Rat Erythrocyte NADH-Cytochrome b₅ Reductase

QUANTITATION AND COMPARISON BETWEEN THE MEMBRANE-BOUND AND SOLUBLE FORMS USING AN ANTIBODY AGAINST THE RAT LIVER ENZYME

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The subcellular distribution of rat erythrocyte NADH-cytochrome b₅ reductase was determined by radioimmunoassay, using a rabbit antibody against the cathepsin D cleaved water-soluble fragment of rat liver microsomal reductase (1-reductase), which is known to be immunologically similar to the red cell enzyme. Erythrocytes contained ~30 ng of reductase/mg of protein, of which 99% were recovered in the hemolsytate supernatant and 2.3% in the ghost fraction. After concentration by precipitation with 70% saturated (NH₄)₂SO₄, the NADH-cytochrome c reductase activity of the soluble enzyme could be assessed in the presence of cytochrome b₅, and was found to be inhibited by anti 1-reductase antibodies.

The sodium dodecyl sulfate-polyacrylamide gel electrophoretic mobilities of erythrocyte membrane-associated and soluble reductase of the liver microsomal enzyme and its cathepsin D cleaved hydrophilic fragment (1-reductase) were examined in crude fractions by blotting followed by specific and highly sensitive immunostaining. The intact microsomal enzyme and the two erythrocyte reductases all had similar mobilities and migrated behind 1-reductase. However, the ghost-associated reductase, which was not attributable to contaminating leukocyte or reticulocyte membranes, was distinguishable from the soluble form by two criteria: (i) a lower dependence on exogenous cytochrome b₅ in the NADH-cytochrome c reductase assay; and (ii) a larger apparent Mₕ upon gel filtration in the presence of Triton X-100, presumably because of detergent binding. Considering these results, possible biogenetic relations between membrane-bound and soluble erythrocyte reductase are discussed.

Over the past years, a number of proteins have been reported to exist both in a soluble and in a membrane-associated form. The best studied example is that of IgM, in which it has been demonstrated that the secretory and membrane-bound forms are products of the same gene (1), but differ in that the membrane form possesses a hydrophobic domain towards its COOH terminus (6). In this tissue, where it has important functions in fatty acid metabolism, it is present on endoplasmic reticulum as well as on outer mitochondrial (7, 8), Golgi (6, 9), and outer nuclear (10) membranes. In contrast, in erythrocytes, this enzyme has been reported to be present as a soluble protein in the cytosol (11, 12), where it is involved in the reduction of methemoglobin via a soluble cytochrome b₅ (13). The soluble erythrocyte enzyme has been purified and characterized as a flavoprotein with a molecular weight, acceptor specificity, and immunological features similar to its membrane-bound counterpart (11, 12, 14-16). Moreover, studies on methemoglobinemic patients suggest that the two forms are products of the same gene (17, 18). It has been proposed that water-soluble reductase is slightly smaller than the membrane-bound protein and derives from the latter by proteolysis during maturation of the erythroblast (11, 19). However, a direct structural comparison between the liver and the erythrocyte enzyme has not been reported to date.

In addition to the soluble form, a membrane-associated form of NADH-cytochrome b₅ reductase has also been reported to be present in the erythrocyte (20, 21). Based on enzyme assay, it has been claimed that as much as 50% of erythrocyte reductase is membrane associated (20). However, quantitative data on the intracellular distribution of erythrocyte NADH-cytochrome b₅ reductase are lacking, because of the difficulty to assay the enzyme in whole hemolysates (22).

Over the past few years, we have been studying the localization, biosynthesis, and turnover of rat liver NADH-cytochrome b₅ reductase, using specific antibodies raised in rabbits as tools for the isolation and quantitation of the enzyme (23-25). In the present study, with the help of these antibodies, we have attempted to gain more information on the relations between membrane-bound and erythrocyte water-soluble NADH-cytochrome b₅ reductase. In particular, we have: (i) developed a RIA1 to obtain reliable quantitative data on the intracellular distribution of rat erythrocyte reductase; and (ii) compared the SDS-PAGE mobilities, and the gel filtration and enzymatic behavior of water-soluble and membrane-bound reductases.

