Regulatory Mechanism of Glutathione Reductase Activity in Human Red Cells

By Yoshihito Yawata and Kouichi R. Tanaka

The mechanism by which glutathione reductase (GR) activity is regulated in relation to flavin metabolism was studied in red cells of normal adults, cord blood, and patients with severe metabolic disorders using spectrophotometric, fluorometric, and radiochemical methods. The increased activity of GR in red cells of adult patients with severe hepatic cirrhosis, chronic uremia, and glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is related to the increased percent saturation of GR with flavin adenine dinucleotide (FAD), and increased red cell flavin level. The percent saturation of GR with FAD correlates with (1) the degree of clinical severity in hepatic cirrhosis or chronic uremia, and (2) total flavin level in red cells. Thus, the increased GR activity in these patients may be regulated actively or passively by the increased flavin level in red cells. This suggests that the increased requirement for enhanced activity of the pentose phosphate pathway may affect GR metabolism secondarily, leading to the association of GR with FAD through an increased uptake of riboflavin. In contrast to the results in adult red cells, cord erythrocytes show an unexpected metabolic pattern of GR and flavin metabolism. Although the total GR activity of cord red cells is considerably higher than in adult red cells, cord red cell GR is only partly saturated with FAD even in the presence of a significantly increased flavin level in cord red cells. Thus, cord red cells may have an additional control mechanism of GR activity.

Glutathione reductase (GR) plays an important role in protecting red cell protein from oxidation. Thus, studies on the mechanisms by which this enzyme is regulated in human red cells are of considerable interest.

Increased activity of GR occurs in red cells of patients with severe uremia, hepatic cirrhosis, glucose-6-phosphate dehydrogenase (G-6-PD) deficiency, and in cord blood. However, the exact mechanism for the increased GR activity in these various states has not been elucidated.

GR is present in at least two forms, an active form associated with flavin adenine dinucleotide (FAD), and an inactive form not bound to FAD. The presence of intermediate form(s) of GR has also been suggested. Extracellular riboflavin may be incorporated into flavin compounds within red cells. Thus, increased GR activity might result from a disproportionately large portion of the enzyme protein being present in the FAD bound (activated) form in the presence of an elevated total flavin level in red cells. The present studies indicate...
that this is so in severe uremia, hepatic cirrhosis, and also in G-6-PD deficiency, but not in cord erythrocytes.

MATERIALS AND METHODS

Venous blood from adults or cord blood was anticoagulated with heparin, centrifuged at 3000 rpm for 15 min, and the plasma and buffy coat were discarded. Red cells were washed three times with cold 0.15 M saline solution, and resuspended in cold 0.15 M saline or in 0.15 M phosphate-buffered saline (pH 7.4) to a concentration of approximately 3 x 10⁶ cells/cu mm as previously described. Hemolysates were prepared by freezing and thawing three times a 1-to-10 dilution of washed red cells in water. All experiments were performed using fresh red cells.

Determination of GR Activity in Hemolysates

GR activity in hemolysates was assayed at 37°C by a slight modification of the method of Long and Carson. The assay system consisted of 0.2 ml of hemolysate, 2.2 ml of 0.177 M Tris-0.0435 M ethylenediaminetetraacetate (EDTA) buffer (pH 7.6), and 0.5 ml of 0.0318 M oxidized glutathione (GSSG). Reagents were added and incubated in order every 10 min, and the reaction was started by adding 0.1 ml of 8.7 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH). The reaction was recorded at 340 mc in the Gilford Automatic Spectrophotometer Model 2000. Activity was expressed in international units, IU, as μmoles NADPH oxidized per min per 10¹⁰ red cells. Total GR activity in hemolysates was measured by preincubating hemolysates with 1 μM FAD at 37°C in the Tris-EDTA buffer for 10 min, before adding other assay reagents. Maximum activation of GR activity in hemolysates by FAD was obtained under these conditions. Further incubation of hemolysates with FAD in higher concentrations up to 1 mM did not increase GR activity. The amount of the inactive form of GR represents the increase in GR activity after incubation with FAD. The fraction of GR associated with FAD (saturation percentage) was calculated by dividing the GR activity of the untreated hemolysate (active form) by the total GR activity (after incubation with FAD). NADPH, GSSG, FAD, and riboflavin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Other chemicals were reagent grade.

