Abnormal Phosphoenolpyruvate Transport in Erythrocytes of Hereditary Spherocytosis

By Hiroshi Ideguchi, Naotaka Hamasaki, and Yukio Ikehara

The rate of phosphoenolpyruvate transport in erythrocytes from patients with hereditary spherocytosis and from healthy individuals was examined in a 0.1 M citrate buffer (pH 6.1 at 37°C) containing 10 mM phosphoenolpyruvate and 10 mM NaF. The rate in erythrocytes from patients with hereditary spherocytosis was 0.09 ± 0.02 μmole/min/ml of cells (mean ± SD, n = 8), whereas the rate in erythrocytes from healthy individuals was 0.23 ± 0.03 μmole/min/ml of cells (mean ± SD, n = 8). The lower rate of transport in erythrocytes of hereditary spherocytosis could not be ascribed to a different glycolytic response to NaF or to a difference in cell age distribution. This phenomenon seemed to be a reflection of some abnormality in the erythrocyte membrane from patients with hereditary spherocytosis.

MATERIALS AND METHODS

Erythrocyte Incubation

The diagnosis of HS was based on clinical features, hematologic data, and family histories. Eight cases from seven different families were examined. Patients had not been splenectomized. Erythrocytes from patients and normal controls were collected in the presence of heparin, washed 3 times with 15 volumes of 0.15 M NaCl containing 5 mM glucose, and once with 15 volumes of an incubation buffer (0.1 M citrate, 10 mM NaF, and 5 mM glucose, pH 6.1 at 37°C). The washed cells were used immediately for experiments. The cells were reincubated with the incubation buffer at 37°C for about 5 min (hematocrit 11%). The incubation was started by the addition of 0.1 M PEP adjusted to pH 6.1. The final PEP concentration and pH of the erythrocyte suspension were 10.3 ± 0.3 mM (mean ± SD, n = 16) and 6.04 ± 0.04 (mean ± SD, n = 6), respectively, at the beginning of the incubation. Erythrocyte suspensions were taken at specified time intervals and directly deproteinized with ice-cold 0.6 M HClO4. The extracts were kept in ice and partially neutralized to pH 2-3 with 5 M KOH. Glycolytic intermediates such as pyruvate, triosephosphates, and fructose 1,6-bisphosphate in the extracts were measured immediately and other glycolytic intermediates except PEP were measured on the next day. The levels of glycolytic intermediates were expressed as μmole/ml of cells, but in the case of pyruvate or lactate the expression means μmole of pyruvate or lactate produced per milliliter of cells in whole suspensions during the incubation, because pyruvate and lactate rapidly penetrate the erythrocyte membrane in the incubation conditions.

For PEP analysis, the erythrocyte suspension was taken at specified time intervals and the supernatant was separated by centrifugation. The supernatant was used for extracellular PEP analysis. The erythrocyte fraction was washed with 20 volumes of ice-cold incubation buffer containing 1 mM 4-acetamido-4'-isothiocyanatosilbene-2,2'-disulphonic acid and resuspended in the incubation buffer (hematocrit about 10%). The resuspended erythrocyte fraction was deproteinized with 0.6 M HClO4. The process of separating and washing the erythrocyte fraction was done as quickly as possible (5-7 min) to minimize the amount of PEP incorporated into the erythrocytes that may be metabolized or leak out. The extract was neutralized to pH 6-7 by 5 M KOH and used for intracellular PEP analysis.

Analytical Procedure

Glycolytic intermediates were measured enzymatically. Because of the high citrate concentration in the samples, extra MgCl2 was added to the assay system for PEP. Hemoglobin was measured by the methods of Drabkin. Hematologic data were performed by standard laboratory methods.
**Reagents**

All enzymes and glycolytic intermediates except PEP were from C.F. Boehringer und Soehne, Mannheim, Germany. PEP (monopotassium salt) was from Sigma Chemical Co., St. Louis, Mo. 4-Acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid was purchased from Nakarai Chemical LTD, Kyoto, Japan. Other reagents were of analytical reagent grade.

**RESULTS**

**Decreased Rate of PEP Transport in Erythrocytes of Hereditary Spherocytosis**

Figure 1 shows the changes in PEP concentrations of erythrocytes from patients with HS and normal erythrocytes when these cells were incubated with 10.4 ± 0.3 mM of PEP (mean ± SD, n = 6) in a 0.1 M citrate buffer (10 mM NaF, 5 mM glucose, 0.1 M sodium citrate, pH 6.1 at 37°C). The hematocrit of the erythrocyte suspension was 11.2% ± 0.6% (mean ± SD, n = 6) at the beginning of incubation, and no hemolysis occurred during the incubation. The initial pH of HS and normal erythrocyte suspensions was 6.04 ± 0.04 at 37°C (n = 6) and changes of pH in the cell suspensions were less than 0.04 pH unit during the incubation.