1 The abbreviations used are: BSA, bovine serum albumin; t-cytochrome b₅, tryptophan solubilized water-soluble fragment of rat liver cytochrome b₅; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; RIA, radioimmunoassay; 1-reductase, water-soluble fragment of rat liver microsomal NADH-cytochrome b₅ reductase cleaved by a lysosomal enzyme (cathepsin D); d-reductase, detergent solubilized rat liver microsomal NADH-cytochrome b₅ reductase; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.
MATERIALS AND METHODS

Materials—In addition to chemicals listed in recent publications (8, 23–25), the following reagents were purchased from the following sources: nitrocellulose paper (type HAWP), Millipore; Bolton-Hunter reagent (125I) (2000 Ci/mmol) and NaI125I, New England Nuclear; BSA (fraction V), polyvinylpyrrolidone-300, dithiothreitol, iodoacetamide, and phenylmethylsulfonyl fluoride, Sigma; Ficol 400, Pharmacia Fine Chemicals, Upsala, Sweden; Affi-Gel blue DEAE, Bio-Rad; keyhole limpet hemocyanin, Calbiochem, La Jolla, CA; Triton X-100 for gas chromatography, Merck, Darmstadt, Germany.

Preparations and Apparatus—Solutions were made in deionized glass distilled water. Solutions indicated in the abbreviated form are: PBS, 0.8% NaCl, 0.02% KCl, 16 mM Na+ phosphate buffer, pH 7.5; RIA buffer, PBS containing 1% BSA, 1% Triton X-100, 5 mM EDTA, 0.02% NaN3, RSA washing buffer, PBS containing 0.2% BSA, 1% Triton X-100, 5 mM EDTA, 0.5% polyethylene glycol 6000; lysis buffer, 5 mM Na+ phosphate buffer, 1 mM EDTA; NaN3, polyvinylmethylsulfonyl fluoride, pH 7.

Ultrafiltrations were carried out in Beckman ultracentrifuges (Beckman Instruments, Palo Alto, CA) and low speed centrifugations in an IEC Centra-7R refrigerated centrifuge (Damon, IEC Division, Needham Heights, MA). Unless otherwise specified, all centrifugations were at 4 °C. For spectrophotometric determinations, a double beam Unicam SP 1800 spectrophotometer (Fye Unicam Ltd, Cambridge, England) was used. Radioactivity counting was performed in a Beckman Gamma 4000 counter.

Rat Erythrocytes—Male Sprague-Dawley rats weighing 250 g were decapitated and blood was collected into a beaker containing 1 ml of 0.1 M Na+ phosphate buffer, 50 mM EDTA, pH 7, for every 10 ml of blood. 3-ml aliquots of blood were distributed in conical glass tubes and centrifuged at 150 × g at room temperature for 10 min. After removal of plasma and of the buffy coat, the red cells were washed four times with eight volumes of PBS by resuspension and centrifugation at room temperature. The last centrifugation was at 350 × g for 10 min. The resulting packed washed red cells were resuspended with an equal volume of 0.1 M Na+ phosphate buffer, 1 mM EDTA, pH 7, lysed with 16 volumes of lysis buffer and centrifuged at 4000 × g for 15 min in the 60 Ti rotor. The resulting pellets were washed several times with lysis buffer by gentle vortexing and centrifugation as described by Dodge et al. (26) and taking care to remove the dark red material which deposited below the fluffy membrane pellet. The final washed pellet was resuspended by gentle vortexing with a small volume of lysis buffer and is indicated as the ghost fraction. The supernatant of the hemolysate was combined with the supernatant of the first wash and is indicated as the soluble fraction of the erythrocyte.

For (NH4)2SO4 precipitation, the soluble fraction was adjusted to a protein concentration of 3.5 mg/ml with lysis buffer and supplemented with solid (NH4)2SO4 to 70% saturation. After incubation for 2 h at 0 °C, the sample was centrifuged at 30,000 × g for 15 min in the 60 Ti rotor. The resulting whitish precipitate, which was distributed along the wall of the Osmonde centrifuge tubes, was resuspended in small volume of PBS + 1 mM EDTA and dialyzed against the same buffer. The resulting sample, clarified by low speed centrifugation, is referred to as (NH4)2SO4 precipitate.

To obtain a Triton extract of erythrocyte ghosts, the ghost fraction was brought to 2% Triton X-100 and centrifuged at 100,000 × g for 30 min in the 40 rotor. The resulting supernatant is referred to as the Triton extract. For some experiments, the Triton-extracted proteins were concentrated by precipitation with 9 volumes of acetone at -20 °C.

Aliquots of the fractions thus obtained were diluted in RIA buffer and stored at -20 °C. Other aliquots were boiled in SDS-PAGE solubilization mixture, alkylated (see below), and stored at -20 °C until use.

