Membrane-bound Cytochrome $b_5$ Reductase (Methemoglobin Reductase) in Human Erythrocytes

STUDY IN NORMAL AND METHEMOGLOBINEMIC SUBJECTS

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Abstract In this study we present evidence that in human erythrocytes NADH-cytochrome $b_5$ reductase (methemoglobin reductase) is not only soluble but also tightly bound to the membrane. The membrane methemoglobin reductase-like activity is unmasked by Triton X-100 treatment, and represents about half of the total activity in the erythrocytes. Like the amphiphilic microsomal-bound cytochrome $b_5$ reductase, the erythrocyte membrane-bound enzyme is solubilized by cathepsin D. Because this treatment is effective on unsealed ghosts but not on resealed (inside-in) ghosts, it is concluded that the enzyme is strongly bound to the inner face of the membrane. The erythrocyte membrane enzyme is antigenically similar to the soluble enzyme. The two forms of enzyme are specified by the same gene, in that both were found defective in six patients with recessive congenital methemoglobinemia. We suggest that the cytochrome $b_5$ reductase of the erythrocyte membrane is the primary gene product. A posttranslational partial proteolysis probably gives rise to the soluble form of the enzyme, which serves as a methemoglobin reductase.

Introduction

The so-called methemoglobin-reductase or NADH-diaphorase has been described long ago as a soluble erythrocyte enzyme that has a major role in the enzymatic reduction of methemoglobin (1). An inherited homozygous defect of this enzyme produces congenital recessive methemoglobinemia (2). The enzyme locus ($\text{DIA}_1$) has been assigned to chromosome 22 (3, 4). It has been demonstrated that this erythrocyte enzyme is actually a soluble NADH-cytochrome $b_5$ reductase (5–8) that reduces a soluble cytochrome $b_5$. The reduced cytochrome $b_5$ interacts directly with methemoglobin (5, 9). In the other cells, the NADH-cytochrome $b_5$ reductase is predominantly bound to the membranes: endoplasmic reticulum (10) and mitochondria (11). However, a soluble form of the enzyme has also been found in the cytosolic fraction of human placenta (8) and rabbit liver (12).

In this paper, we report the finding of a NADH-cytochrome $b_5$ reductase that is strongly attached to the inner face of the erythrocyte membrane and is released either by detergent treatment or by partial digestion by cathepsin D. The immunologic and genetic characterization of the membrane-bound enzyme suggests that it is produced by the same gene ($\text{DIA}_1$) as the soluble erythrocyte diaphorase.

Methods

Preparation of the erythrocyte membranes

The erythrocyte membranes were prepared according to Marchesi et al. (13). Washed human erythrocytes were hemolysed by 30 vol of 5 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, and the 22,000-g pellet was extensively washed in the same medium to obtain a completely white membrane preparation. For the preparation of inside-in resealed ghosts, we used the procedure of Steck and Kant (14) in which hemolysis is initiated by mixing 1 vol of washed erythrocytes with 40 vol of 5 mM sodium phosphate buffer, pH 8, containing 1 mM MgSO$_4$.

Extraction

With detergent. The ghosts were suspended in a 10 mM Tris-HCl buffer, pH 7.4, containing 2% Triton X-100. After 30 min of incubation at 4°C, the suspension was frozen and thawed three times and centrifuged for 10 min at 105,000 g, at 4°C, in a Beckman Airfuge (Beckman Instruments Inc., Fullerton, Calif.) centrifuge to spin down small volumes (170 μL). The supernatant was assayed for enzyme activity.

Preliminary data have been presented at the Twenty-second annual meeting of the American Society of Hematology in 1979. Blood. 541 (Suppl. 1): 342. (Abstr.)

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of Ellman et al. (18); glyceraldehyde-3-phosphate dehydrogenase was assayed according to Beutler et al. (19).

