Purification and Characterization of the Human Neutrophil NADH-Cytochrome $b_5$ Reductase

By Alfred I. Tauber, Jonathan Wright, Frank K. Higson, Susan A. Edelman, and David J. Waxman

NADH-cytochrome $b_5$ reductase is the predominant NADH-diaphorase found in the human neutrophil (Blood 62:152, 1983). Although this reductase segregates with the light membranes of nitrogen-cavitated neutrophils separated on Percoll gradients (which include the plasma membrane markers alkaline phosphatase and NAPDH-oxidase), it is approximately 95% excluded from plasma membrane-enriched phagocytic vacuoles. The reductase constitutes approximately 5% of the light membrane fraction FAD-flavoprotein (14.8 ± 5.5 pmol/mg protein) and was found in equimolar concentration with a high potential b cytochrome also present in this light membrane fraction and tentatively identified as cytochrome $b_5$. Isolation of the reductase from human neutrophils was accomplished by Triton X-114 solubilization of the light Percoll gradient membranes, followed by temperature-dependent phase separation and then affinity chromatography on AMP-Sepharose. The active preparation contained 1.3 mol FAD/mol protein, migrated on sodium dodecyl sulfate-polyacrylamide gels as a single band corresponding to an apparent mol wt of 45,000 daltons, exhibited a pl of 5.7 on chromatofocusing and was obtained in >70% yield, with an overall purification of almost 900-fold. The purified enzyme was characterized by a high specificity for NADH as electron donor (Km = 6.4 μmol/L vs. Km > 1.6 mmol/L for NADPH) and exhibited a maximal turnover of ca. 30,000 min$^{-1}$ at 22°C with either ferricyanide or cytochrome $b_5$ (Km = 10 nmol/L) as electron acceptor. Although the physical characterization and biochemical properties described here demonstrate that this neutrophil NADH $b_5$ reductase is similar to the corresponding liver and erythrocyte enzymes, its unique function in the neutrophil has yet to be determined.

© 1985 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Triton X-100, Triton X-114, disopropyl fluorophosphate (DFP), aminooxyantiprene, 2-hydroxy-1,4-naphthoquinone, 5,5,7-indigo trisulfonic acid potassium salt, phenazine methosulfate, phenazine ethosulfate, N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid (HEPES), piperazine-N, N'-bis (2-ethanesulfonic acid) (PIPES), 3-(N-morpholino) propane sulfonic acid (MOPS), bovine serum albumin (fraction V), and reduced nicotinamide adenine dinucleotide (phosphate) (NAD[P]H) were obtained from Sigma Chemical Co, St Louis; Dextran T-500, Ficoll-Paque, Percoll, and 5 AMP-Sepharose 4B were obtained from Pharmacia Fine Chemicals, Piscataway, NJ; duroquinone (97%) and anthraquinone-2,6-disulfonic acid, disodium salt were purchased from Aldrich Chemical Co, Milwaukee.

Cell isolation and fractionation. Neutrophils were isolated from normal human donors as previously described. Cells were incubated with DFP to inhibit serine protease activity by the addition of 10 μL of 5 mol/L DFP to 5 mL of neutrophils suspended in phosphate-buffered saline (PBS) for five minutes on ice. The inhibitor was subsequently removed by centrifugation of the cell suspension at 400 g for five minutes. The cells were then disrupted by nitrogen cavitation and fractionation on Percoll gradients as described in detail previously. The harvested fractions were characterized by enzyme marker studies as previously described, with an assay for glucose-6-phosphatase by the method of Harper also included.

From the William B. Castle Hematology Research Laboratory, Boston City Hospital; the Departments of Medicine, Biochemistry, and Pathology, Boston University School of Medicine; and the Department of Biological Chemistry and Dana-Farber Cancer Institute, Harvard Medical School, Boston.

Supported in part by grant No. AI-20064 from the National Institutes of Health (A.I.T.), Research Fellowship from the Arthritis Foundation (J.W.), and grant No. BC-462 from the American Cancer Society (D.J.W.).

Submitted Jan 28, 1985; accepted March 18, 1985.