Determination of Flavin Compounds in Plasma and Red Cells

The amount of free riboflavin and flavin mononucleotide (FMN), and of FAD in plasma or red cells was determined fluorometrically using a slight modification of the method of Burch et al. Briefly, heparinized blood was centrifuged, plasma separated, and theuffy coat discarded. Packed red cells were suspended in cold 0.15 M saline without washing at a hematocrit of 50%. Plasma or red cell suspension was deproteinized with ice-cold 20% trichloroacetic acid. Standard riboflavin solution was added to the mixture before deproteinization to assess recovery. After centrifugation, the supernatant was diluted with 0.2 M K₂HPO₄ solution. Fluorescence was determined in triplicate before and after the addition of 10% NaOH in 5% NaHCO₃ using a Turner Fluorometer Model 110 with Corning Filters 5-58 as a primary, and 3-70 as a secondary, respectively. Another aliquot of the supernate was hydrolyzed by incubation at 37°C in the dark overnight.

Uptake of ¹⁴C-Riboflavin by Red Cells

Packed red cells prepared as above were washed three times with cold phosphate-buffered saline with glucose (250 mg glucose in 100 ml of a solution consisting of 1 part of 0.15 M potassium phosphate buffer, pH 7.4, and 9 parts of 0.15 M saline) and resuspended to a hematocrit of 30. Two milliliters of this red cell suspension were incubated at 37°C with 50 µl of 10 µM ¹⁴C-riboflavin solution (1.46 μCi/ml). At zero time and after 5 hr of incubation, 0.3-ml aliquots were removed, and the washed red cells were dried on a 2.1-cm Whatman No. 1 filter paper and radioactivity of red cells on the filter paper was determined according to a slight modification of the method of Lockner utilizing the Packard Liquid Scintillation Counter. Quenching was corrected for using a standard solution of ¹⁴C-riboflavin in each determination.
**GLUTATHIONE REDUCTASE ACTIVITY**

**Effect of Methylene Blue or Sodium Ascorbate on Per Cent Saturation of GR With FAD in Red Cells**

Washed red cells, suspended in the phosphate-buffered saline with glucose, were incubated at 37°C up to 16 hr with 1 μM riboflavin in the presence of methylene blue (12 x 10^-6 M) or sodium ascorbate (12 x 10^-3 M adjusted to pH 7.4). GR activity in the hemolysate of washed red cells was determined with or without the addition of 1 μM FAD, and per cent saturation of GR was calculated.

**Effect of Methylene Blue or Sodium Ascorbate on Uptake of ^14^C-Riboflavin by Red Cells**

Two milliliters of washed red cells, suspended in the buffer (a hematocrit of 30), were incubated up to 16 hr with 50 μl of 10 μM ^14^C-riboflavin solution (1.46 μCi per ml) with no further additive, or with 40 μl of 12 x 10^-6 M methylene blue or 20 μl of 12 x 10^-3 M sodium ascorbate (pH 7.4). Red cell radioactivity following incubation was determined as described above.

**RESULTS**

**Adult Red Cells**

**Effect of FAD on activation of GR in red cells of patients with severe hepatic cirrhosis, severe uremia, or G-6-PD deficiency.** The results of glutathione reductase assays in hemolysates with and without FAD are shown in Table 1. From these data, it is apparent that glutathione reductase is increased as well as almost completely saturated with FAD in red cells of patients with severe cirrhosis, uremia, or G-6-PD deficiency in comparison to normal adult erythrocytes. The degree of reticulocytosis does not correlate with per cent saturation of GR with FAD in red cells (not shown).

**Stability of the active and the inactive forms of GR in red cells.** The stability of the active and the inactive forms of GR was examined in red cells of 36 patients with increased GR activity. During storage of the red cell suspension with physiological saline at 4°C for as long as 10 days, or incubation of the hemolysates with the assay medium at 37°C, no significant changes were noted in total GR activity and in the degree of saturation with FAD. This suggests that the apoenzyme of GR without FAD is quite stable and that the binding of FAD to GR is not easily reversed under these conditions.