If the incubation is carried out in the presence of NaF, the transport rate can be determined from the increment of PEP gained by the erythrocytes, since PEP utilization in the pyruvate kinase reaction would be inhibited by the limited supply of ADP caused by the inhibitory effects of NaF on glycolysis and membrane adenosine triphosphatase. Under these conditions, intracellular PEP increased linearly for 30 min and the transport rate calculated from the PEP accumulation was 0.08 ± 0.01 μmole/min/ml of cells (n = 3) in erythrocytes from patients with HS, whereas the rate in normal erythrocytes was 0.27 ± 0.02 μmole/min/ml of cells (n = 3). However, if the inhibitory effects of NaF were incomplete in HS erythrocytes, the transported PEP would be metabolized to 2,3-bisphosphoglycerate and pyruvate, and the transport rate determined from the increment of PEP remaining inside the cells would be underestimated.

### Table 1. Increases in Concentration of Glycolytic Intermediates During Incubation With or Without PEP for 30 min at 37°C (Mean ± SD, n = 3)

<table>
<thead>
<tr>
<th></th>
<th>Glucose-6-P to 2-Phosphoglycerate (μmole/ml of Cells)</th>
<th>PEP (μmole/ml of Cells)</th>
<th>Pyruvate and Lactate (μmole/ml of Cells)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal</strong></td>
<td></td>
<td></td>
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<tr>
<td>PEP (+)</td>
<td>0.47 ± 0.11</td>
<td>7.94 ± 0.46</td>
<td>1.42 ± 0.10</td>
</tr>
<tr>
<td>PEP (-)</td>
<td>0.35 ± 0.08</td>
<td>-0.01</td>
<td>0.10 ± 0.06</td>
</tr>
<tr>
<td><strong>HS</strong></td>
<td></td>
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<td></td>
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<tr>
<td>PEP (+)</td>
<td>0.83 ± 0.40</td>
<td>2.28 ± 0.31</td>
<td>2.91 ± 0.30</td>
</tr>
<tr>
<td>PEP (-)</td>
<td>0.73 ± 0.12</td>
<td>-0.01</td>
<td>0.08 ± 0.02</td>
</tr>
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</table>

*The levels of glycolytic intermediates were expressed as μmole/ml of cells, but in the case of pyruvate and lactate, the expression means μmoles of pyruvate and lactate produced per milliliter of cells in whole suspensions during the incubation (see text).

Washed erythrocytes were preincubated with the incubation buffer for 5 min at 37°C, and the incubation was started with or without PEP addition. Erythrocyte suspensions were taken at 0, 15, and 30 min and deproteinized with 2 volumes of ice-cold 0.6 M HClO₄. The extracts were kept in ice and partially neutralized to pH 2–3 with 5 M KOH. Glycolytic intermediates such as pyruvate, triose phosphates, and fructose 1,6-bisphosphate, in the extracts were measured immediately and other glycolytic intermediates except PEP were measured on the next day. For PEP analysis, the extracts were prepared as described in Materials and Methods.
In order to examine this possibility, all of the glycolytic intermediates except 1,3-bisphosphoglycerate in the whole suspension were determined when the cells were incubated with or without PEP. Intra- and extracellular PEP concentrations were also determined as described in Materials and Methods. Table 1 shows the increases in concentration of the glycolytic intermediates that took place during a 30-min incubation. In both erythrocytes from HS patients and from normal individuals, the increase in concentration of intermediates from glucose-6-phosphate to 2-phosphoglycerate was almost the same during the incubation with PEP as during the incubation without PEP. This means that the inhibitory effect of NaF on enolase and thereby on glycolysis was sufficient in both erythrocytes from patients and their normal controls. In erythrocytes from HS patients, however, more PEP transported was metabolized to pyruvate and lactate than in normal erythrocytes, suggesting that more ADP was supplied to the pyruvate kinase reaction in erythrocytes from these patients than in normal erythrocytes. In this incubation condition, pyruvate and lactate formed during the incubation quickly equilibrated inside and outside the cells so that to get an accurate accounting of the transported PEP it is necessary to take into consideration not only the intracellular PEP, but also the amounts of pyruvate and lactate in the whole erythrocytes suspension during the incubation.