Purification of Proteins—The water-soluble fragment of rat liver microsomal NADH:cytochrome b5 reductase, here referred to as I-reductase, was purified by the method of Takeue and Omura (27), slightly modified as previously described (22). The detergent solubilized form of rat liver microsomal NADH:cytochrome b5 reductase, here referred to as d-reductase, was purified as described by Spatz and Strittmatter (44), but continuous gradients were used for the elution of ion exchange columns, and Sephadex G-100 was used for the gel filtration step. The trypsin-solubilized fragment of rat liver microsomal cytochrome b5, here referred to as t-cytochrome b5, was prepared as described by Omura et al. (28).

Iodination of Proteins—l-reductase was iodinated with the Bolton-Hunter reagent as described by Bolton (29) to a specific radioactivity of 2·3·108 cpm/μg. To eliminate antigenically inactive iodinated molecules, the 125I-l-reductase preparation was passed through an affinity column containing antireductase antibodies conjugates to Sepharose 4B, and the antigenically active reductase was recovered by subsequent wash (17) followed by elution with 0.1 M glycine-HCl, pH 2.2 as described in Table 1.

Protein A was iodinated by the chloramine T method (30) to a specific radioactivity of -6·108 cpm/μg.

Iodinated proteins were stored at -20 °C in small aliquots in the presence of carrier BSA.

Antibodies—Anti-l-reductase antibodies were raised in rabbits by multiple intradermal injections of the antigen emulsified in complete Freund's adjuvant administered at weekly intervals. Rabbit 1 received a total of 110 μg of l-reductase (2 administrations of 45 μg, followed by two administrations of 10 μg). Rabbit 2 received a total of 300 μg (2 administrations of 100 μg followed by two administrations of 50 μg) of l-reductase coupled to keyhole limpet hemocyanin essentially by the method of Ternynck and Avrameas (51). Blood was collected one week after the last administration and every two weeks thereafter. When necessary, rabbits were boosted by an intramuscular injection of 50 μg of antigen emulsified in Freund's incomplete adjuvant.

Antisera and purified antibodies were purified from antiserum by affinity chromatography as previously described (23), but in the case of rabbit 1, all operations were carried out at room temperature instead of at 4 °C. This procedure, although resulting in a lower recovery, yielded a preparation enriched in high affinity antibodies. The purified antibody obtained from rabbit 1 and rabbit 2 were used for RIA and immunostaining of blots, respectively.

Antirabbit γ-globulin serum was produced in goats injected repeatedly with rabbit IgG purified by Affi-Gel blue DEAE-chromatography. Antisera and purified antibodies were stored in 0.1% NaN3 at 4 °C.

Radioimmunoassay—Red cell fractions, stored at -20 °C in RIA buffer, were clarified by centrifugation (700 × g, 15 min) before use. Preliminary experiments showed that all the enzyme activity was recovered in the supernatant.

Enzyme Assays and Protein Determination—Cytochrome b5, reductase (EC 1.6.2.2) was determined on freshly prepared erythrocyte fractions as described by Sottocasa et al. (7), but the assay was performed in 60 mM Tris-HCl, pH 8.5. Cytochrome b5 was added at the concentrations indicated in the figure legends.

Cytochrome b5 concentration was determined by the difference spectrum between the oxidized and the reduced form as described by Ernst et al. (33), with a window extinction coefficient of 163 for the difference in absorbance between 424 and 409 nm (33).

Protein was determined by the method of Lowry et al. (34) using BSA as standard.

Blotting of Proteins Separated by SDS-PAGE—For SDS-PAGE, samples were brought to a final concentration of 60 mM DL-dithiothreitol, 4.5% SDS, 0.001% bromphenol blue, 0.2 M Tris-HCl, pH 8.9, 0.35 M sucrose, boiled for 2 min, and then alkylation with a 10-fold excess of iodoacetamide. SDS-PAGE was performed essentially as described by Maizel (35) on 10% polyacrylamide slab gels 1.5-mm thick. After electrophoresis, gels were either fixed and stained with Coomassie brilliant blue or subjected to the blotting procedure described by Towbin et al. (36). The transfer onto nitrocellulose paper was carried out at 100 volts (initial current, 0.5 Amp) in a water-cooled apparatus for 90 min.

Immunostaining of Blots—Blots were incubated first for 1 h at 40 °C in a shaker bath in a solution containing 0.9% NaCl, 10 mM Tris-HCl, 15% BSA, 0.2% Ficol 400, 0.2% polyvinylpyrrolidone-360, 0.05% NaN3, and then overnight with constant shaking in the same buffer containing, in addition, 2 pg/μl of antibody conjugates to non-immunoreactive rabbit IgG. After 5 washes of 5 min each with 0.9% NaCl, 10 mM Tris-HCl, 3% BSA, pH 7.5, the blots were further incubated with shaking for 2 h at 4 °C in the initial incubation buffer containing 125I-
of classical purification experiments (27) as well as of our own enzyme. To obtain the 15-fold enrichment shown in Table 1, we isolated a preparation with a good enrichment in reductase.