Address reprint requests to Dr Alfred I. Tauber, FGH-I, Boston City Hospital, 818 Harrison Ave, Boston, MA 02118.

© 1985 by Grune & Stratton, Inc.

0006-4971/85/6603-0031$03.00/0
Phagocytic vacuoles were harvested as described by Stossel et al.\(^{14}\) using paraffin oil; alkaline phosphatase, NADH b, reductase, and myeloperoxidase activities were measured as previously described.\(^{12}\) Yields of these enzymatic activities in sonicated phagocytic vacuoles were calculated based on whole-cell control values. The paraffin oil preparation exhibited no inhibitory activity for the enzymes assayed as assessed by its addition to sonicated control cells.

**b-Type cytochrome redox determinations.** Redox titrations were carried out on samples of well-dispersed sub-cellular fractions of unstimulated neutrophils in 50 mmol/L MOPS, 0.1 mol/L KCl, pH 7.0, under an atmosphere of nitrogen (purified by passage through a column of oxygen-absorbent stored in an Ace-Burlitch inert atmosphere system [Ace Glass, Vineland, NJ]). Oxygen was removed using a BASF catalyst (Ace Glass), which was activated by warming in situ for 30 minutes at 60 °C with 70% nitrogen/30% hydrogen (vol/vol) at a flow rate of three bubbles per second. The integrity of the system was maintained as per the manufacturer’s instructions. A glass redox cuvette, fitted with an inert gas inlet, outlet, and a porthole sealed with a rubber septum for additions by a Hamilton microsyringe was supplied by Dr Owen Jones, Department of Biochemistry, University of Bristol, England. The spectrophotometer cell also contained a ring of brushed platinum and a filled capillary, containing MOPS-KCl buffer linked through a salt bridge of 3% agar/3 mol/L KCl to a calomel/mercury electrode (Fisher Scientific Co, Springfield, NJ). The potential was measured by means of a digital multimeter (Dynascan Corp, Chicago, Model 2810) switched to 1 V full-scale deflection and with resistance much greater than the connection resistance. The circuit was freed of air bubbles, and the capillary dipped 1 cm below the surface of the 7.5-mL sample suspension. Stirring the sample by an Electrothermal Ministrirrer, Model MC4 (Engineering Ltd, London) driven by a Railpower 1100 transformer (Model Rectifier Corp, Edison, NJ) (the paddle just appeared above the light beam), the suspension was allowed to equilibrate with the nitrogen for 30 minutes. Mediators were then added to permit rapid electron transfer between the cytochrome and the other couples added: phenazine methosulfate (12.5 μmol/L), phenazene ethosulfate (12.5 μmol/L), duroquinone (12.5 μmol/L), 5,5,7,7-tindigotisulfonic acid potassium salt (12.5 μmol/L); duroquinone (12.5 μmol/L), and 2-hydroxy-1,4-naphthoquinone (12.5 μmol/L); and 2-hydroxy-1,4-naphthoquinone (12.5 μmol/L), and 2-hydroxy-1,4-naphthoquinone (12.5 μmol/L). These mediations have midpoint potentials (Em) of +80, +55, +5, -70, -145, -184 mV, respectively. Mediator stock solutions were prepared fresh and gassed with purified nitrogen.

A spectrum of the neutrophil suspension over the range from 580 to 530 nm was read at an ambient potential of +245 mV as a baseline on a Beckman (Irvine, Calif) DU7 spectrophotometer (scan mode). Spectra were then run in comparison to this oxidized sample at several points over a range of descending and then ascending potentials. Gassed solutions of sodium dithionite and potassium ferricyanide (both at 1 mg/mL in MOPS-KCl buffer) were introduced to the redox cuvette in microliter aliquots using a Hamilton microsyringe (Fisher Scientific). Dithionite lowered the potential (relative to H+/H- couple) down to -400 mV, while ferricyanide restored it to its starting value. This range could be covered repeatedly by adding a mediator every 30 minutes and then waiting five minutes to enable the potential to stabilize before recording the difference spectrum. The extent of reduction of the cytochrome b at different oxidation-reduction potential (Eo) was calculated by measuring the height of the absorption band at 559 nm above a line joining the troughs on each side of this band. Cytochrome b content was determined by an absolute spectrum against buffer using the absorption peak at 428 nm on a Beckman DU7 spectrophotometer; flavin was determined as previously described.\(^{12}\)

