Endogenous Proteolysis of Membrane-Bound Red Cell Cytochrome-b₅ Reductase in Adults and Newborns: Its Possible Relevance to the Generation of the Soluble “Methemoglobin Reductase”

By Danièle Choury, Abderrezak Reghis, Anne-Lise Pichard, and Jean-Claude Kaplan

The problem of the low activity of so-called methemoglobin reductase in red cells from newborns was re-investigated in view of our current knowledge of this enzyme, i.e., (1) its being cytochrome-b₅ reductase and (2) its presence in two forms: soluble and membrane-bound. We found that red cells from cord blood and newborns exhibited a 50% decrease of soluble cytochrome-b₅ reductase activity, whereas membrane-bound activity was in the adult range. Ghosts from these cells possessed diminished ability to solubilize membrane-bound cytochrome-b₅ reductase in the course of in vitro auto-incubation. This autosolubilizing ability increased with age and reached adult level concomitantly with soluble cytochrome-b₅ reductase activity at 6 mo. We conclude that the relative deficiency of soluble cytochrome-b₅ reductase observed at birth is due to diminished post-translational processing of the membrane-bound enzyme during erythropoiesis of fetal cells. This processing is calcium-dependent related to calmodulin.

MATERIALS AND METHODS

Red cells from the following different categories were investigated: (A) Cord blood samples taken from normal full-term newborns of both sexes after normal pregnancy and delivery. (B) Blood samples from normal infants of different ages between birth and 1 yr (both sexes). (C) Blood samples from normal adults (both sexes).

Preparation of Erythrocyte Membranes

Fresh blood was sampled in heparin, and red cell membranes were prepared according to Marchesi: 5 ml of washed erythrocytes were hemolyzed in 30 vol of 5 mM Tris-HCl buffer, pH 7.5, with 1 mM EDTA and centrifuged at 22,000 g. The pellet was extensively washed with the same buffer, resulting in a white membrane preparation.

Enzyme Assay

NADH cytochrome-b₅ reductase activity was assayed using the method of Hegesh et al. where the substrate is a ferrocyanide-methemoglobin reductase complex.

Erythrocyte Membrane Methemoglobin Reductase Activity

In a noncentrifuged hemolysate. Soluble enzyme activity was tested on a noncentrifuged 1:4 hemolysate. The same hemolysate, treated with 2% Triton X-100, was used to determine total enzyme activity, i.e., membrane-bound plus soluble activities.

In a membrane preparation. Ghosts were suspended in a 10 mM Tris-HCl buffer, pH 7.4, containing 2% Triton X-100. The suspension was frozen and thawed 3 times and spun down for 10 min at 105,000 g at 4°C in a Beckman Airfuge. The supernatant was assayed for enzyme activity.

Determination of Endogenous Processing of Membrane-Bound Cytochrome-b₅ Reductase

Membrane suspensions were incubated 2 hr at 37°C in 0.1 M Tris-maleate, pH 5.6, in the presence of 1% Triton X-100. The 105,000 g supernatants were used for analysis.

Gel permeation chromatography (GPC) was performed on a Chromatex 38 (Touzet et Maltingon, Ivry, France) equipped with a 300 × 7.5 mm Bio Sil TSK 250 column (Biorad) at a flow rate of 0.7 ml/min as described previously. Twenty microliters of extracts containing 10 μg of membrane protein were applied to the column with Tris-HCl 0.05 M, EDTA 1 mM, pH 7.4, containing 1% Triton...
Soluble and Membrane-Bound Cytochrome-b₅ Reductase Activity in Cord Blood Erythrocytes and Comparison With Adult Erythrocytes

The results are shown in Table 1. Soluble cytochrome-b₅ reductase activity is significantly decreased in cord blood erythrocytes as compared to adult erythrocytes. The decrease is 58% if activity is expressed per gram Hb, and 48% if it is expressed per number of cells. In contrast, the membrane-bound cytochrome-b₅ reductase activity of cord blood erythrocytes, measured in total hemolysate, is in the adult range. Similarly, in washed ghosts, the specific activity of cytochrome-b₅ reductase is identical in both cord and adult blood (Table 1).

