

PURIFICATION OF BOVINE LIVER MICROSOMAL NADH-CYTOCHROME b_5 REDUCTASE
USING AFFINITY CHROMATOGRAPHY

Dorothy A. Schafer and Donald E. Hultquist

Department of Biological Chemistry
The University of Michigan
Ann Arbor, Michigan 48109

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Summary: Microsomal NADH-cytochrome b_5 reductase has been purified from bovine liver by an improved procedure which employs affinity chromatography on ADP-agarose in combination with anion exchange chromatography. The reductase was extracted from a 105,000 x g microsomal pellet with Triton X-100. The overall purification from isolated microsomes was 98-fold and the yield was 10%. The preparation was nearly homogeneous on SDS-PAGE. This procedure requires less time and effort than previously described procedures. Partially purified cytochrome b_5 is also obtained.

Purifications of the amphipathic form of microsomal cytochrome b_5 reductase from bovine liver (1,2) and from rabbit liver (3) have been reported. These purification procedures are lengthy, however, and include harsh acetone treatment of microsomes (2,3) or of the partially purified enzyme (1).

In this paper we report an improved purification of NADH-cytochrome b_5 reductase from bovine liver. The purification is accomplished by isolation of microsomes by differential sedimentation, solubilization of the reductase with Triton X-100, and purification of the enzyme by anion exchange chromatography and affinity chromatography on ADP-agarose. Some of these results have been presented in abstract form (4).

EXPERIMENTAL

Materials. Bovine liver was obtained from Kappler Packing Co., Ann Arbor, MI; DEAE-cellulose from Fisher Scientific Co.; DE52-cellulose from Whatman; (N⁶-hexane)-adenosine 5'-diphosphate-agarose (Type II) from P-L Biochemicals; hydroxylapatite from Bio-Rad Laboratories; and NADH, NADP⁺, ADP, and DCIP* from Sigma Chemical Co.

General Methods. NADH-cytochrome b_5 reductase was assayed during the purification according to the procedure of Scott (5) with DCIP as the electron acceptor. The reductase was also assayed by coupling the reduction of cytochrome

*Abbreviation used is DCIP, 2,6-dichlorophenolindophenol.

b₅ to cytochrome c (6). A modified Lowry assay suitable for protein determination in the presence of Triton X-100 was used (7). SDS-PAGE was carried out in 11% gels according to the method of Laemmli (8). All purification steps were performed at 4° C.

Isolation and Extraction of Microsomes. Fresh bovine liver (700g) was homogenized in 0.25 M sucrose, 0.01 M Tris acetate, 1 mM EDTA, pH 8.1 (1.5 l). The homogenate was filtered through cheesecloth and the filtrate centrifuged at 10,800 x g for 10 min. The supernatant fraction was then centrifuged at 13,700 x g for 40 min. Microsomes were obtained from the supernatant fraction by sedimentation at 105,000 x g for 70 min. The microsomes were washed by suspension with a Dounce homogenizer in 0.1 M Tris acetate, 1 mM EDTA, pH 8.1 (1.5 l). Triton X-100 was added to the microsomal suspension to a final concentration of 1% and solubilization was allowed to proceed with gentle stirring for 12 hr. Centrifugation of the Triton suspension at 105,000 x g for 60 min yielded a supernatant fraction which contained about 80% of the DCIP reductase activity.

Purification of Cytochrome b₅ Reductase by Ion Exchange Chromatography.

The initial ion exchange chromatography steps of the purification procedure were essentially those employed by Spatz and Strittmatter (2). The Triton extract was applied to a DEAE-cellulose column (4 x 35 cm) equilibrated with 0.1 M Tris acetate, 1 mM EDTA, pH 8.1. Some of the cytochrome b₅ is retained by this column whereas the flavoprotein does not bind. After charging the sample, the column was washed with 1 l of the equilibration buffer and all fractions containing DCIP reductase activity were pooled and extensively dialyzed against 15 vol of 0.01 M Tris acetate, 1 mM EDTA, pH 8.1.

