Glycolate Kinase Activity in Human Red Cells

By Shinya Fujii and Ernest Beutler

Human red cells manifest glycolate kinase activity. This activity copurifies with pyruvate kinase and is decreased in the red cells of subjects with hereditary pyruvate kinase deficiency. Glycolate kinase activity was detected in the presence of FDP or glucose-1,6-P₂. In the presence of 1 mmol/L FDP, the Kₘ for adenosine triphosphate (ATP) was 0.28 mmol/L and a half maximum velocity for glycolate was obtained at 40 mmol/L. The pH optimum of the reaction was over 10.5. With 10 μmol/L FDP, 500 μmol/L glucose-1,6-P₂, 2 mmol/L ATP, 5 mmol/L MgCl₂, and 50 mmol/L glycolate at pH 7.5, glycolate kinase activity was calculated to be approximately 0.0013 U/ml RBC. In view of this low activity even in the presence of massive amounts of glycolate, the glycolate kinase reaction cannot account for the maintenance of the reported phosphoglycolate level in human red cells.

THE IMPORTANCE of 2,3-diphosphoglycerate (2,3-DPG) in the regulation of oxygen affinity of hemoglobin has been documented repeatedly. The level of 2,3-DPG in the red cells is controlled by a single, bifunctional enzyme that catalyzes both its synthesis and hydrolysis (E.C. 2.7.5.4 1,3-diphosphoglycerate mutase and 2,3-diphosphoglycerate phosphatase). The latter activity is strongly activated by phosphoglycolate,¹ which has been reported to be present at a concentration of approximately 2.5 μmol/L in human red cells,²³ although conflicting results have been reported.⁴⁻⁵ Human red cells contain phosphoglycolate phosphatase.⁶⁻⁹ It has recently been demonstrated that there is no correlation between the phosphoglycolate phosphatase activity and 2,3-DPG level in the red cells of anemic patients, suggesting that the modulation of phosphoglycolate phosphatase activity does not control the level of 2,3-DPG in human red cells.⁰ Therefore, it may be that the formation of phosphoglycolate regulates 2,3-DPG levels in red cells.

Purified pyruvate kinase from rabbit muscle¹¹ and yeast¹² have been demonstrated to have glycolate kinase activity in the presence of adenosine triphosphate (ATP). The incubation of red cells with glycolate was reported to result in the rapid decrease of 2,3-DPG.¹³ It was suggested that this was due to the activation of 2,3-DPG phosphatase by phosphoglycolate synthesized through the phosphorylation of glycolate by pyruvate kinase.

However, there have been no reports of the capacity of human red cell enzymes to phosphorylate glycolate. Muscle-type pyruvate kinase is known to be different in its kinetic, immunologic, and structural properties from red cell–type pyruvate kinase.¹⁴ Although yeast pyruvate kinase is reported to be similar to liver-type pyruvate kinase and red cell–type pyruvate kinase, no details of the kinetics of glycolate phosphorylation by that enzyme have been reported. We have now investigated the possible role of human red cell–type glycolate kinase as the possible physiologic pathway to generate phosphoglycolate.

MATERIALS AND METHODS

Partial Purification of Human Red Cell Glycolate Kinase

Partially purified enzyme was used, rather than a homogeneous preparation, because structural changes have been reported to occur during the total purification procedure of red cell pyruvate kinase.¹⁵⁻¹⁶ Partial purification was performed according to the method for the partial purification of pyruvate kinase recommended by the International Committee for Standardization in Haematology.¹⁷

Glycolate Kinase Assay

Glycolate kinase activity was determined by measuring the formation of adenosine diphosphate (ADP) from ATP in the presence of glycolate. The standard reaction mixture of 0.5 mL contained 100 mmol/L TRIS-HCl buffer, pH 8.0, 0.5 mmol/L EDTA, 100 mmol/L KCl, 15 mmol/L MgCl₂, 5 mmol/L ATP, 1 mmol/L FDP, 25 mmol/L glycolate, and the enzyme (2 IU/mL when measured as pyruvate kinase activity). The ATP and glycolic acid had been neutralized with solid TRIS. Because it was linear for the first 30 minutes, incubation was for 30 minutes at 37 °C. After 30 minutes' incubation, 0.49 mL of ADP assay mixture was added, a baseline recording obtained, and the ADP-measuring reaction started with 10 μL of 250 mmol/L PEP. The ADP assay mixture was composed of 100 mmol/L TRIS-HCl, pH 8.0, 0.5 mmol/L EDTA, 4 mmol/L NADH, 10 IU/mL lactate dehydrogenase (Sigma Chemical Co, St Louis), and 10 IU/mL pyruvate kinase (Sigma). The blank system contained no glycolate.

