Rat hepatic microsomal cytochrome b_5

A simple large-scale purification procedure and antibody production by antigen-containing liposomes

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Cytochrome b_5 from rat liver microsomes (microsomal fractions) was purified in its native form. The procedure described has great capacity, is fast, and the final product is pure as judged from SDS/polyacrylamide-gel electrophoresis. Antibodies to cytochrome b_5 are obtained after administration of the antigen inserted into small unilamellar lipid vesicles.

INTRODUCTION

Cytochrome b_5 , a component of the microsomal fatty acid desaturase system, is an amphiphilic protein with a hydrophilic domain containing the catalytic active haem group and a hydrophobic segment anchoring the protein to the membrane.

Cytochrome b_5 has been purified from a variety of species and the amino acid sequence determined [1]. During the last few years the protein from rat liver has attracted special attention, as cytochrome b_5 from this species has been used in studies concerning biosynthesis, membrane insertion and subcellular localization. However, purification of the native form of cytochrome b_5 from rats by the established procedure is impeded by copurification of a proteolytic activity. Therefore a high concentration of the highly toxic proteinase inhibitor phenylmethanesulphonyl fluoride (PMSF) is needed throughout the six chromatographic purification steps [2]. Recently a simple and rapid one-step procedure for obtaining microgram quantities of cytochrome b_5 from rat liver microsomes (microsomal fractions), without addition of proteinase inhibitor, was described [3]. However, in our hands this method did not result in a pure product.

Here we describe modifications to the one-step procedure described by Burchell [3]. Without modifications it is possible to obtain pure cytochrome b_5 and, furthermore, the procedure allows purification of cytochrome b_5 in milligram quantities. At the same time we describe a method for production of antibodies to native cytochrome b_5 by low-protein-dose immunization of the antigen inserted into small unilamellar liposomes.

MATERIALS AND METHODS

Chemicals

Fractogel TSK DEAE-650(S) was obtained from Merck (Darmstadt, Germany), Sephadex G-75 (Superfine grade) and Sephadex G-50 (Fine grade) from Pharmacia (Uppsala, Sweden). Lubrol 12A-9, polyoxyethylene-[9.5]dodecyl alcohol was a gift from ICI, Copenhagen, Denmark. Bio-Beads SM-2 and Protein Assay Reagent were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Bovine γ -globulin, sodium deoxycholate,

PMSF and dicetyl phosphate were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Distearoyl phosphatidylcholine and cholesterol were from Serdary Research Laboratories (London, Ont., Canada). Freund's adjuvants, complete and incomplete, were from Statens Serum Institute (Copenhagen, Denmark). NaF was of reagent grade; all other chemicals were of analytical grade.

SDS/polyacrylamide-gel electrophoresis (SDS/PAGE)

This was performed in 13% gels as described by Laemmli [4]. The antiserum was tested by immunoblotting after transferring of the proteins to nitrocellulose paper by semi-dry electroblotting [5]. Immuno-electrophoresis was performed in a Tris-buffer system (pH 8.6) containing 1% (w/v) Triton X-100 and 4% (w/v) poly(ethylene glycol).

Assays

In purified fractions cytochrome b_5 was assayed by using a molar absorption coefficient of $117 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 413 nm [6] and in crude fractions by rocket immunoelectrophoresis. Protein was determined by the method of Bradford [7], with bovine γ -globulin as standard.

Preparation and solubilization of microsomes

Rats were killed by decapitation and the liver quickly removed, frozen on solid CO_2 and stored at -80°C until use. The liver (100 g) was thawed within 10 min at 4°C in 0.25 M-sucrose/5 mM-Hepes/0.5 mM-EDTA/0.1 mM-PMSF, pH 7.4. The tissue was cut into three pieces, quickly rinsed with buffer and homogenized in 400 ml of buffer at 0°C by four strokes at 500 rev./min in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10000 g for 20 min and the microsomal fraction was obtained as the supernatant after centrifugation at 105000 g for 60 min. The microsomes were washed once and stored at -80°C until use. For solubilization the microsomal pellet was diluted with ice-cold 62.5 mM-sucrose/20 mM-NaF/5 mM-Hepes/0.1 mM-PMSF (pH 7.4)/1% Lubrol to a final protein concentration of 5 mg/ml, quickly homogenized and immediately centrifuged at 105000 g for 1 h.