Comparison of Endogenous Solubilization of Red Cell Membrane Cytochrome-b₅ Reductase From Adults, Newborns, and Infants

Quantitative study. As described previously, GPC analysis of detergent-treated red cell ghosts gives a single peak, with cytochrome-b₅ reductase activity corresponding to an apparent mol wt of 45,000. When the ghosts are incubated at 37°C for 2 hr prior to analysis, the membrane-bound enzyme is partially converted into a lighter form with an apparent mol wt of 29,000, identical to that of the spontaneously soluble enzyme. As depicted in Fig. 1, the relative amount of soluble (29,000) cytochrome-b₅ reductase obtained after a 2-hr incubation is 66% if the ghosts are from adult red cells and 22% if they are from cord blood red cells. This endogenous solubilization was investigated from birth to 12 mo (Fig. 2). The percentage of...
enzyme processed after 2 hr reached adult level at about 6–7 mo.

Qualitative study. Several typical protease inhibitors were tested in order to determine to which class the suspected red cell membrane protease belonged. No effect was observed when leupeptin (10 μg/ml), pepstatin (10 μg/ml), or PMSF (0.1 mM) was added to the incubation medium. In the presence of EDTA (10 mM) or EGTA (10 mM), there was a 75% inhibition of the solubilization of red cell membrane cytochrome-b₅ reductase (Table 2). This was found only in membranes from adult red cells, whereas those from cord blood were not affected by Ca²⁺-chelating agents (Table 2). Calmodulin (0.3 mg/ml) restored the proteolytic activity of newborn red cell membranes to the level observed in adults, but had no effect on adult red cell membranes (Table 2). Trifluoperazin (10 mM), an antagonist of calmodulin, decreased the solubilizing activity of adult red cell membranes to the level observed in newborns, but did not affect activity in newborn red cell membranes (Table 2).

DISCUSSION

We now know that the enzyme responsible for the reduction of methemoglobin in red cells, designated as methemoglobin reductase or NADH-diaphorase, is actually a soluble form of cytochrome-b₅ reductase. The enzyme locus (DIA₁) has been assigned to chromosome 22. We recently showed that circulating red blood cells from adults contain, in addition to the recognized soluble cytochrome-b₅ reductase, an insoluble cytochrome-b₅ reductase that is tightly bound to the inner face of the membrane. The membrane-bound enzyme has a molecular weight of 45,000 and is solubilized either by Triton X-100 without changing its molecular weight, or by cathepsin-D treatment, which yields an enzyme of 29,000 identical to the spontaneously soluble form. The two forms of enzymes are produced by DIA₁ gene, and we have postulated that the membrane-bound enzyme was the precursor from which the soluble cytochrome-b₅ reductase derived. The endogenous solubilization of the membrane-bound enzyme studied by auto-incubation of washed red cell ghosts results in a lighter enzyme—29,000 instead of 45,000. This cannot be accounted for by dissociation of subunits, since the cytochrome-b₅ reductase is a monomeric enzyme.

Although we have no direct evidence, we consider it very likely that this phenomenon is due to partial proteolytic removal of a hydrophobic anchoring domain, as already demonstrated for the cytochrome-b₅-cytochrome-b₅ reductase system of endoplasmic reticulum. If this model is accepted, one has to demonstrate how and when the phenomenon of post-translational proteolytic solubilization of the membrane cytochrome-b₅ reductase occurs in vivo.

In the present study, we have investigated the cellular distribution of cytochrome-b₅ reductase in newborns. We confirmed the decrease of the soluble fraction and found that, in contrast, the membrane-bound enzyme is in the adult range. This could be due to impaired proteolytic solubilization during the maturation of fetal erythrocytes. Indeed, we found that newborn red cell ghosts exhibited a decreased solubilizing activity recovered at 45,000 daltons without incubation.