<table>
<thead>
<tr>
<th>No of Subjects</th>
<th>Glutathione Reductase Activity (IU)*</th>
<th>Per Cent Saturation of GR With FAD (1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Active Form</td>
<td>Inactive Form</td>
</tr>
<tr>
<td>Normal adults</td>
<td>30</td>
<td>3.04 ± 0.36</td>
</tr>
<tr>
<td>Severe cirrhosis of liver</td>
<td>11</td>
<td>4.70 ± 0.50</td>
</tr>
<tr>
<td>Severe chronic uremia</td>
<td>15</td>
<td>4.81 ± 0.52</td>
</tr>
<tr>
<td>G-6-PD deficiency</td>
<td>5</td>
<td>5.77 ± 0.63</td>
</tr>
<tr>
<td>White with hemolysis</td>
<td>6</td>
<td>4.88 ± 0.40</td>
</tr>
<tr>
<td>Black males without hemolysis</td>
<td>11</td>
<td>5.24 ± 0.63</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>5.45 ± 0.51</td>
</tr>
<tr>
<td>Normal cord blood</td>
<td>15</td>
<td>5.45 ± 0.51</td>
</tr>
</tbody>
</table>

*Mean ± SD, expressed as μ moles NADPH oxidized per min per 10^10 red cells.
Correlation between GR activation by FAD and clinical severity of uremia and cirrhosis. The extent of GR activation by FAD was compared to the clinical severity of chronic uremia and cirrhosis of the liver. Blood urea nitrogen (BUN) level was selected as a measure of the clinical severity in ten patients with uremia, and total bilirubin level in 13 patients with hepatic cirrhosis, respectively. Figure 1 demonstrates a linear relationship between the BUN or total bilirubin level and per cent saturation of GR with FAD.

Content of flavin compounds in red cells. The concentrations of free plus flavin mononucleotide fraction (RF), FAD, and total flavin compounds in red cells are shown in Fig. 2. A markedly increased mean content of these flavin compounds was observed in red cells of 11 patients with hepatic cirrhosis, of ten patients with chronic uremia, and of four patients with G-6-PD deficiency. None of these subjects, including normals, had received riboflavin therapeutically.

Content of flavin compounds in plasma. Flavin compounds were determined in plasma derived from the same whole blood specimens as for red cell flavin determinations. Results are shown in Fig. 3. A significant increase of these flavin compounds was observed in plasma of patients with hepatic cirrhosis and with chronic uremia. In contrast, in white patients with G-6-PD deficiency, the amount of flavin compounds in plasma was normal.

Correlation between total flavins in red cells and per cent saturation of GR with FAD. Figure 4 demonstrates that there is good correlation \( r = 0.723, p < 0.01 \) between the total flavin level in red cells and per cent saturation of GR with FAD.
GLUTATHIONE REDUCTASE ACTIVITY

Fig. 2. Content of flavin compounds in red cells. Concentration of flavin compounds was determined fluorometrically in red cells of ten normal adults, 11 patients with cirrhosis of liver, ten patients with chronic uremia, and four patients with G-6-PD deficiency. RF represents the concentration of free riboflavin and flavin mononucleotide together, FAD indicates flavin adenine dinucleotide, and TOTAL represents the sum of RF plus FAD. Mean values and standard deviation are shown.

Fig. 3. Content of flavin compounds in plasma. Concentration of flavin compounds was determined fluorometrically in plasma derived from the same blood in which flavin level in red cells was determined. RF, FAD, and TOTAL represent the concentration of flavin compounds as shown in Fig. 2.
Correlation between total flavin level in red cells and in plasma. As shown in Fig. 5, there is good correlation ($r = 0.748, p < 0.01$) between the total flavin level in red cells and the level in plasma from ten normal adults, 11 patients with cirrhosis, and ten patients with uremia not on riboflavin supplementation.

Uptake of $^{14}$C-riboflavin by red cells of normal adults, and patients with hepatic cirrhosis and chronic uremia. The mean value ($\pm$ S D) for uptake of $^{14}$C-riboflavin after 5 hr of incubation was $0.31 \pm 0.04$ m$m$oles riboflavin incorporated per ml of red cells in normal adults, and $0.25 \pm 0.05$ in eight patients with uremia or hepatic cirrhosis, respectively. In red cells of two patients with severe uremia and of two patients with severe cirrhosis with marked increases in red cell flavins, the uptake of radioactive riboflavin was decreased to approximately 50%-70% of that observed in the normal subjects.

Effect of methylene blue or sodium ascorbate on per cent saturation of GR with FAD in normal adult red cells. Red cells were incubated with methylene blue or sodium ascorbate in the presence of riboflavin. The per cent saturation of GR with FAD was increased in the presence of riboflavin whether an activator was added or not (Table 2). However, the rate of increase of per cent saturation of GR with FAD was greater in the presence of an activator.

