Characterization of the Purified NADH-Cytochrome \( b_5 \) Reductase of Human Erythrocytes as a FAD-containing Enzyme*

Toshitugu Yubisui and Masazumi Takeshita†§

From the Department of Biochemistry, Kanazawa University School of Medicine, and the Department of Medical Technology, School of Paramedicine, Kanazawa University, Kanazawa, Japan

NADH-cytochrome \( b_5 \) reductase of normal human erythrocytes was purified by procedures including affinity chromatography on Blue-Sepharose to an electroforetically homogeneous protein. The purified enzyme was judged to be a typical flavoprotein based on its absorption spectrum (absorption maxima, 272, 390, and 462 nm; shoulders, 373 and 488 nm) and flavin content (1 mol of FAD/mmol of enzyme). The minimum molecular weight calculated from the flavin content was 32,300.

The purified enzyme showed a distinct negative circular dichroic spectrum at 280 nm and also at 460 to 480 nm. With the best preparations, the molar ellipticities at 460 and 480 nm were well correlated with the enzyme activity and flavin content in the enzyme. A partial loss of flavin from the enzyme led to a concomitant loss of enzyme activity and decrease in the molar ellipticities at 460 and 280 nm. Flavin analogues such as acrinol and proflavine (0.1 mM) strongly inhibited the enzyme activity, and atebirin (0.1 mM) also showed partial inhibition. Complete inhibition was observed with 1 mM of any of these reagents. These results apparently indicate that FAD in the enzyme functions as a prosthetic group, and that circular dichroic spectroscopy is a good measure of the bound form of flavin in the enzyme.

NADH-methemoglobin reductase in human erythrocytes is well known to be the major enzyme to reduce methemoglobin and is deficient in erythrocytes from patients with hereditary methemoglobinemia (1). This enzyme was recently defined as NADH-cytochrome \( b_5 \) reductase (2-4). Scott and McGraw (5), Hegesh and Avron (6), Sugita et al. (3), and Passon and Hultquist (4) described the purification and properties of NADH-dehydrogenase (NADH-cytochrome \( b_5 \) reductase) from human erythrocytes. Their enzymes commonly contained only a small amount of flavin, and some workers claimed that their enzyme does not contain a prosthetic group such as flavin or heme. On the other hand, Kuma and Inomata (7) reported that their purified NADH-methemoglobin reductase contains 1 mol of FAD/mmol of enzyme. These discrepancies concerning the role of flavin in the enzyme prompted us to reinvestigate the NADH-cytochrome \( b_5 \) reductase of human erythrocytes, since it is generally believed that hemoprotein reductases have a prosthetic group such as flavin.

In this paper, we report the properties of NADH-cytochrome \( b_5 \) reductase purified from human erythrocytes, with special attention focusing on the functions of FAD in the enzyme. We found that the molar ellipticity of CD at 460 and at 280 nm is closely correlated with the content of bound flavin and with enzyme activity.

EXPERIMENTAL PROCEDURES

Materials—Normal human red blood cells were obtained from the central laboratory of the Tokyo Red Cross Blood Transfusion Service. NADH, FAD, and FMN were purchased from Boehringer Mannheim, and diethyliothreitol was obtained from Wako Pure Chemicals, Japan. Other reagents were obtained commercially. Cytochrome \( b_5 \) was purified from rabbit liver according to the method of Omura and Takesue (8) after digestion with trypsin, and NADH-cytochrome \( b_5 \) reductase was also purified from rabbit liver by the method of Takesue and Omura (9) after digestion with lysosomal protease. The concentration of cytochrome \( b_5 \) was determined using a difference in the millimolar extinction coefficient of 100 at 424 nm for the reduced minus oxidized form (8).

NADH-Cytochrome \( b_5 \) Reductase of Normal Human Erythrocytes—NADH-cytochrome \( b_5 \) reductase was purified from normal human erythrocytes. The first four steps used here were the same as described previously (10) for the purification of NADPH-flavin reductase from human erythrocytes. The NADH-cytochrome \( b_5 \) reductase eluted from the Ultrogel column was concentrated by 70% ammonium sulfate. After the dialysis against 20 mM phosphate buffer (pH 7.0) containing 0.2 mM dithiothreitol and 1 mM EDTA, the concentrated enzyme was then applied on a Blue-Sepharose column which had been equilibrated with the same buffer. The column  was washed with the same buffer until the absorbance of the effluent at 280 nm became less than 0.1, and the enzyme adsorbed on the Blue-Sepharose was eluted with the same buffer containing 0.1 mM KCl. The eluted enzyme was again concentrated with ammonium sulfate as described above and dialyzed against 10 mM phosphate buffer (pH 7.0) containing 0.1 mM diethyliothreitol and 1 mM EDTA. The dialyzed enzyme was then subjected to preparative electrophoresis using a carrier Ampholine of pH 5 to 8 as described previously (10). After the electrophoresis, most active fractions were collected and applied on a Sephadex G-75 column to remove any impurities and carrier ampholyte. The final preparation was concentrated with 70% ammonium sulfate and dialyzed against 50 mM phosphate buffer (pH 7.5) containing 0.1 mM dithiothreitol and 1 mM EDTA.