**Carbon monoxide binding to b cytochrome.** Carbon monoxide binding to the reduced cytochrome b was determined with a Beckman DU7 spectrophotometer by recording a baseline spectrum for a sample treated with a trace of dithionite; carbon monoxide was then bubbled through the sample at room temperature for 30 seconds and a difference spectrum taken after five to 20 minutes. The extent of CO binding to the cytochrome is given by the ratio of

\[
\text{CO-bound cytochrome b} = \frac{A_{	ext{peak}} - A_{	ext{trough}}}{A_{	ext{peak}}}
\]

The extent of hemoglobin and mitochondrial contamination was estimated as

\[
\text{CO-bound cytochrome b} = \frac{A_{	ext{peak}} - A_{	ext{trough}}}{A_{	ext{peak}}}
\]

Samples whose A/B ratio was less than 0.9 were considered contaminated with such cytochromes and were excluded from this study.

**Neutrophil NADH-b, reductase isolation.** The light membranes (γ fractions) of Percoll gradients were isolated from blood (20 individual units pooled) and then stored at -80 °C.\(^{12}\) These fractions were thawed, solubilized by the addition of Triton X-114 (11.4%) stock, and prepared as previously described\(^{12}\) to a final concentration of 0.7% (vol/vol). After a 30-minute incubation at 4 °C, the solution was centrifuged at 150,000 g for 30 minutes. The supernatant was removed and warmed to 32 °C for five minutes (above the cloud point for Triton X-114). This solution was then centrifuged at 32 °C for 20 minutes at 1,800 g, resulting in precipitation of the Triton-rich fraction. Seventy-five percent to 90% of the NADH b, reductase activity was recovered in the Triton pellet. This Triton X-114-rich pellet was resuspended in 1.2 mL of 20 mmol/L Tris HCl, 0.2% Triton X-100, pH 7.4, and then applied at 4 °C to a column of 5'-AMP-Sepharose (4 mL) equilibrated with the same buffer. The column was washed with 4 vol of equilibration buffer, and the reductase then eluted with the same buffer containing 1 mmol/L NADH.

**Other methods.** NADH b, reductase activity was measured using the ferricyanide (1 mmol/L) reduction assay previously described,\(^{1}\) where a unit of enzyme activity is defined as the reduction of 1 μmol of ferricyanide per minute as assessed at 420 nm, using 1.04 x 10⁶ M⁻¹ cm⁻¹ as the extinction coefficient.\(^{15}\) Protein concentrations were measured by the method of Lowry et al.\(^{14}\) after precipitating the protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 2-mercaptoethanol–reduced samples and stained using the Bio-Rad silver staining technique (Bio-Rad, Richmond, Calif.).\(^{17}\) The isoelectric point of the neutrophil NADH b, reductase was determined by chromatofocusing. The sample in a 1.0-mL vol (6.0 U/mL) was applied to a PBE 94-polybuffer exchange column (30 mL bed volume) (Pharmacia)\(^{18}\) equilibrated for 12 hours at 4 °C with 25 mmol/L imidazole-HCl and 0.2% Triton X-100 at pH 7.4. The column was eluted with Polybuffer 74 (Pharmacia, an amphoteric buffer made for chromatofocusing in the pH 7 to pH 4 range) and 0.2% Triton X-100 at pH 4.0, and the pH and ferricyanide reductase activity of the eluted fractions was then monitored. Purified rat liver cytochrome b,\(^{19}\) its soluble tryptic fragment,\(^{20}\) and purified rabbit liver NADH b, reductase\(^{21}\) were prepared as described previously. NADH b, reductase-catalyzed cytochrome b reduction was measured indirectly (in the presence of 1 to 100 mmol/L cytochrome b) by the subsequent reduction of cytochrome c (50 μmol/L, e - 550 nm - 21,000 M⁻¹ cm⁻¹) in 0.1 mmol/L KPO₄ (pH 7.4), 0.1 mmol/L EDTA, and 0.004% Triton X-100 at 22 °C.