The NADH-cytochrome b5 reductase content of rat erythrocytes measured by RIA was 28 ng/mg of protein (Table I, A), a value approximately 2 orders of magnitude lower than that of rat liver microsomes (1–2 μg/mg of protein on the basis of classical purification experiments (27) as well as of our own RIA data (not shown)). Lysis of red cells with large volumes of hypotonic buffer resulted in the recovery of 90% of the protein as well as of reductase in the soluble fraction (Table I, B). Only 2–3% of the reductase was recovered in the ghost fraction, which was enriched approximately 2-fold compared to whole erythrocytes (Table I, D).

In order to partially purify the soluble reductase, we attempted to apply the DEAE-ion exchange procedure described in the literature for human erythrocyte reductase (11, 12), however, we found that this procedure is not effective for rat hemolysates, whereas (NH₄)₂SO₄ precipitation (70% saturation) yielded a preparation with a good enrichment in reductase. To obtain the 15-fold enrichment shown in Table II, C, it was necessary to reduce the hemoglobin concentration of the hemolysate to 3.5 mg/ml before addition of (NH₄)₂SO₄. At higher protein concentrations, a higher recovery of reductase was obtained but much more hemoglobin precipitated under these conditions.

**Results**

**Quantitation of NADH-Cytochrome b5 Reductase by RIA in Erythrocytes and Erythrocyte Subfractions**—Fig. 1A shows a typical RIA standard curve resulting from the competition between unlabelled and 125I-labeled l-reductase for antireductase antibodies. Since we wanted to develop an assay valid for water-soluble as well as for membrane-bound reductase, it was necessary to check whether the amphipathic form of the enzyme (d-reductase) would compete equally with the cationic D-cleaved water-soluble fragment (l-reductase). That this is indeed the case is shown by the data of Fig. 1B, where it can be seen that, using l-reductase as standard, the assay for d-reductase was linear over the entire range examined. The assay was also linear for erythrocyte samples; however, we found that, with quantities of protein larger than 150 μg, there was an increase in background radioactivity, presumably because of trapping of 125I-l-reductase by nonspecifically precipitated hemoglobin. Therefore, we used samples containing less than 150 μg of protein for our RIA determinations.

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**NADH-Cytochrome c Reductase Activity of Erythrocyte Fractions**—In liver microsomes, NADH-cytochrome b5 reductase activity is usually assayed by the rate of reduction of exogenously added cytochrome c, which received electrons from endogenous reduced cytochrome b5. When the NADH-cytochrome c reductase activity of the (NH₄)₂SO₄ precipitate was assayed, it was found to be dependent upon the addition of t-cytochrome b5 (Fig. 2a), presumably because of the low concentration of endogenous cytochrome b5 in this preparation. In contrast, the ghost fraction showed an NADH-dependent reduction of cytochrome c in the absence of exogenous cytochrome b5. Addition of t-cytochrome b5 stimulated this activity only weakly (Fig. 2b). Thus, the membrane-associated reductase appears to behave like the liver microsomal enzyme, being able to reduce cytochrome c via an endogenous membrane-bound electron acceptor (presumably cytochrome b5). That the enzyme cannot itself reduce cytochrome c.

**Table I**

**Distribution of protein and NADH-cytochrome b5 reductase in rat erythrocyte subfractions**

Protein and NADH-cytochrome b5 reductase were determined by the Lowry assay and by RIA, respectively, in erythrocytes and erythrocyte subfractions (see “Materials and Methods” for experimental details).

| Protein | Reductase | Protein | Reductase |
|---------|-----------|---------|-----------|
| mg/ml packed erythrocytes | % | mg/ml packed erythrocytes | % |
| A. Erythrocytes | 361 ± 42 | 100 | 9.8 ± 0.7 | 100 |
| B. Soluble fraction | 322 ± 37 | 89 | 8.9 ± 0.8 | 91 |
| C. 70% (NH₄)₂SO₄ precipitate of B | 5.4 ± 1.2 | 1.5 | 2.5 ± 0.6 | 25 |
| D. Ghosts | 4.1 ± 0.8 | 1.1 | 0.23 ± 0.06 | 23 |

* Averages ± S.E. Numbers in parentheses indicate the number of experiments.

