THE MOLECULAR WEIGHT OF NADPH-CYTOCHROME C REDUCTASE ISOLATED BY IMMUNO-PRECIPITATION FROM DETERGENT-SOLUBILIZED RAT LIVER MICROSOMES.

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SUMMARY

The molecular weight of detergent-solubilized NADPH-cytochrome c reductase from rat liver microsomes has been estimated to be 79,000. The method used for this determination involves immunoprecipitation of deoxycholate-solubilized enzyme from ^{125}I-labeled microsomal proteins. The antibody was prepared against a purified preparation of Bromelain-solubilized enzyme (molecular weight 71,000). The immunoprecipitate was then subjected to SDS-polyacrylamide gel electrophoresis and the enzyme located by ^{125}I-gamma counting.

INTRODUCTION

Several rat liver microsomal enzymes have been purified following proteolytic solubilization (1-7). Recently, some of these enzymes have been purified after detergent solubilization (8-10). In each case the molecular weight of the detergent-solubilized enzyme has been determined to be greater than the protease-solubilized enzyme, indicating that the enzymes were partially degraded during solubilization (8-10). NADPH-cytochrome c reductase has been solubilized by various methods, all of which appear to involve proteolysis (11). Therefore the possibility exists that this enzyme is also isolated in a partially degraded form. In this paper we wish to report a method by which we have been able to estimate the molecular weight of the native form of this enzyme. Involved in this method are: (1) the enzymatic radioiodination of the native enzyme in microsomes, (2) immunoprecipitation of ^{125}I-labeled NADPH-cytochrome c reductase from sodium deoxycholate-solubilized microsomes using an antibody to a proteolytically solubilized form of the enzyme, (3) solubilization of the immunoprecipitate in sodium dodecyl sulfate (SDS) for polyacrylamide gel electrophoresis, and (4) identification of the molecular weight of the native form of the enzyme by gel fractionation and ^{125}I-gamma counting. Similar techniques have been used previously for the isolation of immunoglobulins (12), Θ-antigens (13), and H-2 alloantigens (14,15) from cell surfaces.

METHODS

The method of Omura and Takesue (2) was used to purify Bromelain-solubilized NADPH-cytochrome c reductase (3) from microsomes isolated from the livers of pheno