Electrophoresis—Electrophoresis of proteins on polyacrylamide gel was carried out by the method of Davis (11). Electrophoresis in the presence of SDS was performed by the method of Weber and Osborn (12).

Enzyme Activity—NADH-cytochrome \( b_5 \) reductase activity was measured by the method of Takesue and Omura (10) and Passon and Hultquist (4) with some modifications. The reaction mixture (2 ml) contained 0.05 M Tris-HCl buffer (pH 8.0), 0.1 mM NADH, 5 μM cytochrome \( b_5 \), purified from rabbit liver microsomes, and an appropriate amount of enzyme. The reaction was started by the addition of

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† Present address, Department of Biochemistry, Medical College of Oita, Hazama-cho, Oita, Japan.

‡ The abbreviation used is: SDS, sodium dodecyl sulfate.
NADH and performed at 25°C. The reaction was measured by:
following the absorbance change at 424 nm and calculated by using a
difference in millimolar extinction coefficient of 100 as described
above.

Analysis of Flavin—Fluorometric analysis of flavin in the purified
enzyme was performed by the method of Burch et al. (14) as described
previously (10). Paper chromatography was carried out as described
by Yagi (15) on Toyofilter paper No. 50 in the dark and at room
temperature. Development of flavins on the filter paper was per-
formed using the upper layer of the solvent system of 1-butanol:acetic
acid:water (4:1:5). After development, flavin on the filter paper was
detected by ultraviolet light.

CD Spectra—CD spectra of the purified enzyme were measured at
25°C with a Jasco J-20 automatic recording spectrophotometer-
polarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The intensity of the CD
spectrum was expressed as molar ellipticity [θ], deg cm²/dmol, assum-
ing a molecular weight of 33,000.

Other spectrophotometric determinations were performed with a
Union SM 401 automatic recording spectrophotometer or with a
Hitachi recording spectrophotometer model 124.

Protein—Protein was determined by the method of Lowry et al.
(16) using serum albumin as a standard.

RESULTS

Purity of the Enzyme—Purity of the final preparation was
examined by electrophoresis on polyacrylamide gel in the
absence and presence of SDS. In the absence of SDS, one
distinct main band and two or three minor bands were
detected with the purified enzyme by staining for protein and
enzyme activity. In the presence of SDS, however, only one
main band stained for protein was observed on the gel.

Absorption Spectrum—The purified enzyme has absorption
maxima at 272, 390, and 462 nm, and shoulders at 373 and 488
nm. The ratio of the absorbance at 272 to 462 nm was 6.4, and
this value is very close to that reported by Kuma and Inomata
(7) for erythrocyte methemoglobin reductase, and those re-
ported by other workers (9, 17) for mammalian liver micro-
somal NADH-cytochrome b₅ reductase.

Prosthetic Group—Flavin analyses were made on different
batches of the purified enzyme, and the results obtained are
shown in Table I. Flavin contained in the purified enzyme was
identified as FAD by paper chromatography. FAD contained in
the purified enzyme was at best 100% of the enzyme purified
from human erythrocytes, and those re-
ported by other workers (9, 17) for mammalian liver micro-
somal NADH-cytochrome b₅ reductase.

Table I

Enzyme activity, flavin content, and molar ellipticity of CD spectra
at 460 nm of purified NADH-cytochrome b₅ reductases

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Enzyme activity</th>
<th>Flavin content</th>
<th>CD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I'²</td>
<td>II'²</td>
<td>mol/mol enzyme</td>
</tr>
<tr>
<td>I'</td>
<td>90.7</td>
<td>2935</td>
<td>1.02</td>
</tr>
<tr>
<td>2'</td>
<td>46.5</td>
<td>2436</td>
<td>0.63</td>
</tr>
<tr>
<td>3'</td>
<td>38.3</td>
<td>2636</td>
<td>0.48</td>
</tr>
<tr>
<td>4'</td>
<td>96.3</td>
<td>3040</td>
<td>0.32</td>
</tr>
<tr>
<td>5'</td>
<td>29.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a NADH-cytochrome b₅ reductase activity was expressed as micro-
moles/min/mg of protein.

b NADH-cytochrome b₅ reductase activity was expressed as moles/
min/mg of FAD.

c Molar ellipticity [θ] of the enzymes was expressed in deg cm²/
dmol of enzyme.

d Molar ellipticity [θ] of the enzymes was expressed in deg cm²/
dmol of FAD.

e Different batches of enzymes purified from human erythrocytes
by our method.

f Enzyme purified from rabbit liver microsomes.