When pH dependence was studied, TRIS-HCl buffer in the reaction mixture was replaced by 50 mmol/L TRIS, 50 mmol/L
Fig 1. Relative activity of pyruvate kinase and glycolate kinase in the various fractions prepared by ammonium sulfate. The fractions were obtained from membrane-free hemolysate. Activities are expressed relative to that in the fraction of 20% to 40% saturation. PK, pyruvate kinase activity; GK, glycolate kinase activity.

Fig 2. DE 52 chromatography of the partially purified enzyme (20% to 40% fraction prepared by ammonium sulfate fractionation) applied to a DEAE cellulose DE 52) column preequilibrated with 10 mmol/L potassium phosphate buffer, pH 7.0, containing 1 mmol/L 2-mercaptoethanol and 0.1 mmol/L EDTA. After washing with the buffer, elution was performed with a 600-mL linear gradient of 0 to 0.3 mol/L KCl in the same buffer. 5-mL fractions were collected, and pyruvate kinase activity, glycolate kinase activity, and protein content were measured.

Fig 3. Effect of FDP concentration on glycolate kinase activity.

Table 1. Glycolate Kinase Activity in Partially Purified Pyruvate Kinase From Pyruvate Kinase–Deficient Donors

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Donor C.W.</th>
<th>Donor B.P.</th>
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</thead>
<tbody>
<tr>
<td>PK activity in the hemolysate</td>
<td>15.5 U/g Hb</td>
<td>6.13 U/g Hb</td>
<td>5.13 U/g Hb</td>
</tr>
<tr>
<td>PK activity in the partially purified preparation</td>
<td>40.0 U/mL</td>
<td>19.3 U/mL</td>
<td>2.3 U/mL</td>
</tr>
<tr>
<td>GK activity in the partially purified preparation</td>
<td>0.059 U/mL</td>
<td>0.026 U/mL</td>
<td>&lt;0.0001 U/mL</td>
</tr>
<tr>
<td>GK/PK x 100</td>
<td>0.14%</td>
<td>0.135%</td>
<td>&lt;0.003%</td>
</tr>
</tbody>
</table>

The partially purified preparations from the normal control and the patients were obtained by the method described in the Materials and Methods section.

PK, pyruvate kinase; GK, glycolate kinase.

(0.14%) of glycolate kinase activity to pyruvate kinase activity as that of the normal control. However, a mutant (B.P.) with 33% of normal pyruvate kinase activity showed no detectable glycolate kinase activity.

RESULTS

Relationship to Pyruvate Kinase

Figure 1 shows pyruvate kinase and glycolate kinase activities in the various preparations from hemolysate obtained by ammonium sulfate fractionation. Most of both activities were present in the fraction of 20% to 40% saturation. Figure 2 shows DE 52 column chromatography of the fraction of 20% to 40% saturation shown in Fig 1. Glycolate kinase activity was coeluted with pyruvate kinase activity.

The red cells of two subjects with a hereditary decrease in pyruvate kinase activity were examined (Table 1). A mutant (C.W.) with 40% of normal pyruvate kinase activity showed the same ratio

glycine, and 50 mmol/L maleate buffer adjusted with KOH or HCl from pH 5.5 to 10.5, as reported previously. Production of phosphoglycolate through the reaction was verified using [γ-32P]-ATP by the method described previously.

Effect of FDP

Figure 3 shows the effect of the concentration of FDP. No activity was observed in the absence of FDP, a finding that is consistent with the results reported using yeast pyruvate kinase. Maximum velocity was observed at 100 μmol/L FDP.