Effect of methylene blue or sodium ascorbate on uptake of $^{14}$C-riboflavin in normal adult red cells. Red cells were incubated with radioactive riboflavin in
Experiments were performed in duplicate on red cells from six different individuals, and average values are shown. See text for details.

GLUTATHIONE REDUCTASE ACTIVITY

Fig. 5. Correlation between total flavin level in red cells and in plasma of normal adults, and patients with cirrhosis of liver and with chronic uremia. Number of subjects studied are shown in parenthesis.

The presence or absence of methylene blue or sodium ascorbate, 14C-riboflavin was incorporated into normal adult red cells incubated with or without an activator of the pentose phosphate pathway. However, the uptake of radioactive riboflavin was increased to a greater degree in those red cells incubated with an activator. The uptake of 14C-riboflavin by red cells of eight subjects was increased 26% ± 9% and 23% ± 5% with methylene blue and sodium ascorbate, respectively.

Cord Red Cells

GR activity and per cent saturation of GR. The activity of GR (the active form) in cord red cells (5.45 IU) is considerably greater than that in normal

Table 2. Effect of Methylene Blue or Sodium Ascorbate on Per Cent Saturation of GR With FAD in Red Cells

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Without Riboflavin</th>
<th>With Riboflavin (1μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Additive</td>
<td>Methylene Blue</td>
</tr>
<tr>
<td>1</td>
<td>64.6</td>
<td>63.8</td>
</tr>
<tr>
<td>2</td>
<td>65.5</td>
<td>66.1</td>
</tr>
<tr>
<td>3</td>
<td>67.2</td>
<td>66.6</td>
</tr>
<tr>
<td>4</td>
<td>58.3</td>
<td>58.0</td>
</tr>
<tr>
<td>5</td>
<td>63.5</td>
<td>62.9</td>
</tr>
<tr>
<td>6</td>
<td>61.9</td>
<td>63.2</td>
</tr>
</tbody>
</table>

*Experiments were performed in duplicate on red cells from six different individuals, and average values are shown. See text for details.
adult red cells (3.04 IU), and is almost equal to the total GR activity (5.21 ± 5.51 IU) observed in adult patients with metabolic disorders, as shown in Table 1. However, FAD could further activate GR markedly to the level of 7.06 IU. It is also to be noted that GR in cord red cells is only partly saturated with FAD (77.2%), even with the clearly high level of the total activity of GR.

**Content of flavin compounds in plasma and red cells of cord blood.** The amount of flavin compounds in plasma and in red cells was determined in cord blood without supplementation of the vitamin. Figure 6 shows a marked increase of flavin compounds in plasma and red cells of cord blood compared to normal adults.

**Incorporation of \(^{14}\text{C}\)-riboflavin into cord red cells.** The mean value (± S D) for incorporation of \(^{14}\text{C}\)-riboflavin into cord red cells was 0.29 ± 0.03 μmole of riboflavin incorporated per ml of red cells after 5 hr of incubation. Thus, there is no difference between normal adult and cord red cells with respect to incorporation of riboflavin.

**DISCUSSION**

Increased GR activity has been observed in the red cells of patients with hepatic cirrhosis, uremia, and G-6-PD deficiency. Surprisingly, there is, in these patients, only a slight increase in total activity of GR, and the increased GR activity is primarily due to a marked increase in the per cent saturation of GR with FAD, that is, most of the enzyme is in its active form. Therefore, GR is almost completely saturated with FAD in the red cells of these patients.

In addition, it is known that red cells of patients with hepatic cirrhosis or chronic uremia are subject to severe metabolic stress. Since it has been suggested that GR may function to maintain the integrity of red cells against metabolic oxidation, the regulatory mechanism of GR activity is of increasing interest in these red cells of patients with severe metabolic disorders.
It was found that the degree of GR activation with FAD correlates well with the degree of clinical severity of these patients, independent of the young mean red cell population.