**Table II**

**NADH-cytochrome b5 reductase content of ghost fractions obtained from erythrocyte preparations differently contaminated with white cells**

Three 6-ml portions of rat blood were centrifuged in order to allow red cells to sediment (see “Materials and Methods”). In preparation A, the buffy coat and a 2-mm layer of red cells were removed. The remaining cells were then washed 6 times (see “Materials and Methods”) and each time a 1-mm layer of cells was removed. In preparation B, the buffy coat was not removed, and the cells were washed four times, taking care not to remove leukocytes. Ghosts were obtained from the 3 preparations as described under “Materials and Methods,” and NADH-cytochrome b5 reductase and protein were determined.

| Preparation | (Leukocytes/erythrocytes) x 10⁻⁶ in red cell preparation | Reductase in ghost fraction |
|-------------|--------------------------------------------------------|---------------------------|
| A | 0.04 | 61 |
| B | 0.23 | 64 |
| C | 0.74 | 63 |

* White and red cells counts were carried out in a Burker chamber, using Turk and Hayem solutions for leukocytes and erythrocytes, respectively.

* NADH-cytochrome b5 reductase was determined by RIA (see “Materials and Methods”).
Liver Reductases by Immunostaining

Liver reductases by immunostaining was carried out on: a, the (NH₄)₂SO₄ precipitate; b, the ghost fraction; c, the ghost fraction treated with Triton X-100 (2%, final concentration). t-cytochrome bs, was either absent (open bars) or present at concentrations of 0.43 μM (dashed bar), 0.86 μM (striped bar), or 1.25 μM (dotted bars). Note change in ordinate scale of groups b and c compared to a.

The reduction in efficiency of the ghost-associated reductase activity became dependent on the addition of exogenous t-cytochrome bs (Fig. 2c). The reduction in efficiency of the ghost-associated reductase to use endogenous cytochrome bs after treatment with detergent can be explained by the diminution of the effective phase. The possibility that erythrocyte membrane-bound reductase could reduce an endogenous membrane-bound cytochrome bs was also put forth by Goto-Tamura et al. (15).

The NADH-cytochrome c assay was linear for both the (NH₄)₂SO₄ precipitate and the Triton-treated ghosts at all concentrations of cytochrome bs tested (not shown). Examination of Fig. 2 shows that the specific activity (nmol of cytochrome c reduced/min/mg of protein) of the (NH₄)₂SO₄ precipitate is 7.7-fold higher than that of Triton-treated ghosts at the same t-cytochrome bs concentration. A similar value (8.2) is obtained when the specific activities determined with RIA of the two fractions are compared (Table I). The good agreement obtained with the two methods strengthens our RIA data.

As expected, and in agreement with other reports (15, 16), both the water-soluble and membrane-associated erythrocyte NADH-cytochrome c reductase activities were inhibited by antibodies raised against liver l-reductase. The inhibition curve for the water-soluble erythrocyte reductase is shown in Fig. 3.

Comparison of SDS-PAGE Mobilities of Erythrocyte and Liver Reductases by Immunostaining of Blots—In order to compare the SDS-PAGE mobilities of membrane and soluble reductase without having to carry through laborious purification procedures of erythrocyte NADH-cytochrome bs, reductase, we applied the recently developed blotting procedure of Towbin et al. (36) followed by immunostaining with antireductase antibodies and ¹²⁵I-protein A. In preliminary experiments, we searched for optimal conditions and succeeded in increasing the sensitivity of immunostaining to the ng level. The staining was highly specific. In fact, with rat liver microsomes, a single band was revealed, which co-migrated with purified d-reductase. The preparations that we compared with this method and whose Coomassie brilliant blue staining patterns are shown in Fig. 4A, were: lane 1, purified l-reductase diluted in carrier BSA (only the latter component is revealed by the Coomassie stain); lane 2, (NH₄)₂SO₄ precipitate contained

![Fig. 3. Inhibition of rat erythrocyte NADH-cytochrome bs reductase activity by antibodies raised against rat liver l-reductase. 120 μg (NH₄)₂SO₄ precipitate were incubated in a total volume of 100 μl in the presence of PBS + 1 mm EDTA and increasing concentrations of antireductase IgG purified by affinity chromatography. After incubation for 10 min at room temperature, samples were assayed for NADH-cytochrome c reductase activity (see "Materials and Methods") in the presence of 0.83 μM t-cytochrome bs. 100% (=17 nmol of cytochrome c reduced/min/mg of protein) refers to the value obtained when the (NH₄)₂SO₄ precipitate was incubated in the presence of 150 μg/ml of nonimmune IgG. Incubation with nonimmune IgG did not decrease the enzyme activity compared to that of samples without IgG.]