**RESULTS**

**Subcellular localization.** Studies of the subcellular localization of neutrophil NADH b, reductase, which is
membrane-bound, were attempted by analysis of four fractions corresponding to four intracellular compartments obtained from nitrogen-cavitated neutrophils on Percoll gradients. More than 80% of the NADH ferricyanide reductase cosedimented with the plasma membrane markers alkaline phosphatase and NADPH-oxidase in the \( \gamma \) fraction, but this light membrane fraction also contained approximately 25% of the total cellular glucose-6-phosphate, an endoplasmic reticulum marker. Thus the so-called \( \gamma \) fraction of the Percoll gradient likely contains heterogeneous light membranes, in addition to the plasma membrane. The plasma membrane could be excluded as the subcellular site of neutrophil b\(_5\) reductase, since phagocytic vacuoles (a preparation of plasma membrane-coated ingested paraffin oil) do not contain the \( b_5 \) reductase. Thus, although these phagocytic vacuoles were enriched in the plasma membrane marker alkaline phosphatase (containing 47.5% \( \pm \) 9.3% \( n = 4 \) of the cellular total), they contained only 5.2% \( \pm \) 1.3% \( n = 4 \) of the total cellular NADH ferricyanide reductase activity. The myeloperoxidase and cytochrome b content of these vacuoles were 2.5% \( \pm \) 1.0% and 7.35% \( \pm \) 2.3% \( n = 4 \) of the cellular totals, respectively. These results indicate that the \( b_5 \) reductase is not bound to the plasma membrane, but to another membrane of similar density. Further resolution of the \( \gamma \) Percoll fraction has not been accomplished, and thus the precise site of the reductase localization awaits further study.

**Associated b cytochrome.** The Percoll gradient \( \gamma \) fraction was also found to contain a b-type cytochrome of decidedly higher potential than the Percoll \( \beta \) fraction b-type cytochrome previously identified as the low-potential cytochrome b\(_{245}\). The specific content of this high-potential cytochrome was 17.4 \( \pm \) 5.5 pmol/mg protein (mean \( \pm \) SD, \( n = 3 \)), corresponding to approximately 9% of the \( \gamma \) fraction b cytochrome obtained from resting neutrophils. The redox potential of this high-potential b-type cytochrome was estimated to be between +40 and -50 mV. Cytochrome b\(_5\) has a midpoint redox potential of +2 mV, a value consistent with the tentative assignment of the high-potential neutrophil b-type cytochrome as cytochrome b\(_5\).

**Purification of neutrophil NADH b\(_5\) reductase.** The \( \gamma \) fraction of the Percoll gradient subfractionated neutrophils contained 80% of the cellular NADH b\(_5\) reductase activity and was therefore used as the starting material for isolation of this enzyme. Neutrophils were treated with DFP before cell disruption, since this agent has been shown to protect endogenous neutrophil proteins from proteolytic digestion. As shown in Table 1, solubilization with Triton X-114 was highly efficient and preserved enzyme activity; recovery of b\(_5\) reductase activity from AMP-Sepharose, an affinity absorbent with a group specificity for NAD-requiring enzymes, was routinely greater than 50% (Fig 1). Fractions eluting from the affinity column exhibiting activities greater than 2 U/mL were pooled, concentrated in a Micro-ProDiCon apparatus (Biomolecular Dynamics, Beaverton, Ore), and analyzed on 10% polyacrylamide gels in the presence of 2% SDS followed by silver staining. A single band was observed for the purified b\(_5\) reductase (mean \( \pm \) SD of three preparations), corresponding to a mol wt of 45,000 \( \pm \) 2,000 daltons. The flavin-protein ratio for the purified enzyme was 1.30 \( \pm \) 0.06 (mean \( \pm \) SD, \( n = 2 \)). The reductase was applied to a chromatofocusing column (see Materials and Methods) and individual fractions each corresponding to \( \approx \)2.5% of the bed volume collected and then assessed for enzymatic activity. The activity migrated to equilibrium at pH 5.7 \( \pm \) 0.1 (\( n = 2 \)).