Proteins were estimated by the method of Lowry et al. When large amount of Triton X-100 produced a precipitate in the final mixture, it was discarded by 5-min centrifugation at 10,000 rpm.

**Electrophoresis**

Horizontal starch gel electrophoresis was performed as described by Kaplan and Beutler (20) using a Tris-EDTA borate buffer, pH 8.6. Polycrylamide gel isoelectrofocusing was carried out in a pH 6–8 linear gradient (Ampholines; LKB Instruments, Inc., Orsay, France) according to the method of Drysdale et al. (21). In all the electrophoretic studies specific staining for NADH-diaphorase activity was performed with a mixture containing 1.2 mM NADH, 0.06 mM dichlorphenol indophenol, and 1.2 mM MTT in a 0.25 M Tris-HCl buffer (pH 8.4) (20).

**Immunological studies**

**Inactivation by antiserum.** The different preparations were incubated with a chicken antiserum prepared against human erythrocyte-soluble cytochrome b₅ reductase (8) in the presence of 5% (vol/vol) of polyethylene glycol. Increasing amounts of antiserum were added to constant amounts (expressed as units of methemoglobin-ferrocyanide reducing activity) of enzyme. After incubation for 1 h at 4°C followed by centrifugation (20 min at 22,000 g) the residual activity was measured in the supernatant.

**Double immunodiffusion.** Double immunodiffusion was performed according to Ouchterlony (22). Antigens and antiserum were incubated for 2–3 d and then the plate was extensively washed with isotonic saline solution for 2 d to remove the excess enzyme that had not reacted with the antiserum. The precipitation lines were specifically visualized using the NADH-diaphorase staining method (20).

**Chemicals**

NADH, 2,6-dichlorophenol indophenol, MTT, Triton X-100, potassium ferricyanide, cathepsin D, D-t-gluceraldehyde-3-phosphate, adenosine 5-triphosphate, 5,5-dithiobis

(2-nitrobenzoic acid), acetyl thiocione iodide were from Sigma Chemical Co., St. Louis, Mo. DEAE-cellulose 52 was from Whatman Inc., Clifton, N. J.

**RESULTS**

**Erythrocyte membrane methemoglobin reductase activity.** Treating a noncentrifuged 1:4 hemolysate by 2% Triton X-100 (final concentration) doubles the activity of the enzyme assayed by the method of Hegesh (15) (Table I).

The detergent had no effect on the soluble enzyme present in a membrane-free hemolysate (105,000 g supernatant). Therefore, the presence of the enzyme as a component of the erythrocyte membrane was investigated. The washed membranes were found to contain a methemoglobin reductase-like activity that was increased 15-fold by detergent treatment (Table I). In contrast, extensive washing of the membranes by
1 M NaCl did not result in the appearance of this enzyme activity in the 105,000 g supernatant.

**Accessibility of various electron acceptors.** The accessibility of each electron acceptor was determined by measuring the enzyme activity with each of them in the absence and presence of detergent. As shown in Table II, the membranes exhibit little accessibility to the ferrocyanide methemoglobin complex (64,000 mol wt) used in the Hegesh assay (15). In contrast, with smaller acceptors, such as ferricyanide, dichlorophenol indophenol, or MTT the accessibility was dramatically increased.

**Membrane solubilization by Triton X-100 and detergent/protein ratio.** The membranes were suspended in 1 vol of 10 mM Tris-HCl buffer, pH 7.4, and the protein concentration adjusted to 4 mg/ml. Triton X-100 was added to obtain a final concentration varying between 0.05 and 10% (wt/vol). Each preparation was centrifuged for 10 min at 105,000 g at 4°C. The supernatant was assumed to contain the solubilized fraction of the membrane. The precipitate contains the insoluble part of the enzyme. The solubilization is dependent on the Triton X-100 concentration and maximal solubilization is reached at 2% Triton (wt/vol). At this concentration 75% of the enzyme activity is recovered in the supernatant, 25% remaining in the precipitate.