Fig. 1. (a) Ion-exchange chromatography on Fractogel TSK DEAE-650(S) and (b) gel chromatography on Sephadex G-75 (Superfine grade)

After the wash as described in the text the column (2.5 cm $\times$ 76 cm) was eluted with 5 mM-Hepes/20 mM-NaF/20 % (v/v) glycerol/70 mM-(NH₄)₂SO₄/0.05 % Lubrol, pH 7.4. Fractions (18.5 ml) were collected and assayed for protein (A_{280} , Δ) and cytochrome b_5 (A_{413} , $\circ$). (b) The column (2.5 cm $\times$ 85 cm) was eluted with 20 mM-Tris/acetate/0.2 mM-EDTA/1 % sodium deoxycholate, pH 8.1. Fractions (6.0 ml) were collected and assayed as described in (a).

Ion-exchange chromatography

The supernatant obtained from solubilized microsomes from 300 g of liver were applied directly to a Fractogel TSK DEAE-650(S) column (2.5 cm $\times$ 76 cm) equilibrated in 5 mM-Hepes/20 mM-NaF/20 % (v/v) glycerol/0.05 % Lubrol, pH 7.4. Chromatography was performed at 4 °C at a constant rate of 60 ml/h and the absorbance of the effluent was recorded at 280 nm. The column was washed with 800 ml of equilibration buffer until no protein could be detected. The column was further washed with 1200 ml of 5 mM-Hepes/20 % (v/v) glycerol/70 mM-(NH₄)₂SO₄, pH 7.4, and two protein peaks emerged after 300 and 600 ml. By adding Lubrol to a final concentration of 0.05 % to this last washing buffer, cytochrome b_5 was eluted as a broad peak. The column was regenerated with 600 ml of 5 mM-Hepes/0.5 M-NaF, pH 7.4, and re-equilibrated with 1200 ml of 5 mM-Hepes/20 mM-NaF, pH 7.4, before another purification cycle.

Gel chromatography

The fractions from the Fractogel column with an A_{413}/A_{280} ratio better than 1 were collected. The combined fractions were concentrated and the buffer changed to

20 mM-Tris/acetate/0.2 mM-EDTA on an Amicon PM 10 filter before residual Lubrol was removed on a filter with 1 ml of Bio-Beads. To the final solution was added sodium deoxycholate to 1 % before applying it to a Sephadex G-75 (Superfine grade) column (2.5 cm $\times$ 85 cm) equilibrated and eluted with 20 mM-Tris/acetate/0.2 mM-EDTA/1 % sodium deoxycholate, pH 8.1, at a constant rate of 8 ml/h. Pure cytochrome b_5 was eluted in the second peak.

Preparation of antigen-containing liposomes

Liposomes containing cytochrome b_5 were prepared by a method similar to the cholate-dialysis method described by Takagaki *et al.* [8]. A lipid mixture of distearoyl phosphatidylcholine, cholesterol and dicetyl phosphate in the molar proportions 7:2:1 was dried to a thin film in a flask. To the lipid (2.7 μ mol) was added 30 nmol of cytochrome b_5 dissolved in 700 μ l of 20 mM-Tris/acetate buffer, pH 8.1, containing 0.2 mM-EDTA and 1 % sodium deoxycholate. The mixture was heated to 37 °C for 10 min, followed by sonication for 2 $\times$ 30 s in a bath sonicator (Laboratory Supplies Co., Hicksville, NY, U.S.A.) and incubated for 15 min at 37 °C. The mixture

Table 1. Purification of cytochrome b_5

Values are means of duplicate determinations in a typical experiment (starting with 300 g of rat liver).

Fraction	Volume (ml)	Protein (mg/ml)	Cytochrome b_5 (μM)	Specific activity (nmol/mg)	Purification (fold)	Yield (%)
Solubilized microsomes	1200	4.13	1.28	0.3	1	100
Fractogel eluate	185	0.52	4.30	8.3	28	52
Sephadex eluate	24	0.90	28.12	31.2	104	44

Fig. 2. (a) SDS/PAGE and immunoblotting in 13%-(w/v)-polyacrylamide gel and (b) crossed immunoelectrophoresis of 20 μl of solubilized microsomes (right) and 0.5 μg of purified cytochrome b_5 (left)

(a) Lane 1, 50 μl of solubilized microsomes; lane 2, 50 μl of Fractogel eluate; lane 3, 10 μl of Sephadex eluate; lane 4, molecular-mass markers (all stained with Coomassie Blue). Lane 5, 2.5 μl of Fractogel eluate; lane 6, 0.3 μl of Sephadex eluate (both transferred to nitrocellulose paper). Rabbit anti-(cytochrome b_5) serum was diluted 1:300 and incubated overnight, followed by development with horseradish-peroxidase-conjugated second antibody. (b) The antibody-containing area was 4.6 cm $\times$ 3 cm, and the concentration of the antiserum was 14 $\mu\text{l}/\text{ml}$. The precipitates were stained with Coomassie Blue.