The concentration of NADH b\(_5\) reductase in the Percoll gradient \( \gamma \) fraction was calculated to determine what percentage of the FAD-flavoproteins are accounted for by this activity. With three separate preparations, a concentration of 14.8 \( \pm \) 5.5 pmol/mg \( \gamma \) fraction protein was found, which corresponds to approximately 5% of the FAD-containing proteins of this fraction and closely approximates the concentration of the high-potential b cytochrome (17.4 \( \pm \) 5.5 pmol/mg) also found in this fraction.

**Biochemical characterization of the neutrophil b\(_5\) reductase.** This purified human neutrophil diaphorase, isolated on the basis of its NADH-dependent ferricyanide reductase activity, was identified as an NADH b\(_5\) reductase both by its physical similarities to the corresponding liver and erythrocyte enzymes, and by its ability to reduce purified cytochrome b\(_5\). Because only small quantities of the purified neutrophil enzyme were available, its cytochrome b\(_5\) reductase activity was monitored indirectly, i.e., in the presence of

Table 1. Purification of Neutrophil NADH b\(_5\) Reductase

<table>
<thead>
<tr>
<th>Percoll ( \gamma ) fraction</th>
<th>Triton X-114 supernatant</th>
<th>Triton X-114 pellet</th>
<th>AMP-Sepharose eluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Recovery (U/mg)</td>
<td>Total Recovery (U/mg)</td>
<td>Total Recovery (U/mg)</td>
<td>Total Recovery (U/mg)</td>
</tr>
<tr>
<td>ml U/mL mg/mL U/mg (%)</td>
<td>ml U/mL mg/mL U/mg (%)</td>
<td>ml U/mL mg/mL U/mg (%)</td>
<td>ml U/mL mg/mL U/mg (%)</td>
</tr>
<tr>
<td>26.5 1.52 1.73 0.88</td>
<td>25.5 1.66 1.08 1.54 103 1.75</td>
<td>22.0 1.71 .41 4.17 93 4.74</td>
<td>1.8 15.7 .02 785.0 73 892.0</td>
</tr>
</tbody>
</table>

Enzyme activity was assessed by reduction of ferricyanide as detailed in Materials and Methods.
cytochrome c. These experiments were performed in the presence of 0.004% Triton X-100, which has been shown to facilitate productive interactions between cytochrome b\textsubscript{5} and liver NADH b\textsubscript{5} reductase\textsuperscript{24} and at concentrations of pyridine nucleotide (NADH, 0.16 mmol/L) and terminal electron acceptor (cytochrome c, 50 μmol/L) well above their respective K\textsubscript{m} values.\textsuperscript{25} Under these conditions, electron transfer from cytochrome b\textsubscript{5} to cytochrome c is not expected to be rate limiting.\textsuperscript{25} As shown in Table 2, the reduction of cytochrome c catalyzed both by the purified neutrophil b\textsubscript{5} reductase and by the corresponding liver b\textsubscript{5} reductase was obligatorily dependent on the presence of cytochrome b\textsubscript{5}. No activity was observed using a soluble tryptic fragment of cytochrome b\textsubscript{5}, consistent with the observations of Mihara and Sato using NADH b\textsubscript{5} reductase isolated from rabbit liver.\textsuperscript{24} Virtually no cytochrome c reduction was observed in the absence of the nicotinamide cofactor. NADPH (1.6 mmol/L) exhibited only ~7% to 9% of the activity of NADH under standard assay conditions, suggesting a K\textsubscript{m} for NADPH of >1.6 mmol/L. This can be compared with a K\textsubscript{m} (NADH) of 6.4 μmol/L for the neutrophil enzyme (data not shown) (cf K\textsubscript{m} [NADH] = 2.7 to 6.0 μmol/L for the enzyme from calf liver\textsuperscript{26,27}), in agreement with the pyridine nucleotide specificity reported for the liver and erythrocyte enzymes.\textsuperscript{26-29} Both the neutrophil and liver b\textsubscript{5} reductases exhibited apparent K\textsubscript{m} values for cytochrome b\textsubscript{5} that were on the order of 8 to 10 nmol/L with the V\textsubscript{max} for cytochrome c reduction indistinguishable from the maximal rates of ferricyanide reduction obtained in the presence of saturating (1 mmol/L) K\textsubscript{3}Fe(CN)\textsubscript{6} (Table 3). These rates correspond to turnover numbers of approximately 30,000 at 22 °C for both enzymes.