The dialysate was applied to a DE52-cellulose column (4 x 35 cm) equilibrated with 0.01 M Tris acetate, 1 mM EDTA, pH 8.1. The column was developed with a step-wise gradient using 1 l each of 0.01 M, 0.025 M, 0.05 M, 0.075 M, 0.10 M, and 0.20 M Tris acetate, pH 8.1, all containing 1 mM EDTA, 0.2% sodium deoxycholate, and 0.5% Triton X-100. The reductase was eluted in a sharp peak by the 0.20 M Tris buffer (Fig. 1). NADH-cytochrome c reductase activity paralleled the peak of DCIP reductase activity. Cytochrome b₅ was subsequently eluted

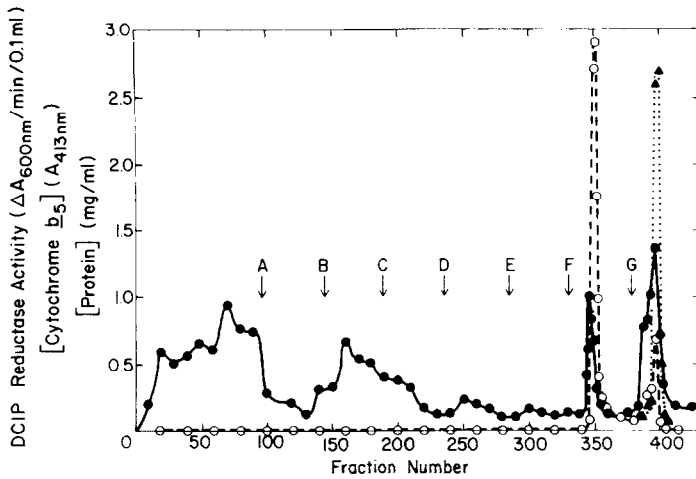


Fig. 1. Purification of cytochrome b_5 reductase by DE52-cellulose chromatography. Arrows indicate when each elution buffer was started. All buffers are Tris acetate, pH 8.1, containing 1 mM EDTA, 0.5% Triton X-100, and 0.2% sodium deoxycholate. A, 0.01 M buffer; B, 0.025 M buffer; C, 0.05 M buffer; D, 0.075 M buffer; E, 0.10 M buffer; F, 0.20 M buffer; G, 0.01 M buffer containing 0.25 M NaSCN but no Triton X-100 or deoxycholate. Fractions contained 18.8 ml. ●—●, protein concentration; ○—○, DCIP reductase activity; ▲.....▲, cytochrome b_5 concentration. For details see Experimental.

with 0.25 M NaSCN in 0.01 M Tris acetate, 1 mM EDTA, pH 8.1. The reductase-containing fractions were pooled and concentrated to 1/10 the original volume by ultrafiltration on an XM-50 Amicon membrane.

Purification of Cytochrome b_5 Reductase by Affinity Chromatography. The reductase fraction was dialyzed against 0.02 M Tris acetate, 1 mM EDTA, pH 7.5, and applied to an ADP-agarose affinity column (1 x 5 cm) equilibrated with the dialysis buffer. Some reductase activity did not bind to the column, but the bulk of the activity bound and a bright yellow color appeared throughout the entire column. The column was washed with 50 ml of 0.1 M Tris acetate, 1 mM EDTA, pH 8.1, followed by 25 ml of the same solution containing 0.1% Triton X-100. The reductase was eluted in a sharp peak with 1 mM ADP in 0.1 M Tris acetate, pH 8.1, containing 1 mM EDTA and 0.1% Triton X-100 as shown in Fig. 2. Fractions 22-30 were pooled, concentrated to 6.0 ml on an XM-50 membrane, dialyzed against 0.02 M Tris acetate, 1 mM EDTA, pH 7.5, and applied to a second affinity column equilibrated similarly to the first. After washing with 0.1 M Tris acetate, 1 mM EDTA, pH 8.1, the column was eluted with a 0-1.0 mM linear