**Configuration of the erythrocyte membrane preparations.** To check the configuration of the membranes prepared by Marchesi's method we have assayed two enzyme markers, one of the inner face of the membrane (glyceraldehyde-3-phosphate-dehydrogenase) and the second of the external face of the membrane (acetylcholinesterase). The membranes that we used were unsealed because both enzymes were found to be equally accessible (94% accessibility). The percentage of accessibility was derived from the following ratio: activity minus detergent vs. activity plus detergent, times 100.

**Solubilization of the membrane-bound cytochrome b₅ reductase by cathepsin D.** The ghosts were incubated 2 h at 37°C with 0.05% cathepsin D in 0.1 M, Tris-maleate, pH 5.6. Assays were performed on 105,000 g supernatants and on pellets. The extent of solubilization of the enzyme is expressed as the percentage of enzyme activity in the supernatant relative to the total values recovered in the supernatant and the pellet.

It was not possible to solubilize by cathepsin D the membrane-bound enzyme of sealed (inside-in) ghosts (Table III). This experiment shows that the erythrocyte membrane methemoglobin reductase is bound to the inner face of the membrane.

**Immunological comparison with soluble erythrocyte cytochrome b₅ reductase.** Double immunodiffusion showed that the detergent-treated erythrocyte membrane enzyme and the soluble erythrocyte enzyme react similarly toward an antiserum directed against the soluble erythrocyte cytochrome b₅ reductase. The electrophoretic behavior of the membrane-bound enzyme in starch gel followed by staining for NADH-diaphorase activity was also investigated. As seen on Fig. 2 the membrane enzyme migrates more slowly than the soluble enzyme. However it is completely

### Table II

**Accessibility to Various Electron Acceptors of the Erythrocyte Membrane Methemoglobin Reductase**

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Without Triton X-100</th>
<th>With Triton X-100</th>
<th>Accessibility %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrocyanide methemoglobin complex (mol wt = 64,000)</td>
<td>6.37</td>
<td>62.00</td>
<td>7.8</td>
</tr>
<tr>
<td>Dichlorophenol indophenol (mol wt = 290)</td>
<td>37.4</td>
<td>66.00</td>
<td>57</td>
</tr>
<tr>
<td>MTT (mol wt = 414)</td>
<td>37.5</td>
<td>55.50</td>
<td>68</td>
</tr>
<tr>
<td>Ferricyanide (mol wt = 329)</td>
<td>0.409</td>
<td>0.405</td>
<td>100</td>
</tr>
</tbody>
</table>

All enzyme activities determinations were carried out at 25°C. The incubations in the presence of detergent were performed at +4°C with 2% Triton X-100. The percent accessibility is determined by the without Triton X-100/with Triton X-100 ratio × 100.

### Table III

**Solubilization by Cathepsin D of the Erythrocyte Membrane Methemoglobin Reductase**

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Methemoglobin reductase activity</th>
<th>Pellet vs. Supernatant</th>
<th>Percent solubilized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>105,000 g supernatant</td>
<td>Triton + Triton</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsealed ghosts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- cathepsin D</td>
<td>57</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unsealed ghosts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ cathepsin D</td>
<td>21.3</td>
<td>12.3</td>
<td>36.6</td>
</tr>
<tr>
<td>Resealed ghosts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(inside-in) + cathepsin D</td>
<td>34</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Ghosts prepared as described in text were incubated for 2 h at 37°C with 0.05% cathepsin D in 0.1 M, Tris-maleate, pH 5.6. Assays were performed on 105,000 g supernatants and on pellets. The extent of solubilization of the enzyme is expressed as the percentage of enzyme activity in the supernatant relative to the total values recovered in the supernatant and the pellet.
abolished by pretreatment with the antiserum directed against the soluble cytochrome b₅ reductase. Under these conditions the soluble enzyme is also completely inhibited (not shown) (8).