was added directly to a Sephadex G-50 (Fine grade) column (0.9 cm $\times$ 28 cm), which was eluted with 20 mM-Tris/acetate buffer, pH 8.1, containing 0.2 mM-EDTA, for removal of deoxycholate and formation of lipid vesicles. The void volume was collected and the cytochrome b_5 concentration adjusted to 200 μ g/ml.

The incorporation of cytochrome b_5 into lipid vesicles was analysed by gradient centrifugation in a linear sucrose gradient (0–38 %, w/w) at 284 000 g for 18 h. Fractions were collected and analysed for lipid by measuring radioactivity ($[^3\text{H}]$ phosphatidylcholine) and for protein by measuring A_{413} . The lipid and the protein profile coincided and banded at a peak density of 1.05 g/cm^3 .

Production of antisera

Antiserum against rat cytochrome b_5 was produced by immunization of rabbits. The antigen inserted into liposomes was mixed with an equal volume of Freund's adjuvant. For the first injection the complete adjuvant was used and for the following injections the incomplete adjuvant. A 500 μ l portion of the suspension, containing 50 μ g of protein, was injected subcutaneously into the rabbit's back, the immunization scheme of Harboe & Ingild [9] being followed.

RESULTS AND DISCUSSION

We were not able to obtain pure cytochrome b_5 by following the original one-step procedure using solubilized microsomes from 10 g of liver and a 1.5 cm $\times$ 45 cm Fractogel TSK DEAE-650(S) column as described previously [3]. In our hands this procedure resulted in a preparation contaminated with impurities corresponding to about 50 % of the total protein. During our unsuccessful attempts to refine the conditions, we observed, however, that the degree of purification and the elution profile (Fig. 1) were identical when the column load was increased. The modified procedure described here is still very simple, and our method allows amphiphilic cytochrome b_5 to be obtained pure with high yield and in milligram quantities within a few days.

As indicated in Table 1, about 700 nmol of cytochrome b_5 could be isolated in a two-step chromatographic procedure from 300 g of rat liver, with an overall yield of 44 %. The isolated cytochrome b_5 was pure as judged by SDS/PAGE (Fig. 2). After tryptic digestion the purity and homogeneity was also confirmed by gel chromatography and SDS/PAGE (result not shown). The specific content of cytochrome b_5 was 31 nmol/mg of protein, and the A_{280}/A_{413} ratio was 0.34.

Cytochrome b_5 is a small well-conserved protein, and antibodies are not easily obtained [2]. Thus antibody production from rat liver cytochrome b_5 has only been reported in a few cases [10, 11] and the methods employed have caused unnecessary discomfort to the animal by injection of high amounts of antigen into the foot pads [10] or into lymph nodes of knee joints [11], preswollen after administration of Freund's complete adjuvant into the foot pads.

Here we describe a method for production of antibodies to the native form of cytochrome b_5 by subcutaneous administration of the antigen inserted into small unilamellar liposomes through hydrophobic interaction of part of the protein with the lipid bilayer. In this way cytochrome b_5 acts as an immunogen in small quantities, thus ensuring specificity of the antibody. Liposomes as promoters of antigen production have been described for other proteins [12], and the mechanism by which liposomes enhance the immunogenicity is probably that liposomes present the antigen to the immune system on a membrane-like structure and enhance the antibody response by acting as adjuvant.

Antibodies were produced in four out of six animals by using the described procedure. The titre of the antisera collected after the second booster was 1–3 nmol/ml, estimated by immunoelectrophoresis [9]. In comparison it should be mentioned that we were not able to raise antibodies against cytochrome b_5 by using the tryptic form of the protein coupled to methylated bovine serum albumin [13], in spite of a 5-times-higher antigen dose.

The specificity of the antiserum was demonstrated in two ways. First, when detergent-solubilized microsomes from rat liver were subjected to crossed immunoelectrophoresis against cytochrome b_5 antisera, a single precipitation arc, corresponding to that of pure cytochrome b_5 , was obtained (Fig. 2). Secondly, immunoblotting after SDS/PAGE of the fractions during the purification procedure revealed only one band corresponding to pure cytochrome b_5 (Fig. 2).

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