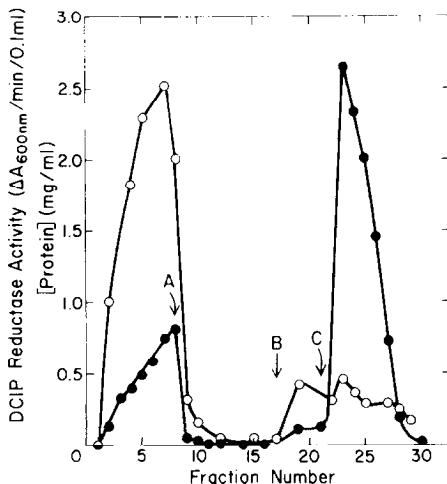


Fig. 2. Purification of cytochrome b_5 reductase by affinity chromatography on ADP-agarose. Arrows indicate when each elution buffer was started. A, 0.1 M Tris acetate, 1 mM EDTA, pH 8.1; B, 0.1 M Tris acetate, 1 mM EDTA, pH 8.1, containing 0.1% Triton X-100; C, 0.1 M Tris acetate, 1 mM EDTA, pH 8.1, containing 0.1% Triton X-100 and 1 mM ADP. Fractions contained 9.0 ml. \circ — \circ , protein concentration; \bullet — \bullet , DCIP reductase activity. For details see Experimental.

gradient of NADP^+ that was constructed with 15 ml of 0.1 M Tris acetate, 1 mM EDTA, 0.1% Triton X-100, pH 8.1, and 15 ml of 1 mM NADP^+ in this same solution. Subsequently, 50 ml of 2 mM ADP in 0.1 M Tris acetate, 1 mM EDTA, 0.1% Triton X-100, pH 8.1, were applied and the reductase was eluted in a sharp peak.

Before the purified reductase was employed in some studies, residual Triton X-100 and ADP were removed by chromatography on hydroxylapatite. The sample was concentrated to 3 ml on an XM-50 membrane, dialyzed against 0.02 M Tris acetate, 1 mM EDTA pH 7.5, and applied to a hydroxylapatite column (1.7 x 16 cm) equilibrated in 0.01 M potassium phosphate, pH 7.5. The column was washed with the equilibration buffer until Triton X-100 was no longer eluted. Triton X-100 was monitored by its absorbance at 275nm. The reductase was eluted with 0.1 M potassium phosphate, pH 7.5.

RESULTS AND DISCUSSION

The improved method for purification of the amphipathic form of bovine liver microsomal cytochrome b_5 reductase is summarized in Table 1. The procedure gives a 98-fold purification from the microsomal preparation with a 10%

Table 1
Summary of Purification of Bovine Microsomal Cytochrome b_5 Reductase

Step	Protein (mg)	Reductase Activity (units)	Specific Activity (units/mg protein)	Overall Purification (fold)	Overall Yield (%)
Microsomes	8508	8874	1.04	1.00	100
Triton extract	7462	6688	0.90	0.87	76
DEAE-cellulose	3488	4959	1.42	1.37	56
DE52-cellulose	199	2978	15.0	14.4	34
ADP-agarose					
First	11.4	973	85.4	82.1	11
Second	8.8	899	102	98.1	10

Protein concentration and DCIP reductase activity were determined as described in General Methods. 1 unit of reductase activity is defined as the change of 1 absorbance unit at 600 nm/min.

yield. The purified reductase preparation shows a visible spectrum (Fig. 3) characteristic of the flavoprotein. Electrophoresis on SDS-polyacrylamide gels shows one major band with minor bands that represent only a few percent of the Coomassie-stained protein (Fig. 4).