Inactivation test by increasing amounts of antiserum was performed upon (a) the semipurified soluble cytochrome b₅ reductase in the absence and presence of cathepsin D; (b) the detergent-treated membrane enzyme, and (c) the cathepsin D-treated membrane enzyme. The inactivation curves obtained were similar in the four cases (Fig. 3).

Status of membrane cytochrome b₅ reductase from patients with recessive congenital methemoglobinemia. We investigated six patients with re-

DISCUSSION

It has been established that the erythrocyte NADH-methemoglobin reductase is a soluble form of cytochrome b₅ reductase (5–8). In the present study we show that the addition of a detergent to the total unspun hemolysate doubles the methemoglobin reductase activity. Inasmuch as the detergent has no activating effect on a membrane-free hemolysate, this phenomenon strongly suggests that the enzyme could be an intrinsic component of the human erythrocyte membrane, as previously proposed by Goto-Tamura et al.
for rabbit erythrocyte ghosts (6). Indeed, thoroughly washed human erythrocyte membranes contain a methemoglobin reductase-like activity that is unmasked by detergent treatment, but cannot be released by high ionic strength treatment. Like the microsomal cytochrome \( b_5 \) reductase, which is released as a soluble entity by lysosomal cathepsin (25–27), the erythrocyte membrane-bound enzyme is solubilized by cathepsin D treatment. This treatment is fully effective if ghosts are unsealed, whereas it is ineffective if they are resealed "inside-in." This indicates that the enzyme is actually bound to the inner face of the membrane. In the absence of detergent, the unsealed ghosts exhibit almost no activity on the ferrocyanide-methemoglobin complex. In contrast, with a small substrate such as potassium ferricyanide they exhibit maximal activity even in the absence of detergent. Similarly other small xenobiotic acceptors, such as dichlorophenol indophenol and MTT behave in the same way, whereas their activity was only sub-maximal in the absence of detergent (57 and 67%, respectively of the maximal activity obtained with detergent). This suggests that the membrane-bound enzyme is more accessible to small substrates than to the much larger molecule of methemoglobin.

The presence of such an enzyme on the inner face of the erythrocyte membrane raises several questions: (a) Is it a membrane-bound cytochrome \( b_5 \) reductase? (b) If so, is it related to the soluble erythrocyte cytochrome \( b_5 \) reductase? (c) Is it also implicated in the mechanism of methemoglobin reduction? It is possible to give positive answers to questions (a) and (b). The proteolytic release of the erythrocyte membrane bound enzyme by cathepsin D is reminiscent of similar results obtained with liver microsomes (15, 26). It has been established that the microsomal-bound enzyme is an amphiphilic protein consisting in a hydrophilic moiety carrying the active site and a hydrophobic domain at the COOH-terminal end by which the protein is anchored to the endoplasmic reticulum (28–30). Treatment by cathepsin D causes the cleavage of the molecule at the junction between the hydrophilic and hydrophobic domains and releases an active soluble enzyme (29–32). In the case of the erythrocyte membrane-bound enzyme the site of cleavage by cathepsin D must be different from that of the putative endogenous protease, because of the observed differences in pl between the cathepsin D solubilized enzyme and the spontaneously soluble enzyme (Fig. 1).

Immunological studies confirm the relationship between the erythrocyte membrane-bound enzyme and the soluble cytochrome \( b_5 \) reductase. Double immunodiffusion electrophoresis and inactivation curve, after exposure to an antiserum directed against the soluble erythrocyte cytochrome \( b_5 \) reductase, show an identical behavior. Because the soluble enzyme has been previously demonstrated to be immunologically similar to the microsomal cytochrome \( b_5 \) reductase in human (8) and animals (6, 7), it can be deduced that the enzyme that is strongly bound to the erythrocyte membrane is also a cytochrome \( b_5 \) reductase. The finding that in six subjects with congenital recessive methemoglobinemia (type I and type II) the deficiency of erythrocyte-soluble cytochrome \( b_5 \) reductase is invariably associated with a deficiency of the erythrocyte membrane enzyme provides an ultimate proof of their common genetic origin.