**DISCUSSION**

The biochemical properties that we initially described for the membrane-bound NADH-ferricyanide reductase activity of human neutrophils\textsuperscript{1} were virtually identical to those reported previously for both the microsomal NADH b\textsubscript{5} reductase of liver and the soluble methemoglobin reductase of erythrocytes.\textsuperscript{24-28} This suggested that the majority, if not all, of the membrane-bound ferricyanide reductase activity of human neutrophils is NADH b\textsubscript{5} reductase. The physical and biochemical properties of the purified human neutrophil enzyme reported here (isolated in >70% yield based on total neutrophil ferricyanide reductase activity) provide firm support for the identity of this activity with the previously characterized b\textsubscript{5} reductases isolated from other tissues, including liver and erythrocytes.

The apparent mol wt of the neutrophil NADH b\textsubscript{5} reductase (45,000 daltons) corresponds to that of the liver enzyme.\textsuperscript{23} The mol wt reported for the purified erythrocyte membrane b\textsubscript{5} reductase (36,000 to 45,000 daltons) can be compared with a value of 29,000 to 33,000 daltons for its proteolyzed, soluble form.\textsuperscript{18-20} These values refer to monomeric mol wt; the detergent-solubilized enzyme exists as an oligomeric aggregate (sedimentation coefficient of 135), compared with a value of 29,000 to 33,000 daltons for its proteolyzed, soluble form.\textsuperscript{18-20} These values refer to monomeric mol wt; the detergent-solubilized enzyme exists as an oligomeric aggregate (sedimentation coefficient of 135), having an estimated mol wt of about 360,000.\textsuperscript{22} This erythrocyte enzyme, as well as the corresponding liver enzyme,\textsuperscript{27,31-33} is highly susceptible to proteolytic digestion,\textsuperscript{30} and we have therefore attempted to protect against proteolysis by the neutrophil enzyme by DFP treatment of the neutrophils before cell disruption. The purified neutrophil b\textsubscript{5} reductase eluted in the void volume of a Sephadex G-75 column and just behind the exclusion volume of a Sephadex S-200 column in the presence of 0.2% Triton X-100 (data not shown), suggesting that it had not undergone proteolytic cleavage. Consistent with this conclusion is our observation that the purified neutrophil b\textsubscript{5} reductase could reduce cytochrome b\textsubscript{5}, but not a soluble, tryptic fragment of cytochrome b\textsubscript{5} at the same maximal rate observed for ferricyanide reduction. The flavin content of approximately 1 mol FAD/mol enzyme is the same reported for the erythrocyte\textsuperscript{28} and the liver enzymes\textsuperscript{24} and supports the identity of this reductase with that found in other tissues. That the human neutrophil b\textsubscript{5} reductase purified in this study may be distinct from the corresponding enzyme purified from other sources is suggested by the more stringent conditions required for its solubilization\textsuperscript{1} and by the pl of 5.7 determined for the neutrophil enzyme in the present study, a value that differs from the pl values of 6.4 to 6.7 determined for NADH b\textsubscript{5} reductase isolated from human erythrocyte,\textsuperscript{24} placenta,\textsuperscript{31} and rabbit liver.\textsuperscript{31} It should be noted, however, that these values might not be directly comparable, since the technique used in this study (chromatofocusing) differs from the ones used

<table>
<thead>
<tr>
<th>Table 2. NADH b\textsubscript{5} Reductase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reaction Mixture</strong></td>
</tr>
<tr>
<td>Complete system +</td>
</tr>
<tr>
<td>- cytochrome b\textsubscript{5}</td>
</tr>
<tr>
<td>+ tryptic b\textsubscript{5}†</td>
</tr>
<tr>
<td>- NADH, + NADPH (1.6 mmol/L)‡</td>
</tr>
</tbody>
</table>

*An estimated 0.5 to 0.6 pmol of b\textsubscript{5} reductase purified from either human neutrophils or rabbit liver was diluted into 0.1 mol/L KPO\textsubscript{4} (pH 7.4) containing 50 mmol/L rat liver cytochrome b\textsubscript{5}, 50 μmol/L cytochrome c, and 0.004% Triton X-100 (wt/vol), and the rate of cytochrome c reduction at 22 °C monitored at 550 nm after addition of NADH to 0.16 mmol/L.

