)
Table 3. Substrate Specificity of the Inhibitory Factor

<table>
<thead>
<tr>
<th>Addition to Peroxidation System</th>
<th>GSH Formed (%)</th>
<th>With GSH</th>
<th>Without GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 mM mercaptan alone</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 mM mercaptan + IF</td>
<td>82.7</td>
<td>37.4</td>
<td></td>
</tr>
<tr>
<td>1.0 mM mercaptan alone</td>
<td>87.1</td>
<td>41.7</td>
<td></td>
</tr>
<tr>
<td>1.0 mM mercaptan + IF</td>
<td>87.1</td>
<td>41.7</td>
<td></td>
</tr>
<tr>
<td>0.5 mM mercaptan alone</td>
<td>95.7</td>
<td>44.6</td>
<td></td>
</tr>
<tr>
<td>0.5 mM mercaptan + IF</td>
<td>95.7</td>
<td>44.6</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. The Effect of Highly Purified GSH-S Transferase on Malonyldialdehyde Formation

<table>
<thead>
<tr>
<th>Addition to Peroxidation System</th>
<th>MDA Formed (%)</th>
<th>With GSH</th>
<th>Without GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) None</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2) GSH (2.5 mM)</td>
<td>100</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>(3) Pure GSH-S-T (a) (2.8U)</td>
<td>88.8</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>(4) Pure GSH-S-T (2.8U) + GSH</td>
<td>83.2</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>(5) Crude GSH-S-T (b) (0.28U)</td>
<td>100</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>(6) Crude GSH-S-T (0.28U) + GSH</td>
<td>54.5</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>(7) Pure GSH-S-T (1.4U) + crude GSH-S-T (0.28U)</td>
<td>90.7</td>
<td>16.8</td>
<td></td>
</tr>
<tr>
<td>(8) Pure GSH-S-T (1.4U) + crude GSH-S-T (0.28U) + GSH</td>
<td>93.5</td>
<td>16.8</td>
<td></td>
</tr>
<tr>
<td>(9) Crude GSH-S-T (1.7U) + GSH</td>
<td>90.7</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>(10) Crude GSH-S-T (1.7U) + GSH</td>
<td>16.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Highly purified GSH-S transferase (pure GSH-S-T) (a) was obtained by affinity chromatography using epoxy-activated Sepharose 6B, as described in Materials and Methods. Crude GSH-S-T (b): GSH-S transferase activity contained in the active fractions pooled after G75 gel filtration shown in Fig. 4. MDA: 100% = 5.7 nmole of malonyldialdehyde/ml of reaction mixture.

nyldialdehyde formation. These results suggest that a GSH-dependent inhibitory activity is different from known GSH-S transferase activity.

Using the active fractions pooled after G75 gel filtration, the substrate specificity of the inhibitory activity for thiol reagents was studied (Table 3). In the presence of 2.5 mM 2-mercaptoethanol, malonyldialdehyde formation was more inhibited than in the presence of 2.5 mM GSH. The extent of inhibition was almost the same in 0.5 mM 2-mercaptoethanol and 0.5 mM GSH. Cysteine inhibited malonyldialdehyde formation even in the absence of the factor, and such inhibition was enhanced further in its presence.

Protection against lipid peroxidation by the inhibitory factor was also examined at increased concentrations of ascorbate and FeCl3. As shown in Fig. 5, the increase in their concentration did not prevent GSH-dependent inhibition.

DISCUSSION

It has been generally accepted that protection against the oxidative damage of red cell membrane lipids is mediated by the reduction of endogenously formed hydroperoxides of lipids to hydroxy derivatives through GSH peroxidase. However, McCay et al.7 and Burk et al.9 suggested that the rat liver cytosolic factor, which protects against peroxidation of microsomal lipids in the presence of GSH, is not GSH peroxidase. The former group showed that peroxides of microsomal lipids were very poor substrates for GSH peroxidase activity and suggested that the protection was conferred by prevention of peroxidation rather than by reducing lipids hydroperoxides formed. On the contrary, Ursini et al.10 purified a cytosolic inhibitory protein from pig liver and heart and found it to be distinct from both known GSH peroxidases and GSH-S transferases. This enzyme exhibited GSH peroxidase activity on hydroperoxide groups present in polyunsaturated fatty acid residues of phosphatidylcholine liposomes as well as with cumene and t-butyl hydroperoxides as substrates.

In our studies, as shown in Fig. 3, the main peak of GSH peroxidase activity after DE 52 chromatography manifested no inhibitory effect against malonyldialdehyde formation. The peak of GSH-dependent inhibitory factor contained no GSH peroxidase activity with MDA, Malonyldialdehyde (100% = 6.6 nmole malonyldialdehyde/ml of reaction mixture).
t-butyl hydroperoxide as substrate (Fig. 4). In our system, GSH peroxidase is clearly not the enzyme responsible for the protection against lipid peroxidation of red cell membrane.

Our results (Table 2) strongly suggest that GSH-S transferase is also not the protective factor. This conclusion is consistent with those of McCay et al. and Ursini et al., but it contradicts the observation of Burk et al. Purification by epoxy-activated Sepharose 6B has been reported to be effective on multiple forms of GSH-S transferases from human and rat livers, and one from human red cells. Therefore, it seems unlikely that there exists another form of GSH-S transferase in human red cells that does not bind to this affinity chromatography column. In addition, several isozymes from rat liver, one from human red cells, and one from bovine lens are reported to be inactive when such mercaptans as 2-mercaptoethanol and t-cysteine replace GSH, whereas in our peroxidation inhibition (Table 3), these reagents were able to replace equimolar quantities of GSH. These findings make it very unlikely that GSH-dependent inhibition against lipid peroxidation is due to an as yet unidentified isozyme of GSH-S transferases in red cells. Thus far, we have been unable to purify the protective factor sufficiently to be certain whether it represents a single protein or whether multiple factors are required, as has been suggested previously in the case of liver.

Lipid peroxidation is considered to occur in several steps, via free radical formation, peroxidative attack upon lipids by free radicals formed, and subsequent lipid peroxidation, initiated by attack of free radicals and degradation of peroxidized lipids. Which step is involved in the GSH-dependent protection by the inhibitory factor described here is unknown. As Fig. 5 showed that the increase in concentration of ascorbate and FeCl₃ has no effect on the degree of the inhibition against lipid peroxidation, it would seem apparent that the inhibition is not due to direct interaction with ascorbate or iron itself. The elucidation of the mechanism will require the further purification and characterization of the inhibitory factor.

Red cells are known to generate hydrogen peroxide by various mechanisms, such as the interaction between ascorbate and oxyhemoglobin and the decomposition of oxygen anion by superoxide dismutase. It may be that GSH peroxidase plays a role in the defense mechanism by converting H₂O₂ to water, as discussed by McCay et al. However, even greatly lowered levels of red cell GSH peroxidase are not associated with hemolytic anemia, and it may be that the novel factor(s) that we have described is(are) important in protecting cells against peroxidation. The inhibitory factor(s) may account for the fact that decreased GSH peroxidase activity is well tolerated.

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thione peroxidase activity on phosphatidylcholine hydroperoxides. Biochim Biophys Acta 710:197, 1982
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