The identity between the erythrocyte membrane cytochrome \( b_5 \) reductase assayed according to Hegesh et al. (15) and the NADH-diaphorase stained by 2,6 dichlorophenol indophenol after electrophoresis (20) is demonstrated by the strict parallelism of the results obtained with the two methods. Conversely the identity between the erythrocyte membrane cytochrome \( b_5 \) reductase and the NADH erythrocyte membrane oxidoreductase described by Zamudio (16, 33) is still not demonstrated but is very likely. An atebrin-sensitive NADH-oxidoreductase has been recently described in mouse liver plasma membranes (34). This enzyme should be different from the cytochrome \( b_5 \) reductase that is bound to the endoplasmic reticulum and to the erythrocyte membrane since the latter is not affected by atebrin (data not shown).

Regarding the possible role of the membrane enzyme in methemoglobin reduction (question c), we cannot answer yet. It is noteworthy that cytochrome \( b_5 \) has already been found as a component of the erythrocyte membrane (35). To what extent the other classical functions of the cytochrome \( b_5 \)-mediated

### Table IV

**Membrane-bound and Soluble Methemoglobin Reductase from Patients with Recessive Congenital Methemoglobinemia**

<table>
<thead>
<tr>
<th>Case</th>
<th>Membrane enzyme activity*</th>
<th>Soluble enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg protein</td>
<td>nmol/mg hemoglobin</td>
</tr>
<tr>
<td>Type I disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(without</td>
<td>15</td>
<td>0.3</td>
</tr>
<tr>
<td>mental retardation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHIR</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>PIER</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>BOUR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type II disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mental retardation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NASS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BOU</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>LLE</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Controls</td>
<td>75±10</td>
<td>2.7±0.5</td>
</tr>
</tbody>
</table>

The enzyme activity was assayed according to Hegesh et al. (15).
* Measured in the presence of 2% Triton X-100.
electron transport system are operating in the erythrocyte membrane is also still unexplored. Concerning methemoglobin reduction, it should be noted that we have found that, in spite of the absence of detectable soluble methemoglobin-reductase in chicken erythrocytes, the intact cells can promote methemoglobin reduction after exposure to nitrite (unpublished data). Board et al. (36) have found that in nucleated erythrocytes from birds and reptiles the enzyme is only membrane-bound. It is therefore possible that the membrane-bound cytochrome \( b_5 \) reductase of the human nucleated erythrocytes does play a role in the reduction of methemoglobin.

Finally, whatever its metabolic role, the membrane-bound erythrocyte cytochrome \( b_5 \) reductase seems to represent the primary gene product of the \( DIA_4 \) locus (3, 4). This is suggested by its deficiency in recessive congenital methemoglobinemia, which indicates that the erythrocyte membrane cytochrome \( b_5 \) reductase is under the same genetic control as the soluble erythrocyte enzyme. Their respective distribution, about half and half, is probably a unique situation proper to the mature circulating erythrocyte. The membrane-bound enzyme could serve as a precursor of the soluble form that would be released by partial proteolysis of the former. Preliminary results, obtained by sucrose gradient centrifugation and high speed gel filtration showed us that the soluble enzyme and the cathepsin D solubilized membrane enzyme display an identical molecular weight lower than that of the membrane bound detergent treated enzyme (manuscript in preparation). A similar model has been suggested by Hultquist (37) for the production of the soluble cytochrome \( b_5 \) from its reticulum-bound precursor during the erythroid maturation. Concerning the reductase it would be important to identify the proteolytic enzyme involved in its maturation, and to determine at which stage the post-translational processing of the membrane-bound enzyme occurs. These problems are under current investigation.

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