Purification and Characterization of the Human Neutrophil NADH-Cytochrome b5 Reductase

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

NADH-cytochrome b5 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 NADPH-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 b5. 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 pI 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 v 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 b5 (Km ~ 10 mmol/L) as electron acceptor. Although the physical characterization and biochemical properties described here demonstrate that this neutrophil NADH b5 reductase is similar to the corresponding liver and erythrocyte enzymes, its unique function in the neutrophil has yet to be determined.

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MATERIALS AND METHODS

Triton X-100, Triton X-114, diisopropyl fluorophosphate (DFP), aminooxyacetic acid, 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), piperezine-N, N'-bis (2-ethanesulfonic acid) (PIPES), 3-(N-morpholino) propene 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, Ficolli-Paque, Percoll, and 5 AMP-Sepharose 4B were obtained from Pharmacia Fine Chemicals, Piscatway, 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 fractionated 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.

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Phagocytic vacuoles were harvested as described by Stossel et al.\textsuperscript{14} using paraffin oil; alkaline phosphatase, NADH b, reductase, and myeloperoxidase activities were measured as previously described.\textsuperscript{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.

\textbf{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 KCI to a calomel/mercury electrode (Fisher Scientific Co, Springfield, NJ). The potential was measured by means of a digital multimeter (Dynascop 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 Electrophoretic Miniristrir, 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 mmol/L), phenazine ethosulfate (12.5 mmol/L), duroquinone (12.5 mmol/L), 5,5,7-indigotrisulfonic acid potassium salt (12.5 mmol/L), duroquinone (12.5 mmol/L), and 2-hydroxy-1,4-naphthoquinone (12.5 mmol/L), anthraquinone-2,6-disulfonate disodium salt (12.5 mmol/L). These mediators 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 assessed at 22 °C.\textsuperscript{12} The extent of hemoglobin and mitochondrial contamination was estimated as

\[
\text{A} = \frac{\text{peak}_{580} - \text{peak}_{540}}{\text{peak}_{540}}
\]

The extent of hemoglobin and mitochondrial contamination was estimated as

\[
\text{B} = \frac{\text{peak}_{540} - \text{peak}_{420}}{\text{peak}_{420}}
\]

\textbf{RESULTS}
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 coexistulated with the plasma membrane markers alkaline phosphatase and NADPH-oxidase in the γ fraction, but this light membrane fraction also contained approximately 25% of the total cellular glucose-6-phosphatase, an endoplasmic reticulum marker. Thus the so-called γ 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% ± 9.3% of the cellular total), they contained only 5.2% ± 1.3% (n = 4) of the total cellular NADH ferricyanide reductase activity. The myeloperoxidase and cytochrome b content of these vacuoles were 2.5% ± 1.0% and 7.35% ± 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 γ Percoll fraction has not been accomplished, and thus the precise site of the reductase localization awaits further study.

Associated \(b\) cytochrome. The Percoll gradient γ fraction was also found to contain a \(b\)-type cytochrome of decidedly higher potential than the Percoll β fraction. This \(b\) cytochrome previously identified as the low-potential cytochrome \(b_{266}\). The specific content of this high-potential cytochrome was 17.4 ± 5.5 pmol/mg protein (mean ± SD, n = 3), corresponding to approximately 9% of the γ fraction \(b\) cytochrome obtained from resting neutrophils. The redox potential of this high-potential \(b\) 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 γ 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-ProDisCon 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 ± SD of three prepara- tions), corresponding to a mol wt of 45,000 ± 2,000 daltons. The flavin-protein ratio for the purified enzyme was 1.30 ± 0.06 (mean ± SD, n = 2). The reductase was applied to a chromatofocusing column (see Materials and Methods) and individual fractions each corresponding to ~2.5% of the bed volume collected and then assessed for enzymatic activity. The activity migrated to equilibrium at pH 5.7 ± 0.1 (n = 2).

The concentration of NADH \(b_5\) reductase in the Percoll gradient γ 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 ± 5.5 pmol/mg γ 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 ± 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