![Fig. 4. SDS-PAGE analysis of erythrocyte and liver fractions, followed by blotting and specific immunostaining for NADH-cytochrome bs reductase. A shows the Coomassie brilliant blue staining pattern of the preparation which were used for blotting and immunostaining. The autoradiogram of such a blot is seen in B (see "Materials and Methods" for experimental details). Blotting of the lower 1/3 and upper 1/3 of the gel were carried out separately because of the limited dimensions of our blotting apparatus. Lanes contained: lane 1, purified l-reductase (10 ng) plus BSA (1 μg); lane 2, (NH₄)₂SO₄ precipitate (33 μg of protein); lane 3, rat liver microsomes prepared by differential centrifugation (5.5 μg of protein); lane 4, acetone precipitate of the Triton X-100 extract of erythrocyte ghosts (150 μg of protein). Numbers on the left indicate M, values × 10⁻⁹ of protein standards (Bio-Rad SDS-PAGE low molecular weight standards).]
tate; lane 3, a rat liver microsomal fraction; and lane 4, the Triton X-100 extract of erythrocyte ghosts concentrated by acetone precipitation. Immunostaining of a blot of a gel like the one of Fig. 4A showed, as expected, that the reductase present in microsomes migrated more slowly than its water-soluble fragment detached by proteolytic digestion (l-reductase; compare lanes 1 and 3 of Fig. 4B). The ghost-associated reductase co-migrated with microsomal reductase (Fig. 4B, lane 4). Unexpectedly, also the water-soluble erythrocyte reductase was found to migrate more slowly than l-reductase very close to the position of microsomal and ghost reductase (Fig. 4B, lane 2). Based on the position of protein standards, the apparent $M_r$ values of l-reductase and of microsomal uncleaved reductase were ~30,000 and 32,000, respectively.

**Comparison of Erythrocyte Membrane-associated and Soluble Reductases by Gel Filtration in the Presence of Detergent**—If the erythrocyte membrane-associated NADH-cytochrome $b_5$ reductase is really an integral membrane protein similar to the enzyme in liver microsomes, it should bind mild detergents and, therefore, show a larger apparent molecular weight than its soluble counterpart in gel filtration experiments in the presence of Triton X-100. This hypothesis was born out in the experiment shown in Fig. 5. Using a Sephadex G-100 column equilibrated with 2% Triton X-100, the enzyme activity present in the Triton extract of erythrocyte ghosts eluted close to the void volume in the same position as purified rat liver microsomal d-reductase. In contrast, the activity present in the (NH$_4$)$_2$SO$_4$ precipitate (erythrocyte water-soluble reductase) eluted much later, at the position expected for l-reductase (Fig. 5A). Based on the ratio of the elution volumes of reductase and of blue dextran the apparent $M_r$ values of ghost reductase and water-soluble reductase were ~100,000 and ~30,000, respectively. When Triton solubilized ghost proteins were mixed with (NH$_4$)$_2$SO$_4$ precipitated erythrocyte cytoplasmic proteins and the mixture was analyzed on the same Sephadex G-100 column, two NADH-cytochrome $b_5$ reductase peaks were obtained at the expected positions (Fig. 5B).

**Table III**

| Reticulocytes in red cell preparation | Whole red cells | Ghost fraction |
|--------------------------------------|-----------------|----------------|
| %                                   | μg/ml packed red cells | ng/mg protein | μg/ml packed red cells | ng/mg protein |
| A: 1.3                               | 11.6            | 40             | 0.27                   | 60            |
| B: 41.6                              | 20.1            | 67             | 0.36                   | 111           |

*NADH-cytochrome $b_5$ reductase content of erythrocyte ghost fractions obtained from red cell preparations differing in reticulocyte content*

2 rats (body weight, 220 g) were injected intraperitoneally with 0.6 ml of 1.6% phenylhydrazine (pH 7.0) 3 times at 12-h intervals and sacrificed 6.5 days after the first injection (group B). Two untreated rats were sacrificed at the same time (group A). Red cells were prepared as described for preparation A in Table II. The percentage of reticulocytes in the preparations was determined on smears after staining with brilliant cresyl blue. Ghosts were obtained from the two preparations as described under "Materials and Methods," and NADH-cytochrome $b_5$ reductase and protein were determined by RIA and the Lowry assay, respectively.