Effect of Glucose-1,6-P2

Figure 4 shows the effect of the concentration of glucose-1,6-P2 on glycolate kinase activity. In the absence of glucose-1,6-P2, no activity was detected. At 500 μmol/L glucose-1,6-P2, glycolate kinase was stimulated to 60% of the maximum. The maximum velocity was obtained at 2 mmol/L.
Other Kinetic Properties

Figure 5 shows the effect of the concentration of ATP. A $K_m$ of 0.28 mmol/L was computed. Figure 6 shows the function of the concentration of glycolate. A half maximum velocity was obtained at about 40 mmol/L. Figure 7 shows the pH dependence of glycolate kinase activity. The glycolate kinase was much more active in the very alkaline range. The activity at pH 7.5 was only 6% of that at pH 10.5. Dependence of glycolate kinase on divalent metal ions was also examined (data not given). The enzyme showed 10 to 20 times higher activity in the presence of 15 mmol/L MnCl₂ than in the presence of 15 mmol/L MgCl₂.

MnCl₂ was an effective activator of the enzyme even at the concentrations of only 2 to 10 μmol/L (the physiologic concentration = 4 μmol/L). However, in the presence of the physiologic concentrations of $Mg^{2+}$ (5 mmol/L), further addition of even 10 μmol/L MnCl₂ had little effect on the activity.

DISCUSSION

Although purified pyruvate kinase from rabbit muscle and yeast manifest glycolate kinase activity, the physiologic importance of this in vitro reaction was uncertain. Phosphoglycolate was shown to be present in the red cells by some investigators. Therefore, it seemed reasonable to speculate that phosphoglycolate might be synthesized in human red cells from glycolate by pyruvate kinase. We examined the properties of human red cell glycolate kinase to determine whether it might be the source of the putative red cell phosphoglycolate.

We have now demonstrated that human red cells contain glycolate kinase activity. As shown in Figs 1 and 2, most of pyruvate kinase and glycolate kinase activities emerged in the same preparation fractionated by 20% to 40% saturation of ammonium sulfate and comigrated on the chromatography. In addition to the fact that rabbit muscle and yeast pyruvate kinases have a glycolate kinase activity, these results suggest that glycolate kinase reaction is catalyzed by red cell pyruvate kinase. This conclusion is further confirmed by the fact that partially purified preparations from donors with pyruvate kinase deficiency manifested correspondingly decreased glycolate kinase activity (Table I).

As shown in Fig 7, the optimal pH for red cell glycolate kinase is high, well beyond the physiologic range. About 40 mmol/L glycolate was required to obtain the half maximum velocity. The exact intracellular concentration of glycolate is unknown but is clearly several orders of magnitude lower. Taking into account the physiologic concentrations of FDP and glucose-1,6-P₂, our calculation indicated that human red cell glycolate kinase showed only 0.02% of pyruvate kinase activity under conditions of 10 μmol/L FDP, 500 μmol/L glucose-1,6-P₂, 2 mmol/L ATP, 50 mmol/L glycolate, 5 mmol/L MgCl₂, and pH 7.5. Because pyruvate kinase activity in human red cells is about 5 U/mL RBC, glycolate kinase activity is com-
GLYCOLATE KINASE ACTIVITY

483

puted as 0.0013 U/mL RBC even in the presence of 50 mmol/L glycolate. At a 100-μmol/L concentration of glycolate, then, the rate of formation of phosphoglycolate would be only $2.6 \times 10^{-12}$ mol/min/mL RBC. Phosphoglycolate phosphatase activity measured under conditions similar to those in the human red cell is about 0.2 to 0.4 U/mL RBC. With a $K_m$ of 0.76 mmol/L, a steady state would be achieved at a phosphoglycolate concentration of 0.005 to 0.01 mol/L. From these results, it seems unlikely that human red cell glycolate kinase has physiologic importance for the synthesis of phosphoglycolate, although there may be the possibility that an as-yet-unknown effector activates the enzyme sufficiently strongly to maintain the phosphoglycolate level.

Our present work suggests that the pathway other than glycolate kinase reaction may exist to account for the maintenance of phosphoglycolate level in human red cells.

REFERENCES


Glycolate kinase activity in human red cells

S Fujii and E Beutler