Table 2. Effect of Calcium Effectors on Endogenous Proteolytic Solubilization of Red Cell Membrane Cytochrome-b₅ Reductase

<table>
<thead>
<tr>
<th>Effector</th>
<th>Adult (Ghosts)</th>
<th>Newborn (Ghosts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>56</td>
<td>19</td>
</tr>
<tr>
<td>EGTA (10 mM) alone</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>Ca²⁺ (100 μM) alone</td>
<td>55</td>
<td>26</td>
</tr>
<tr>
<td>Ca²⁺ (100 μM) + calmodulin (0.3 mg/ml)</td>
<td>54</td>
<td>51</td>
</tr>
<tr>
<td>Ca²⁺ (100 μM) + trifluoperazin (10 mM)</td>
<td>20</td>
<td>23</td>
</tr>
</tbody>
</table>

Red cell membranes were incubated at 37°C in 0.1 M Tris-maleate, pH 5.6, and 1% Triton X-100, with and without effector. After 2 hr, the preparation was passed through a GPC column (see text), and the relative amount of solubilized cytochrome-b₅ reductase was derived from the ratio: enzyme activity recovered in the peak at 29,000 daltons to total activity recovered at 45,000 daltons without incubation.
effect of the membrane cytochrome-b₉, reductase during auto-incubation at 37°C in the presence of Triton X-100. After birth, the endogenous proteolysis occurring in ghosts under such conditions increased progressively to reach adult level at about 6 mo. Interestingly, the curve of maturation of the soluble cytochrome-b₉ reductase content in circulating red cells of infants of increasing age was parallel (Fig. 2). It must be emphasized that in our system, the endogenous processing activity was studied in mature peripheral blood erythrocytes. The finding of such an activity in detergent-solubilized ghosts does not necessarily mean that it is operative in mature circulating cells. In fact, we have evidence that this is not the case, since there is no modification of the membrane versus soluble enzyme ratio during in vivo aging of circulating mature red cells.¹⁷ Instead, the solubilization of cytochrome-b₉ reductase occurs during erythroid maturation, most likely at a late stage.¹⁸ However, since we have found a correlation between low soluble cytochrome-b₉ reductase content and low endogenous proteolysis in cord blood cell ghosts, together with a parallel curve of evolution of both parameters after birth, we believe that the in vitro endogenous proteolysis test is meaningful.

The nature and number of proteases in maturing red cells is still controversial, although several different proteases have already been described in mature red cells and reticulocytes.¹⁶,¹⁹-²¹ Since the proteolytic solubilization of membrane cytochrome-b₉ reductase was not sensitive to leupeptin, PMSF, or pepstatin, the protease involved cannot be a cathepsin-like protease, although erythrocyte membrane-bound cytochrome-b₉ reductase is solubilized by cathepsin-D treatment;²² nor can it be the acid protease described by Hultquist in rabbit reticulocytes.²³ A strong inhibitory effect of EGTA and trifluoperazin on the in vitro solubilization of red cell membrane cytochrome-b₉ reductase was observed only in ghosts from adults. It suggests that the Ca²⁺-calmodulin system is involved in the proteolysis of the membrane enzyme. Moreover, there is a striking equivalence between the low proteolytic activity observed in cord blood ghosts and the residual activity in adult ghosts in the presence of EGTA or trifluoperazin. The fact that in cord blood ghost activity is restored to adult level by calmodulin, together with the fact that added calmodulin does not stimulate adult ghosts, would suggest that the primary defect in fetal ghosts is a lack or a decrease of calmodulin. However, this was not found to be the case (data not shown). Further investigation is required to elucidate this phenomenon and its potential developmental significance.

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REFERENCES

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