**DISCUSSION**

It has been known for quite some years that erythrocytes of many animal species possess a soluble NADH-cytochrome $b_5$ reductase, structurally, enzymatically, and immunologically similar to the liver microsomal enzyme (11-16). The presence in erythrocyte ghosts of a membrane-bound form of the enzyme (20, 21), as well as of its electron acceptor cytochrome $b_5$ (37), has also been reported. However, up to the present,
Rat Erythrocyte NADH-Cytochrome b₅ Reductase

13859

rigorous quantitative studies on the distribution of the enzyme between the membrane and cytosolic compartments had not been carried out. The lack of quantitative data in the literature is due to the difficulty of assaying reductase activity in whole hemolysates, on the one hand because of its extremely low concentration, on the other because of the presence in hemolysates of electron acceptors which interfere with the assay when low molecular weight substrates are used (22). In order to obtain an accurate picture of the subcellular distribution of NADH-cytochrome b₅ reductase in erythrocytes, we therefore developed a RIA in the presence of detergent which would permit the determination at the ng level of reductase both in the membrane and in the soluble fraction. The results indicated that nearly all erythrocyte reductase is in the soluble form and that only a small amount (2-3%) is recovered in the ghost fraction. The ghost-associated reductase represented such a low percentage of the total as to cause the suspicion that it was due either to the presence of membranes of white cells and/or reticulocytes, or to contaminating soluble reductase. The first possibility was ruled out by experiments in which the reductase content of ghost fractions obtained from red cell preparations containing different proportions of leukocytes and of reticulocytes were compared. In fact, a nearly 20-fold increase in the leukocyte concentration of blood cell preparations resulted in no change in the reductase concentration in the corresponding ghost fraction, while a 30-fold increase in reticulocyte concentration resulted in only a 2-fold increase in ghost-associated reductase specific activity, indicating that the contribution of reticulocyte membrane-associated reductase to the total reductase recovered in normal erythrocyte ghost fractions is approximately 5%. The higher content of the reductase in the reticulocyte-enriched red cell population is in agreement with the reported age-dependent decay of the enzyme in human erythrocytes (38).

The second possibility, that the membrane-associated reductase is due to the contaminating water-soluble form, seems extremely unlikely on the basis of two different observations. 1) The enzymatic behaviour of the membrane-associated protein is different from that of the soluble form, and similar to that of liver microsomal reductase. 2) Although migration rates of the membrane-associated and water-soluble enzymes in SDS-PAGE were nearly indistinguishable, the two forms of the enzyme were clearly resolved by Sephadex G-100 gel filtration in the presence of Triton X-100. The membrane-associated enzyme, as well as purified d-reductase, eluted at a position corresponding to an apparent Mr, much larger than that found by SDS-PAGE. In contrast, the apparent Mr values of water-soluble erythrocyte reductase found by gel filtration and SDS-PAGE were approximately the same. The high apparent Mr of membrane reductase found by gel filtration in the presence of 2% Triton X-100 is presumably due to detergent binding of the hydrophobic domain of the molecule. Membrane and secretory IgM have also been distinguished by this experimental approach (2). The gel filtration behavior of purified human erythrocyte membrane reductase has recently been analyzed by others (21) in the presence of 0.2% Triton X-100. An apparent Mr of 144,000 was found, which the authors attributed to self-association between reductase molecules. The slightly different results of these workers could be due to the lower detergent concentration used in that study (21) compared to the present work.

On the basis of our experiments, it seems safe to conclude that, in rat erythrocytes, NADH-cytochrome b₅ reductase exists in two forms: a water-soluble protein which represents the majority of reductase molecules, and a small amount of membrane-associated, amphipathic enzyme. It is of interest that in the liver exactly the opposite situation exists, inasmuch as only about 2% of the reductase is found in a membrane-free supernatant. Thus, different cell types appear to be strong favor the synthesis of one of the two forms of the enzyme in accordance with functional requirements.

Our results are in disagreement with other reports that human erythrocyte ghosts contain a conspicuous percentage of the reductase activity of the red cell (50% in Ref. 20 and 20% in Ref. 21). However, as already pointed out, previous studies on the subcellular distribution of erythrocyte reductase were not rigorously quantitative. For example, in Ref. 21, soluble reductase activity was assayed on a fraction partially purified by DEAE-cellulose chromatography. Since the recovery of reductase in this preparation compared to the total soluble fraction of the hemolysate was not determined, it is possible that substantial amounts of soluble reductase were lost during the ion exchange chromatographic step. Another possibility is that soluble reductase was partially inactivated during the purification procedure. In the liver, we have indeed observed that NADH-cytochrome b₅ reductase activity is differentially affected by aging in different locations (8). Lastly, it cannot be excluded that real differences exist between the intracellular distributions of reductase in human and rat erythrocytes.