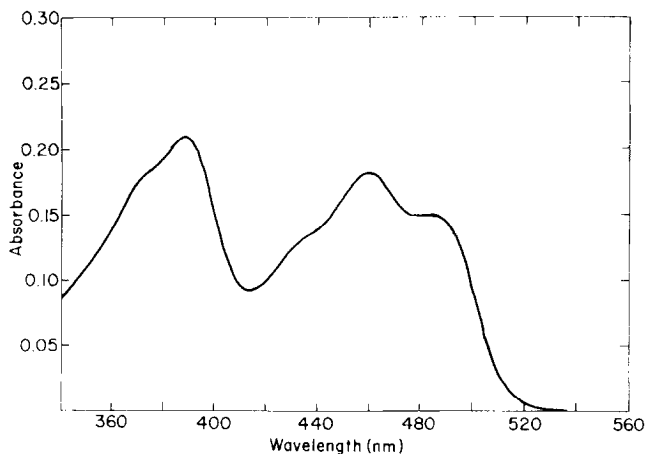


Fig. 3. Visible absorbance spectrum of oxidized cytochrome b_5 reductase in 0.1 M Tris acetate, pH 8.1, containing 1 mM EDTA and 0.1% Triton X-100. Reductase from the second ADP-agarose column was concentrated to 2 ml on an XM-50 membrane. Spectrum was obtained at 25°C on a Cary model 18 spectrophotometer.

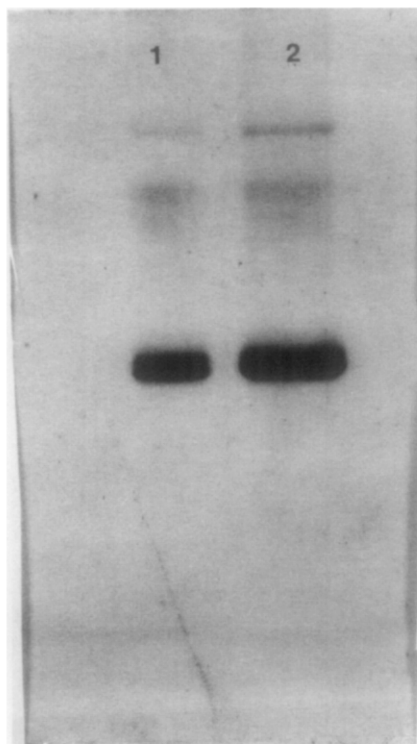


Fig. 4. SDS-polyacrylamide gel electrophoresis of cytochrome b_5 reductase. Lanes 1 and 2 contain 3 μ g and 12 μ g of protein, respectively. Migration is from top to bottom.

The key steps responsible for improving the procedure are affinity chromatography and the use of differential sedimentation for isolation of microsomes. Preparation of microsomes by sedimentation, rather than by a relatively lengthy and non-specific ammonium sulfate fractionation of crude liver homogenate, is a more efficient and preferred initial step and yields a microsomal fraction with much higher cytochrome b_5 reductase specific activity.

The affinity chromatography step achieves considerable purification and yields a reductase preparation which appears spectrally pure and is nearly free of other proteins. The ADP-agarose column, which in our procedure replaces the final DEAE-cellulose column in the preparation of Spatz and Strittmatter (2) requires less time and effort. It is possible to simplify the affinity chromatographic procedure by using only the second of the two columns.

The interaction of the flavoprotein with the ADP affinity ligand probably involves the pyridine nucleotide binding region of the enzyme. Strittmatter (9) has studied the nature of the interaction of pyridine nucleotides with microsomal cytochrome b_5 reductase and the inhibition of NADH oxidation by various substrate analogs. ADP is an effective inhibitor of the enzyme. Absence of the amino group from the adenine moiety of NADH has no effect on the rate of NADH oxidation, hence, the amino-linked ADP-resin is suited for efficient interaction with the substrate binding site. It is also possible that the hydrophobic surfaces of reductase have some affinity for the 6-carbon spacer arm of the resin, since low amounts of Triton X-100 (0.1%) are required for elution by ADP. Triton X-100 alone causes the reductase to leak off the column in a very broad peak.

The ADP-agarose column should be useful for other studies that require specific removal of reductase from a reaction mixture, and may also be employed for the purification of the soluble cytochrome b_5 reductase of erythrocytes.

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