†Tryptic cytochrome b\textsubscript{5} (50 nmol/mL) added in place of uncleaved cytochrome b\textsubscript{5}.

‡Reaction initiated by NADPH (1.6 mmol/mL) in place of NADH.
previously for PI determination (cf reference 36). We are unable to explain the large difference in Km values for cytochrome b5 obtained with both our liver and neutrophil preparations (approximately 9 nmol/L, Table 3) as compared with previous studies using purified calf and rabbit liver b5 reductase, where the Km for b5 is reported as 45 to 15 μmol/L, respectively.27,31 It should be noted, however, in our study that the liver and neutrophil enzymes exhibit the same kinetic parameters (Table 3).

With regards to subcellular localization, NADH b5 reductase is found in the endoplasmic reticulum of liver and in both the plasma membrane and the cytosol in the erythrocyte22,28,30. Although the human neutrophil b5 reductase cosediments with the plasma membrane markers alkaline phosphatase and NADPH-oxidase,12 it is excluded from plasma membrane-containing phagocytic vacuoles, indicating that the reductase is localized in an unidentified light membrane, which is distinct from the plasma membrane. In liver, NADH b5 reductase is present largely in the endoplasmic reticulum but is also found in the outer mitochondrial membrane and in the Golgi apparatus12,31. Each of these components is sparsely represented in the neutrophil.17 Glucose-6-phosphatase, an enzyme found in the endoplasmic reticulum, does not localize to the γ fraction of the Percoll gradient in the same proportion as does the b5 reductase, suggesting that the two enzymes are found in distinct compartments in the intact cell. Experiments pursuant to resolution of this issue are underway, as are studies related to the possible role of the reductase in neutrophil lipid metabolism.38

Neutrophil b5 reductase accounts for approximately 5% of the flavoprotein content of the γ Percoll fraction. Its concentration of 15 pmol/mg of γ fraction protein is roughly equivalent to that of the high-potential b cytochrome (17 pmol/mg) present in this same fraction and tentatively identified as cytochrome b5. We have previously postulated that the low NADH cytochrome c reductase activity catalyzed by the particulate neutrophil b5 reductase might reflect the presence of low levels of endogenous cytochrome b5, which serves as intermediate for this electron transfer reaction.25,39,40 However, the present suggestion of equimolar concentration of b5 and b5 reductase suggests instead that the neutrophil b5 reductase and the endogenous high-potential b-type cytochrome may be incapable of effectively reacting in the subcellular membrane preparation we used.3 With the isolation of the neutrophil b5 reductase, this problem may be explored more fully.

The functional role of the neutrophil b5 reductase has not been determined. Although ferricyanide reductase activity does not change on neutrophil activation,1 it is plausible that the neutrophil b5 reductase may function in stimulated cells in a different fashion from that observed in resting cells, since the stimulated neutrophil's lipid requirements are dramatically altered.37 Reconstitution experiments with purified components should enable more sophisticated study of these questions. Determination of the biochemical role of the neutrophil NADH b5 reductase rests on determining its physiologic electron acceptor(s) and a comprehensive assessment of its lipid substrate specificity in resting and activated neutrophils.

REFERENCES

19. Waxman DJ, Walsh C: Phenobarbital-induced rat liver


27. Spatz L, Strittmatter P: A form of reduced nicotinamide adenine dinucleotide-cytochrome b5 reductase containing both the catalytic site and an additional hydrophobic membrane-binding segment. J Biol Chem 248:793, 1973


40. Rogers MJ, Strittmatter P: The binding of reduced nicotinamide adenine dinucleotide-cytochrome b5 reductase to hepatic microsomes.
Purification and characterization of the human neutrophil NADH-cytochrome b5 reductase

Al Tauber, J Wright, FK Higson, SA Edelman and DJ Waxman