Whether electron transport chains characteristic of the endoplasmic reticulum are present also on plasma membranes is a question which has been the object of much debate over the past years (39, 40). Our findings, that erythrocyte ghosts possess an amphipathic NADH-cytochrome b₅ reductase different from the soluble form and not attributable to contamination by leucocyte endomembranes, indicate that this enzyme is localized on the plasma membrane of at least one cell type. Whether its recovery in plasmalemmal fractions of other cells, i.e. hepatocytes, is due entirely to contamination by endomembranes, as put forth by some authors (40), reflects the presence of true plasma membrane-associated reductase, as suggested by others (37, 39, 41), remains to be established by further work.

A novel observation of this study concerns the SDS-PAGE mobilities of the different forms of NADH-cytochrome b₅ reductase revealed by specific immunostaining of nitrocellulose blots. By inclusion of polyvinylpyrrolidone and Ficol as well as high concentrations of BSA in the incubation buffers, it was possible to reduce background and to increase sensitivity of the immunostaining to the ng level. It was thus possible to detect NADH-cytochrome b₅ reductase in mixtures where it represented as little as 1/10,000 of total protein. Using this method, we could compare the SDS-PAGE mobilities of the different forms of reductase present in crude fractions, such as the (NH₄)₂SO₄ precipitate of the soluble fraction of the erythrocyte, the Triton extracted proteins of erythrocyte ghosts, and total liver microsomes. The results showed that erythrocyte water-soluble reductase migrates close to the microsomal and ghost-associated form, more slowly than the cathepsin-D solubilized fragment of the enzyme (I-reductase). This result is in variance with a recently published paper, in which it was reported that the purified human erythrocyte cytoplasmic reductase co-migrates with the cathepsin-D cleaved water-soluble fragment of the membrane enzyme (21). Although this different result may be due to a difference between the rat and the human system, we feel that it is more likely that the smaller apparent Mr of human cytoplasmic reductase was due to cleavage at a sensitive site during the purification procedure. In fact, we observed that upon storage of the (NH₄)₂SO₄ precipitate of the soluble fraction of the erythrocyte, a smaller form of soluble reductase was gener-

2 N. Borgese, D. Macconi, L. Parola, and G. Pietrini, unpublished RIA data.
ated, which co-migrated with l-reductase. Other examples of proteins which lose a fragment during purification are offered by peroxisomal catalase (42) and glyoxysomal isocitrate lyase (43). Thus, our results illustrate the importance of the development of micromethods suitable for the analysis in complex mixtures of specific macromolecules which have undergone a minimum of manipulation.

A favored hypothesis for the origin of erythrocyte water-soluble reductase as well as for soluble cytochrome b₅ has been that of proteolytic cleavage of membrane reductase during maturation of the erythroblast (19). Since the membrane enzyme is a two-domain protein, consisting of a hydrophilic, cytoplasmically located moiety and a hydrophobic, membrane-associated part, it is thought that proteolytic cleavage at the junction between the two domains could generate the water-soluble enzyme, which would thus resemble the artificially generated, cathepsin-D cleaved water-soluble fragment of microsomal reductase (l-reductase). Consistent with this hypothesis, membrane associated NADH-cytochrome b₅ reductase and cytochrome b₅ have been identified in Friend erythroleukemia cells (44) and recently, proteolytic activities have been recognized in erythrocyte (45) as well as reticulocyte (46) membrane fractions, which are capable of detaching a water-soluble fragment from membrane reductase at acid pH. Moreover, it has been reported that, in the case of bovine cytochrome b₅, liver lysosomal enzymes can convert the microsomal form to a water-soluble form identical to the erythrocyte water-soluble fragment. Therefore, if erythrocyte water-soluble reductase derives from the membrane-bound form by proteolysis, the processing enzyme(s) involved must have different specificities than liver lysosomal cathepsins. On the other hand, since it has never been demonstrated that membrane reductase is indeed the precursor of the soluble enzyme, it is possible that, as in the case of secretory and membrane proteins, which have the large hydrophilic NH₂-terminal part in common, but have distinct COOH termini, responsible for their different locations. Thus, the synthesis of the soluble form could be independent from that of the membrane enzyme and triggered by a switching event occurring during erythrocyte maturation. This hypothesis is not necessarily inconsistent with the idea that the two forms of reductase are products of the same gene. In fact, recent studies have demonstrated that a single gene can yield different mRNAs by differential splicing of a precursor RNA (1, 48), and also that DNA rearrangements in somatic cells may permit the expression of different combinations of exons at different stages of differentiation (49).

With the available data, it is not possible to decide which of the possible biogenetic pathways is the correct one. Whatever the pathway, the switch from the membrane enzyme to the soluble form must occur before the reticulocyte state since, in the latter cell type, nearly all of the reductase is found in the cytosol. Clearly, experiments on the biosynthesis and on the gene structure of NADH-cytochrome b₅ reductase are required to gain further insight into this problem.

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