ND, not determined.

* Enzyme purified from human erythrocytes by the method of
Sugita et al. (3).
by the method of Sugita et al. (3) and with rabbit liver microsomal enzyme as shown in Table I. These results apparently indicate that FAD contained in the NADH-cytochrome b₅ reductase purified from human erythrocytes functions as a prosthetic group, and that the CD measurement of the enzyme is useful to determine the amount of bound flavin or of the functionally active enzyme.

Effects of Flavins and Flavin Anallogues—Table II shows the effects of flavins and some flavin analogues on NADH-cytochrome b₅ reductase activity. By preincubation of the purified enzyme with FAD (10 μM), about 20 to 30% activation was observed, but the effect was smaller. The activation by flavin was, however, not always reproduced, and this may be due to the instability of the apoprotein of the enzyme as suggested by Strittmatter (18). Atebrin showed partial inhibition, but acrinol and proflavine strongly inhibited the enzyme activity. Complete inhibition was observed with 1 mM of any of these reagents.

DISCUSSION

One of the important problems to be clarified concerning the nature of NADH-cytochrome b₅ reductase of human erythrocytes is whether or not the enzyme contains flavin as a prosthetic group. Enzymes purified from human erythrocytes by many workers (3-6) contained only small amounts of flavin, but Kuma and Inomata (7) described a preparation which contained only 1 mol of FAD/mol of enzyme. In the present studies, we purified the NADH-cytochrome b₅ reductase by procedures including affinity chromatography on Blue-Sepharose and found that our enzyme contained up to 1 mol of FAD/33,000 g of protein. We found that the cytochrome enzyme is unstable in the absence of EDTA and dithiothreitol in the buffer solution, and the binding of flavin in the enzyme seems to be weaker than that in the microsomal enzyme. This may be the reason that many workers (3-6) obtained an enzyme which contained only small amounts of flavin.

Preincubation of the purified enzyme with a flavin or with a flavin analogue in the reaction mixture caused slight activation, or significant inhibition of the enzyme activity, respectively, as shown in Table II. Although high concentration of flavin or overnight preincubation of the enzyme with flavin at 0°C did not cause significant activation, results shown in Table II support well the flavoprotein nature of the enzyme. Moreover, we found that the CD spectrum of the cytochrome enzyme is closely similar to that of liver microsomal enzyme, which is characterized as a FAD-enzyme. These results apparently show that the NADH-cytochrome b₅ reductase in human erythrocytes exists as a FAD-containing enzyme, although these results do not exclude completely the possibility of the existence of a flavin-free enzyme.

Many workers (19-23) reported that CD measurements of flavoproteins can provide information concerning the protein-flavin interaction. Free flavins (FAD and FMN) and apoprotein of many flavoproteins have only weak or no CD in the visible region. On binding flavin with apoprotein, however, a distinct CD spectrum in the visible region is observed. NADH-cytochrome b₅ reductase purified from human erythrocytes and rabbit liver microsomes was found to have a large negative CD band at 460 nm and also at 280 nm as shown in Fig. 1. Negative CD at around 460 nm was reported by Becvar et al. (22) on a FMNH₂-bacterial luciferase complex of Benechella harveyi and by Hiwatashi et al. (24) on NADPH-adrenodoxin reductase. Flavoproteins which have a distinct negative CD at 460 nm may have a different environment around the flavin binding site in the enzyme compared with other groups of flavoproteins which have positive CD such as d-amino acid oxidase of pig kidney (19, 20) and flavodoxins of Clostridium pastorianum and Desulfovibrio vulgaris (23).

Another interesting finding observed in this study is that the negative CD at around 460 nm of the cytochrome NADH-cytochrome b₅ reductase changed to positive CD on reduction of flavin in the enzyme with NADH under anaerobic conditions (data not shown). Similar change of positive CD of a FMN: luciferase complex (21) to negative CD on binding FMNH₂ to the enzyme was reported by Becvar et al. (22). The molar ellipticity at 280 nm as well as at 460 nm seems to correlate well with the enzyme activity and flavin content as shown in Fig. 1. This finding suggests that tryptophan or tyrosine may participate in the binding of flavin in the enzyme. Strittmatter (18) indicated that 1 specific tyrosine residue in liver microsomal NADH-cytochrome b₅ reductase participates in the binding of flavin. To clarify the interesting points described above, further studies must be done.

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