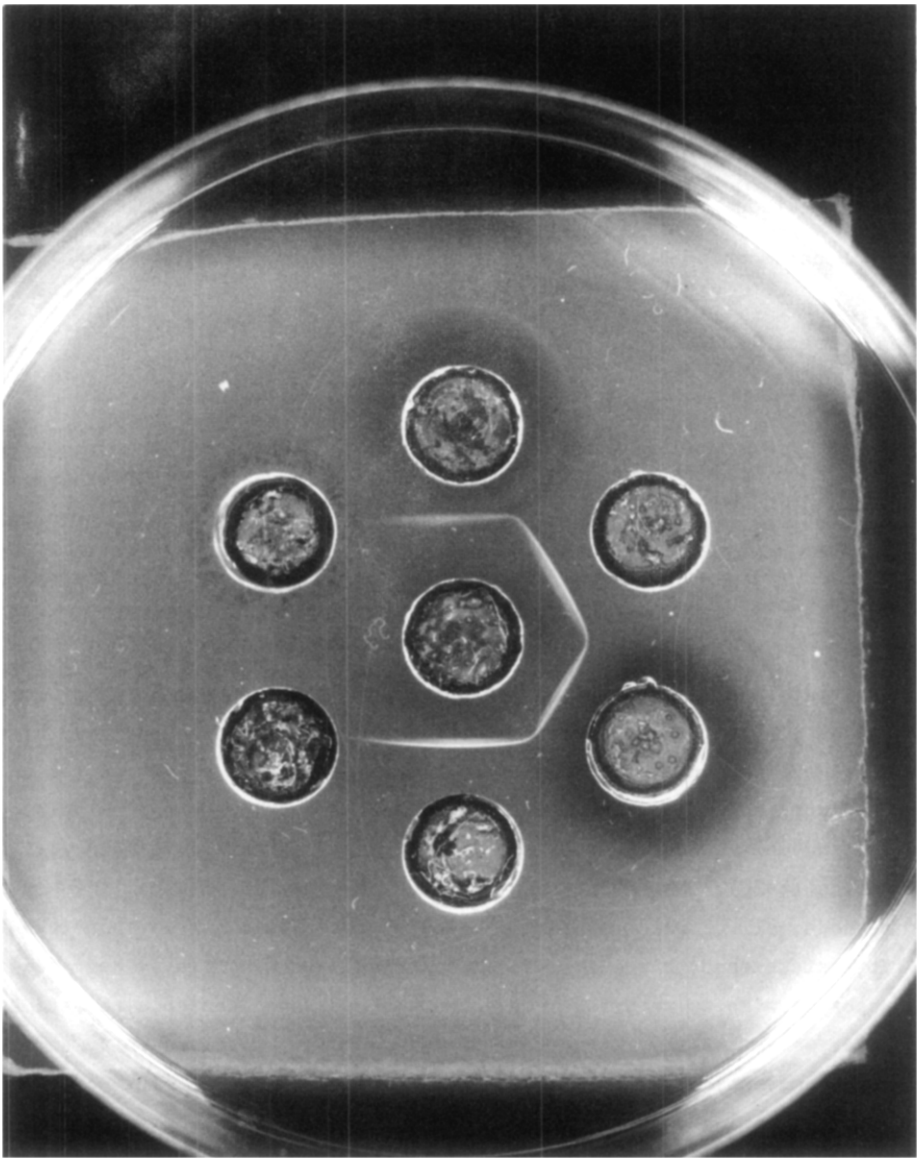

Figure 1. Immunoprecipitation of the antibody to purified, Bromelain-solubilized NADPH-cytochrome c reductase with the purified enzyme and with detergent-solubilized microsomes in Ouchterlony double diffusion agar plates. The center well contains anti-NADPH-cytochrome c reductase γ-globulin (1.62 mg protein). The outer wells are numbered clock-wise from the top (No. 1). Wells No. 1 and 3 contain 0.2 and 0.4 mg of microsomal protein, respectively (5 mg/ml in 2% sodium deoxycholate). Wells No. 2 and 4 contain 3 and 10 μg of purified, Bromelain-solubilized NADPH-cytochrome c reductase, respectively. Well No. 5 contains buffer and well No. 6 contains 2% sodium deoxycholate.

barbital pre-treated rats (16). The enzyme was assayed for its ability to reduce cytochrome c and ferricyanide as described by Omura and Takesue (2). The preparation of a γ-globulin fraction from pre-immune and immune rabbit serum has been described elsewhere (17). This procedure involves ammonium sulfate fractionation and DEAE-chromatography to remove complement and other contaminating proteins (18). The ability of the antibody in the γ-globulin fraction of immune serum to precipitate the proteolytically-solubilized enzyme and the native form of this enzyme in sodium deoxycholate-solubilized microsomes was assessed by Ouchterlony double diffusion agar plates (19).

Since NADPH-cytochrome c reductase is known to be exposed to the outside of the microsomal membrane (20), the native form of this enzyme was easily labeled in liver microsomes by lactoperoxidase-catalyzed iodination. The iodination procedure (21) involved suspending washed microsomes at a protein concentration of 0.5 mg/ml in 0.1 M Tris-HCl (pH 7.5 at 25°C), 10^{-6} M KI (containing 10 μC/ml of ^{125}I obtained from New England Nuclear), 5.0 x 10^{-7} M lactoperoxidase (Sigma) and 0.0001% butylated hydroxytoluene. Iodination was carried out at 25°C by addition of 5 μM H_2O_2 at 1 minute intervals over a 3 minute reaction period. The iodination mixture was then diluted with cold 0.1 M Tris-HCl (pH 8.0 at 4°C) and centrifuged at 105,000 xg for 90 minutes. As has been previously noted iodination does not inhibit the activity of NADPH-cytochrome c reductase (21).