![Fig 1](https://example.com/fig1.png)
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 b5 and liver NADH b5 reductase and at concentrations of pyridine nucleotide (NADH, 0.16 mmol/L) and terminal electron acceptor (cytochrome c, 50 μmol/L) well above their respective Km values. Under these conditions, electron transfer from cytochrome b5 to cytochrome c is not expected to be rate limiting. As shown in Table 2, the reduction of cytochrome c catalyzed both by the purified neutrophil b5 reductase and by the corresponding liver b5 reductase was obligatorily dependent on the presence of cytochrome b5. No activity was observed using a soluble tryptic fragment of cytochrome b5, consistent with the observations of Mihara and Sato using NADH b5 reductase isolated from rabbit liver. Both the neutrophil and liver b5 reductases exhibited apparent Km values for cytochrome b5 that were on the order of 8 to 10 nmol/L with the Vmax for cytochrome c reduction indistinguishable from the maximal rates of ferricyanide reduction obtained in the presence of saturating (1 mmol/L) K3Fe(CN)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 were virtually identical to those reported previously for both the microsomal NADH b5 reductase of liver and the soluble methemoglobin reductase of erythrocytes. Both the neutrophil and liver b5 reductases that were on the order of 8 to 10 nmol/L with the Vmax for cytochrome c reduction indistinguishable from the maximal rates of ferricyanide reduction obtained in the presence of saturating (1 mmol/L) K3Fe(CN)6 (Table 3). These rates correspond to turnover numbers of approximately 30,000 at 22 °C for both enzymes.

**Table 2. NADH b5 Reductase Activity**

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th>Neutrophil b5 Reductase (nmol/min)</th>
<th>Liver b5 Reductase (nmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system*</td>
<td>11.71</td>
<td>8.46</td>
</tr>
<tr>
<td>− cytochrome b5</td>
<td>0.24</td>
<td>0.36</td>
</tr>
<tr>
<td>+ tryptic b5†</td>
<td>0.31</td>
<td>0.26</td>
</tr>
<tr>
<td>− NADH, + NADPH</td>
<td>0.78</td>
<td>0.76</td>
</tr>
<tr>
<td>(1.6 mmol/L)‡</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*An estimated 0.5 to 0.6 pmol of b5 reductase purified from either human neutrophils or rabbit liver was diluted into 0.1 mol/L KPO4 (pH 7.4) containing 50 mmol/L, rat liver cytochrome b5, 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 b5 (50 nmol/L) added in place of uncleaved cytochrome b5.
‡Reaction initiated by NADPH (1.6 mmol/L) in place of NADH.

**Table 3. Kinetic Analysis of NADH b5 Reductase Activity**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Neutrophil b5 Reductase</th>
<th>Liver Reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km (b5) (nmol/L)</td>
<td>9.9 ± 3.2</td>
<td>7.9 ± 1.7</td>
</tr>
<tr>
<td>Vmax (cytochrome c) (nmol/min)</td>
<td>27.6 ± 9.0</td>
<td>32.4 ± 6.6</td>
</tr>
<tr>
<td>Vel (K3Fe(CN)6) (nmol/min)</td>
<td>24.0 ± 2.2 (n = 3)</td>
<td>33.0 ± 1.5 (n = 6)</td>
</tr>
</tbody>
</table>

Km values (± SD) for cytochrome b5 were obtained from cytochrome c reductase assays performed under the standard assay conditions (Table 2) using an estimated 1 pmol enzyme per assay sample. Maximal velocities for cytochrome c reduction are compared with the rates obtained for ferricyanide reduction ("Vel"; mean ± SD for three to six determinations) using saturating K3Fe(CN)6 (1 mmol/L, final concentration) in place of cytochrome b5 plus cytochrome c.

of human neutrophils is NADH b5 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 b5 reductases isolated from other tissues, including liver and erythrocytes.

The apparent mol wt of the neutrophil NADH b5 reductase (45,000 daltons) corresponds to that of the liver enzyme. The mol wt reported for the purified erythrocyte membrane b5 reductase (36,000 to 45,000 daltons) can be compared with a value of 29,000 to 33,000 daltons for its proteolized, soluble form. These values refer to monomeric mol wt; the detergent-solubilized enzyme exists as an oligomeric aggregate (sedimentation coefficient of 13S), having an estimated mol wt of about 360,000. This erythrocyte enzyme, as well as the corresponding liver enzyme, is highly susceptible to proteolytic digestion, and we have therefore attempted to protect against proteolysis of the neutrophil enzyme by DFP treatment of the neutrophils before cell disruption. The purified neutrophil b5 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 b5 reductase could reduce cytochrome b5, but not a soluble, tryptic fragment of cytochrome b5, 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 b5 and the liver enzymes and supports the identity of this reductase with that found in other tissues. That the human neutrophil b5 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 and by the pi of 5.7 determined for the neutrophil enzyme in the present study, a value that differs from the pi values of 6.4 to 6.7 determined for NADH b5 reductase isolated from human erythrocyte, placenta, and rabbit liver. 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
previously for pl 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 erythrocyte.22,28,29 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 apparatus32,33; each of these components is sparsely represented in the neutrophil.34 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.35

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.36 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.

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