It appears, therefore, that PEP transport should be measured by the sum of the PEP increase in erythrocytes and the pyruvate and lactate increases in whole erythrocyte suspension during the incubation. The true transport rate calculated by this procedure in HS erythrocytes was 0.17 \pm 0.01 \text{ mmole/min/ml of cells} \left( n = 3 \right) and was almost half of the rate in normal erythrocytes, which was 0.31 \pm 0.01 \text{ mmole/min/ml of cells} \left( n = 3 \right). The lower PEP transport in HS cells was also verified by measuring decreases of extracellular PEP concentration during a 30-min incubation, though the transport rate calculated by the decrements was less accurate than the rate calculated by increments of intracellular PEP. The decrease of extracellular PEP concentration during a 30-min incubation was 1.4 \pm 0.1 \text{ mM} \left( n = 3 \right) in the normal cell suspension, while it was 0.9 \pm 0.1 \text{ mM} \left( n = 3 \right) in the HS cell suspension.

The PEP transport in both normal and HS erythrocytes was completely inhibited with 1 \text{ mM 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid} (Fig. 1). The possibility that a portion of PEP incorporated into the cells may leak out during the process of washing the erythrocyte fraction was eliminated by washing the cells with ice-cold incubation buffer containing 1 \text{ mM} of the inhibitor.

Although the reduced PEP transport rate in HS erythrocytes was exaggerated by only measuring the PEP increment during the incubation, the apparent transport rate was useful as a screening device for the presence of an abnormal PEP transport system. Accordingly, we have determined the PEP transport rate from the PEP increment in the following experiments.

**PEP Transport Rates in Erythrocytes From Patients With Various Anemias**

It is natural to assume that the erythrocyte population in HS, particularly in erythrocytes from unsplen-

### Table 2. PEP Transport Rates in Erythrocytes From Patients With Various Anemias

<table>
<thead>
<tr>
<th>Reticulocytes (%)</th>
<th>Transport Rate (\text{\mu m mole/min/ml of Cells})</th>
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<tbody>
<tr>
<td>Normal control (n = 8)</td>
<td>0.23 \pm 0.03 (mean \pm SD)</td>
</tr>
<tr>
<td>Hereditary spherocytosis (unsplenectomized)</td>
<td>\begin{tabular}{l} 1.7 \text{ (mean \pm SD)} \ 3.3 \text{ (mean \pm SD)} \ 3.5 \text{ (mean \pm SD)} \ 3.7 \text{ (mean \pm SD)} \ 4.0 \text{ (mean \pm SD)} \ 6.0 \text{ (mean \pm SD)} \ 16.5 \text{ (mean \pm SD)} \ 16.2 \text{ (mean \pm SD)} \end{tabular}</td>
</tr>
<tr>
<td>Autoimmune hemolytic anemia</td>
<td>\begin{tabular}{l} 1.1 \text{ (mean \pm SD)} \ 1.9 \text{ (mean \pm SD)} \ 5.0 \text{ (mean \pm SD)} \end{tabular}</td>
</tr>
<tr>
<td>Rh incompatibility</td>
<td>\begin{tabular}{l} 8.8 \text{ (mean \pm SD)} \end{tabular}</td>
</tr>
<tr>
<td>Paroxysmal nocturnal hemoglobinuria</td>
<td>\begin{tabular}{l} 1.2 \text{ (mean \pm SD)} \end{tabular}</td>
</tr>
<tr>
<td>(\beta)-thalassemia</td>
<td>\begin{tabular}{l} 1.2 \text{ (mean \pm SD)} \end{tabular}</td>
</tr>
<tr>
<td>Aplastic anemia</td>
<td>\begin{tabular}{l} 0.2 \text{ (mean \pm SD)} \ 0.7 \text{ (mean \pm SD)} \end{tabular}</td>
</tr>
<tr>
<td>Others</td>
<td>\begin{tabular}{l} 6.2 \text{ (mean \pm SD)} \end{tabular}</td>
</tr>
<tr>
<td>Iron deficiency anemia</td>
<td>\begin{tabular}{l} 1.6 \text{ (mean \pm SD)} \ 2.4 \text{ (mean \pm SD)} \ 2.9 \text{ (mean \pm SD)} \ 4.0 \text{ (mean \pm SD)} \ 5.5 \text{ (mean \pm SD)} \end{tabular}</td>
</tr>
<tr>
<td>1 wk after iron treatment</td>
<td>\begin{tabular}{l} 0.3 \text{ (mean \pm SD)} \ 1.6 \text{ (mean \pm SD)} \end{tabular}</td>
</tr>
<tr>
<td>3 mo after iron treatment</td>
<td>\begin{tabular}{l} 0.24 \text{ (mean \pm SD)} \end{tabular}</td>
</tr>
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</table>