After iodination, 2.0 mg of ^{125}I-labeled microsomal membranes, mixed with 7 mg of unlabeled microsomes, were suspended at a concentration of 4 mg/ml in 1.5% sodium deoxycholate containing 0.05 M Tris-HCl (pH 7.5 at 25°C) and 10 mM EDTA. A small amount of material, not solubilized by the detergent, was removed by centrifugation at 130,000 xg for 90 minutes. γ-Globulin from immune serum was added to the supernatant in a ratio of 1 mg γ-globulin to 1 mg of microsomal protein and incubated at 0-4°C for 12 hours. A control using pre-immune γ-globulin was run in parallel but this γ-globulin did not form an immunoprecipitate. The precipitate resulting from the antibody was centrifuged at 500 xg for 10 minutes and washed 3 times with 1% sodium deoxycholate in 0.05 M Tris-HCl (pH 7.5 at 25°C) containing 10 mM EDTA. The immunoprecipitate was then either suspended in 0.05 M Tris-HCl (pH 7.5 at 25°C) and 10 mM EDTA for enzymatic assay or dissolved in 1% SDS containing 10% sucrose, 10 mM Tris-HCl (pH 8.1 at 25°C), 1 mM EDTA, 40 mM dithiothreitol, and 10 μg/ml pyronin B tracking dye before heating at 100°C for 15 minutes prior to electrophoresis. 1% SDS-polyacrylamide gel electrophoresis was performed according to the method of Fairbanks, et al., (22). After electrophoresis the gels were either stained with Coomassie blue (23) and scanned for protein at 550 nm using a Gilford spectrophotometer or immediately fractionated using a Savant Autogel Divider for ^{125}I-

TABLE I. NADPH-CYTOCHROME C AND NADPH-FERRICYANIDE REDUCTASE ACTIVITIES.

Fraction	μ moles of receptor reduced/min	
	cytochrome c	ferricyanide
Microsomes	2.1	3.1
Sodium deoxycholate-solubilized microsomes	2.1	3.0
Immunoprecipitate	0.1	1.0
Supernatant from immunoprecipitation	0.0	0.0

As described in the "Methods", microsomes were solubilized in sodium deoxycholate, centrifuged, and anti-NADPH-cytochrome c reductase was added to the supernatant to form an immunoprecipitate. After washing, the immunoprecipitate was suspended in 0.05 M Tris-HCl (pH 7.5 at 25°C) and 10 mM EDTA. All fractions were assayed for NADPH-cytochrome c reductase and NADPH-ferricyanide reductase as described by Omura and Takesue (2).

gamma counting on a Nuclear Chicago gamma spectrometer. Molecular weight markers phosphorylase a, bovine serum albumin, carbonic anhydrase, alcohol dehydrogenase and ribonuclease-A were run in parallel with membrane protein samples.

RESULTS AND DISCUSSION

As demonstrated in Figure 1, both Bromelain-solubilized NADPH-cytochrome c reductase and sodium deoxycholate-solubilized microsomes gave positive precipitin reactions to the γ-globulin fraction of immune serum prepared against the Bromelain-solubilized enzyme. This suggested the possibility of precipitating native NADPH-cytochrome c reductase from detergent-solubilized microsomal proteins using this γ-globulin.

Enzymatic assays were performed to test for the solubilization of NADPH-cytochrome c reductase from microsomes by sodium deoxycholate and the presence of this enzyme in the immunoprecipitate formed from the detergent-solubilized microsomal proteins and the anti-reductase-γ-globulin. This data is presented in Table I. The detergent solubilized all of the enzymatic activity from microsomes. Addition of antibody inhibited the ability of the detergent-solubilized enzyme to reduce cytochrome c by 97% and to reduce ferricyanide by 66%, however all of the residual activity was in the immunoprecipitate.

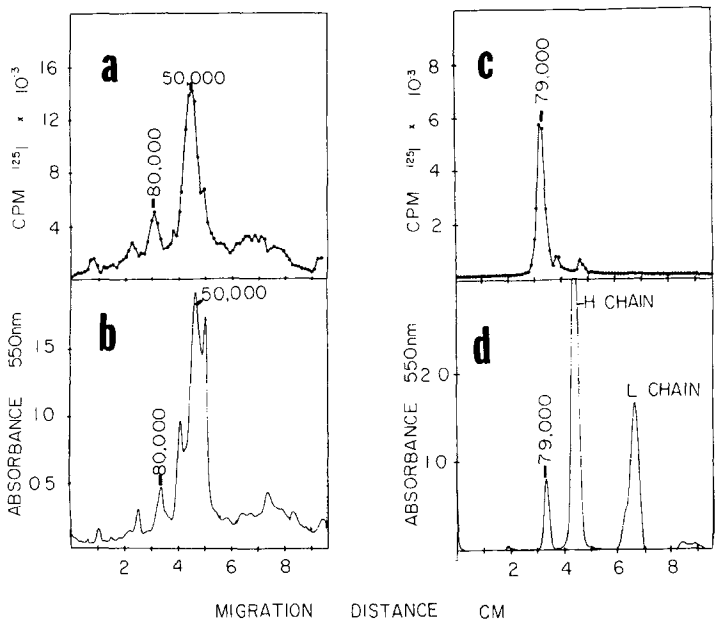

Figure 2. Distribution of ^{125}I and protein on SDS-polyacrylamide gels of total microsomal protein and the immunoprecipitate formed from sodium deoxycholate-solubilized microsomal proteins and the reductase antibody. (a) ^{125}I-distribution obtained after enzymatic iodination of rat liver microsomal proteins; (b) protein scan of total microsomes; (c) ^{125}I-distribution in immunoprecipitate; and (d) protein scan of immunoprecipitate. Molecular weight markers, based on R_f values, were determined independently for each gel.