It has been reported that flavin level in red cells is affected by the plasma flavin level, and that extracellular riboflavin can be incorporated into red cells and converted to FAD enzymatically. Therefore, it may be suggested that the level of flavin compounds in plasma or in red cells may regulate the degree of association of GR with FAD in red cells. In red cells of patients with hepatic cirrhosis, chronic uremia, and G-6-PD deficiency, flavin levels were markedly increased, corresponding to the increased per cent saturation of GR with FAD in these patients. There are at least two possibilities concerning the increase of flavin content in red cells. It may be due to a passive incorporation of increased plasma flavins, or due to the increased selective uptake of riboflavin in plasma by red cells, even though the amount of flavin compounds is not elevated in plasma. Because of these considerations the amount of flavin compounds in plasma of normal adults and these patients was determined.

It is noteworthy that the amount of flavins in plasma is significantly increased in patients with hepatic cirrhosis, and extremely elevated in patients with uremia, in spite of no riboflavin supplementation in these patients. Furthermore, there is a linear relationship between total flavin level in red cells and that in plasma in normal adults and in these patients. Thus, the increased red cell flavin compounds in patients with cirrhosis or uremia may be due to the increased flavin compounds in plasma. In addition, it is interesting that uptake of riboflavin by red cells is decreased rather than increased in these patients, especially in the severest cases. This may be interpreted as a feedback mechanism due to the marked increase of intracellular flavin compounds in red cells.

On the other hand, it is known that red cells of patients with G-6-PD deficiency represent a metabolic defect in the activity of the pentose phosphate pathway (PPP). In patients with G-6-PD deficiency, an increased saturation percentage of GR with FAD and an enhanced level of FAD in red cells have been described. We have also found that the amount of other flavin compounds, in addition to FAD, is markedly increased in these red cells. It is postulated that red cells of patients with G-6-PD deficiency can maintain the integrity of their function by the maximum association of GR with FAD due to marked increase of red cell flavins.

However, the amount of flavins in plasma was not increased in G-6-PD deficiency. This suggests that a different mechanism is operative in these red cells. Therefore, it is suggested that the increased amount of flavins in red cells may be due to the increased active uptake of riboflavin by red cells in order to enhance GR activity in red cells even at the normal level of plasma flavins. In patients with G-6-PD deficiency, there may be an increased requirement for the enhanced activity of the PPP, due to the metabolic defect of the enzyme. In addition, some uremic red cells demonstrate a defect of carbon-2-glucose recycling through the PPP. Thus, the defect of the shunt activity might lead to increased GR activity due to a compensatory mechanism. This may be the case in G-6-PD deficiency. In G-6-PD deficiency, as well as in some cases of hepatic cirrhosis without renal failure, increased red cell flavins cannot be explained by such retention of flavins. Thus, it is suggested that an active concentrating
mechanism in these red cells may exist and serves to increase riboflavin uptake from the plasma and thereby to increase flavin coenzyme levels in these cells. Our results of the effect of the increased activity of the PPP on the degree of the association of GR with FAD in red cells may suggest the presence of the mechanism. There is a significant increase of per cent saturation of GR with FAD in red cells incubated with riboflavin plus an activator of the PPP (methylene blue or sodium ascorbate) over that with riboflavin alone, through the significantly increased uptake of extracellular riboflavin by red cells. Thus, although it has been shown that glutathione reductase itself may not play a regulatory role in the PPP, the present results suggest that the increased requirement of the PPP might affect secondarily the degree of the association of GR with FAD.

Our results in human adult red cells may be summarized schematically as shown in Fig. 7. Mature adult red cells cannot synthesize enzyme protein to meet increased needs. However, increased GR activity can be achieved by activation of the GR apoenzyme through mechanisms which increase passively or actively the amount of the cofactor, FAD.

In contrast to these results in human adult red cells, red cells of human cord blood show a peculiar metabolic pattern of GR and flavin metabolism.

The active form of GR in cord red cells is considerably higher than in normal adult red cells. The level of the active form in cord red cells is almost equal to the total GR activity observed in adult patients with the metabolic disorders. Furthermore, the total GR activity in cord red cells is clearly higher than the total GR activity in normal or abnormal adult red cells. It should be noted that GR in cord red cells is only partially saturated with FAD, in spite of a marked increase of flavin compounds in plasma and red cells. This suggests that a different mechanism for regulation of GR activity should be considered for cord red cells. Although we have no direct evidence, our recent results of kinetic
properties of GR in intact cord red cells suggest the existence of functionally less active GR enzyme in cord red cells. Thus, GR activity in cord red cells might be controlled by additional unknown mechanisms other than flavin metabolism.

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REFERENCES

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