Experimental details are given in the text.
ectomized patients, is relatively younger than the cell population in normal individuals. The decreased rate of the PEP transport in erythrocytes of HS could therefore be a phenomenon commonly observed in a younger cell population. Thus, the transport rates in erythrocytes of patients with various anemias, which are expected to have relatively a younger erythrocyte population, were determined (Table 2). The rate in HS erythrocytes was 0.09 ± 0.02 μmole/min/ml of cells (n = 8), whereas the rate in normal erythrocytes was 0.23 ± 0.03 μmole/min/ml of cells (n = 8). The rates in erythrocytes from patients with autoimmune hemolytic anemia, Rh incompatibility, paroxysmal nocturnal hemoglobinuria, β-thalassemia, and aplastic anemia were all within normal range.

We also examined the transport rate in erythrocytes of iron deficiency anemia, whose erythrocytes are believed to be younger than the cells of normal individuals. The younger cell population of erythrocytes from these patients was confirmed by separating the cells using the differences of their density (data were not shown) and hematologic data. The PEP transport rate in erythrocytes from patients with iron deficiency anemia was within the normal range and was not changed by normalizing the cell age distribution to that of healthy individuals by iron treatment. In about a week after the beginning of iron treatment (reticulocyte level: 1.6%-5.5%), the transport rate of erythrocytes from these patients was 0.24 ± 0.01 μmole/min/ml of cells (n = 5) and 3 mo after the treatment was started, the transport rates in erythrocytes from two of these patients whose hematologic data were nearly normalized to those of healthy individuals, were 0.22 and 0.24 μmole/min/ml of cells (Table 2).

DISCUSSION

HS is an autosomal dominant disorder characterized by anemia, spherocytic erythrocytes, and increased osmotic fragility of the cells. The existence of a primary defect in the cell membrane is suggested by experimental evidence, such as abnormal composition of membrane proteins, reduced membrane protein phosphorylation, and abnormal lipid composition. None of these results, however, has been accepted generally and only the evidence that the HS erythrocyte membrane is abnormally permeable to sodium has been confirmed. Despite the controversy as to the basic defect in the cell membrane, the prevailing working concept appears to hold that a membrane abnormality exists in HS erythrocytes. The present investigation showed that there was a lower rate of PEP transport in erythrocytes from patients with HS, when this rate was determined in a 0.1 M citrate buffer with 10 mM PEP and 10 mM NaF at pH 6.04 and 37°C.

The lower rate of transport in HS erythrocytes was not ascribed to the difference in cell age between normal and affected cells. Thus, the transport rate in erythrocytes of iron deficiency anemia, whose cell population was younger than normal, was in the normal range and was not changed significantly during iron treatment (Table 2). Furthermore, the transport rates in erythrocytes of patients with various anemias, all of which except aplastic anemia are expected to have relatively a younger erythrocyte population, were also within the normal range (Table 2).

When the incubation was carried out with NaF, we could approximate the PEP transport rate by measuring the increment of PEP that was accumulated inside the cells, since more than 80% of the PEP transported into normal erythrocytes remained as PEP in the presence of NaF (Table 1). The slow degradation of PEP may be explained by the limited supply of ADP available for the pyruvate kinase reaction. The situation was somewhat different in HS erythrocytes. Half of the PEP transported was metabolized to pyruvate and lactate in the affected erythrocytes (Table 1). The inhibitory effect of NaF on enolase and glycolysis was sufficient in both cases, because the increase in concentration of the intermediates from glucose-6-phosphate to 2-phosphoglycerate during the incubation with PEP was the same as noted during the incubation without PEP (Table 1). Therefore, the different amounts of PEP metabolized between normal and the affected cells may be explained by the different amounts of ADP supplied to the pyruvate kinase reaction, suggesting that ADP-generating reactions such as membrane adenosine triphosphatase may be more active in HS cells than in normal cells under the experimental conditions employed in this study. Although the rate calculated from the sum of the intracellular PEP and pyruvate and lactate in HS erythrocyte suspension (the true transport rate) was higher than the rate calculated from the intracellular PEP increment alone, the true transport rate in HS erythrocytes was still lower than the corresponding measurement in normal erythrocytes. We are unable to conclude the lower transport rate is specific to HS erythrocytes, until the extensive study will be done on other hemolytic anemias. At the present time, however, we may suggest the lower rate may be a reflection of some abnormality of HS erythrocyte membrane.
In summary, the lower rate of PEP transport seemed to be a reflection of some abnormality in the affected cell membrane but not a mere reflection of a younger cell population. The lower rate appeared to be specific for HS erythrocytes, suggesting that this finding could be of potential diagnostic value.

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