The protein and ^{125}I profiles obtained on SDS-polyacrylamide gels for iodinated microsomes and the immunoprecipitate formed from sodium deoxycholate-solubilized ^{125}I-labeled microsomal proteins are shown in Figure 2. Both profiles are very complex for total microsomal proteins. The ^{125}I profile for the immunoprecipitate indicates that native NADPH-cytochrome c reductase has an apparent molecular weight of 79,000 $\pm$ 1,500 (average and standard deviation of 5 determinations). Coomassie blue protein staining indicates one protein band corresponding to the ^{125}I label in this region. Two other unlabeled polypeptide chains are also present in the immunoprecipitate. These have molecular weights of 52,000 and 25,000, approximately the molecular weights of heavy and light γ-globulin polypeptide chains (24).

Figure 3 compares the Coomassie blue protein banding patterns for gels run on Bromelain-solubilized NADPH-cytochrome c reductase, the solubilized immunoprecipitate, and a mixture of these two samples. The Bromelain-solubilized enzyme has an apparent molecular weight of 71,000 $\pm$ 500 (average and standard deviation of 5 determinations) in comparison to the 79,000 molecular weight of

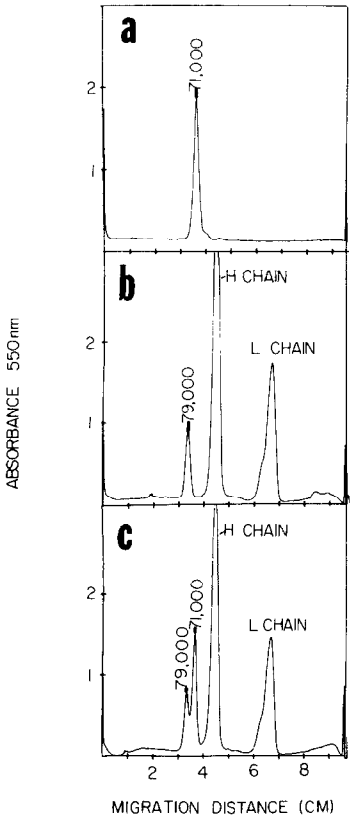

Figure 3. Protein scans from SDS-polyacrylamide gels of (a) purified Bromelain-solubilized NADPH-cytochrome c reductase; (b) the immunoprecipitate formed from sodium deoxycholate-solubilized microsomal proteins and the reductase antibody; and (c) a mixture of a and b. Molecular weight markers, based on R_f values, were determined independently for each gel.

the native enzyme. Others have determined molecular weights for proteolytically solubilized forms of NADPH-cytochrome c reductase which range from 68,000 to 74,000 (25,4).

It is likely that native NADPH-cytochrome c reductase is an amphipathic microsomal membrane protein, similar to cytochrome b_5 and NADH-cytochrome b_5 reductase. These proteins have been shown to be attached to the microsomal membrane by a hydrophobic tail of 40 to 44 amino acids (8), and of 99 amino acids (9), respectively. Proteolytic digestion cleaves the major portion of these proteins from the microsome while leaving behind the hydrophobic tails (8,9,10). If this is also the case with NADPH-cytochrome c reductase, this enzyme would appear to have a tail of approximately 70 amino acids on the basis of the molecular weight differences of the native and proteolytically solubilized

enzymes. Spatz and Strittmatter (9) have reported that SDS gel electrophoresis gives a 20% underestimation of the molecular weight of detergent-solubilized NADH-cytochrome b_5 reductase. They attribute such a disparity to the ability of the hydrophobic tail of this protein to bind extra SDS, increasing its mobility during electrophoresis and therefore decreasing its apparent molecular weight. It is possible that this may also be the case with NADPH-cytochrome c reductase. In any case, the results presented in this paper demonstrate that the detergent-solubilized enzyme is larger than the protease-solubilized enzyme.

ACKNOWLEDGEMENT

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