PURIFICATION OF CYTOCHROME P.450 FROM BOVINE ADRENOCORTICAL MITOCHONDRIA

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Received November 9,1972

SUMMARY

One soluble cytochrome P.450 from bovine adrenocortical mitochondria has been purified to near homogeneity. The purified enzyme catalyses side-chain cleavage of cholesterol and to a much lesser extent 11β-hydroxylation (<13% side-chain cleavage) but shows no 18-hydroxylase activity. The molecular weight of this P.450 is approximately 800,000.

Cytochrome P.450 from adrenocortical mitochondria catalyses a number of reactions in which steroid substrates are hydroxylated at various positions e.g. 11β, 18 and the side-chains of cholesterol and cholesterol sulfate (1-4). The cytochrome is usually released from membranes by sonication and has recently been obtained in soluble form (5,6). Exploration of the spectral and enzymatic properties of P.450 is hampered by the inhomogeneity of present preparations. We report here the purification of cytochrome P.450 from bovine adrenocortical mitochondria to near homogeneity. Certain properties of the purified enzyme are given.

EXPERIMENTAL PROCEDURE

Adrenocortical mitochondria were prepared according to Omura and coworkers (1) and extracted with cholic acid, as described by Mitani and Horie (Step 7) (7). The middle supernatant layer (7) was brought to 60% saturation with $(NH_4)_2SO_4$ and the precipitate was dialysed against phosphate buffer (pH 7.6, 50 mM) containing EDTA (1 mM) and cysteine-HCl (10 mM). The preparation was centrifuged at 105,000 x g (60 min) and the supernate was dialysed against phosphate buffer (pH 7.6; 1 mM) containing EDTA (0.1 mM) and dithioerythritol (DTE) (0.1 mM) before being applied to a column of DEAE

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cellulose. The P.450 was eluted with 0.1 M phosphate buffer (pH 7.6) with EDTA (0.1 mM) and dithioerythritol (0.1 mM). This fraction was applied to a column of hydroxylapatite eluted by means of a gradient of the phosphate buffer mixture described above. The eluate reveals two peaks containing P.450 of which the second (eluted between 150 and 300 mM phosphate buffer), was applied to a column of Biogel A-15 M and eluted with 0.1 M phosphate buffer (pH 7.6) containing DTE (0.1 mM) and EDTA (0.1 mM). Four standard proteins were later separately applied to the same column and eluted with the same buffer. The sources and molecular weights of these proteins are as follows:

glutamate dehydrogenase: M.W. 640,000; beef liver; Boehringer Mannheim, GmbH;
alcohol dehydrogenase: M.W. 151,000; yeast; Sigma Chemical Company;
catalase: M.W. 250,000; beef liver; Boehringer Mannheim GmbH;
bovine serum albumin: M.W. 65,000 Sigma Chemical Company.

The first three of these proteins were detected by standard enzyme assays (8) while bovine serum albumin was detected by A_{280} nm.

Side-chain cleavage of cholesterol and hydroxylase activities was measured by published methods (2). The ultracentrifugal properties of the enzyme were examined in the Beckman Model E.

RESULTS AND DISCUSSION

Figure 1 shows exclusion volumes for adrenocortical P.450 and four other proteins. The P.450 was eluted as a single symmetrical peak (V/V_o = 1.78). A linear relationship between log M.W. and V/V_o over the range tested is observed and the molecular weight of P.450 is approximately 800,000. Side-chain cleavage enzyme activity was symmetrically distributed throughout the peak of A_{280}; the ratio of enzyme activity to A_{280} was a straight line of zero slope throughout the peak (data not shown).

The sedimentation coefficient of P.450 is seen in Figure II as a function of protein concentration. This relationship is linear and extrapolation to zero concentration shows a value for S^o_{20W} of 21 x 10^{-13} which

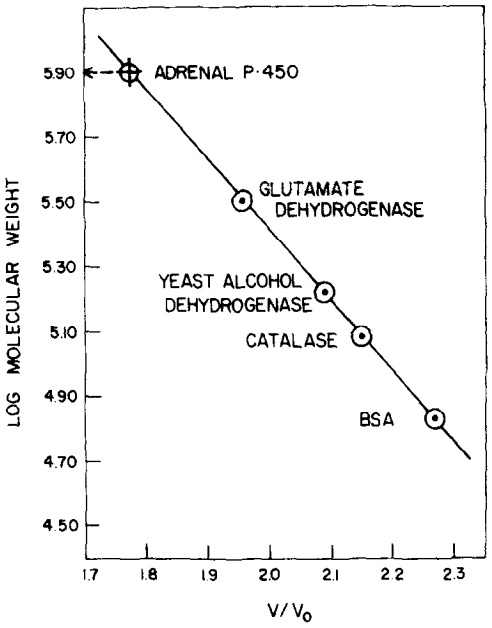

Fig. 1.

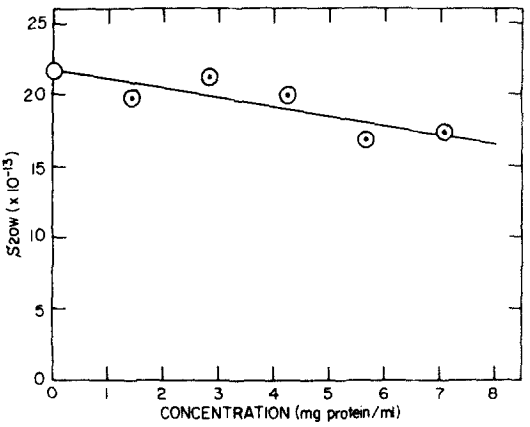

Fig. 2.

FIGURE I:

Exclusion volumes (expressed as V/V_o where V is the exclusion volume for the protein and V_o is the void volume of the column), as a function of log M.W. Under the conditions used the molecular weight of catalase was 125,000. BSA: bovine serum albumin.

FIGURE II:

Sedimentation coefficient of adrenal P.450 as a function of protein concentration determined in the analytical ultracentrifuge.

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is compatible with the molecular weight determined from exclusion chromatography More accurate values will be obtained after further examination of the properties of P.450.

The relative homogeneity of the P.450 was demonstrated by three criteria:

i) the protein (A_{280}) and enzyme activity were eluted from Biogel as a single symmetrical peak.

ii) P.450 appeared as a single band on disc electrophoresis in polyacrylamide gel (3.7%).

iii) P.450 appeared as a single peak during analytical ultra-centrifugation.

The cytochrome P.450 showed the following enzyme activities:

	nmoles/min/nmole P.450
side-chain cleavage (cholesterol)	1.84
11β-hydroxylation	0.20
18-hydroxylation	<0.03

Evidently one cytochrome P.450 from bovine adrenocortical mitochondria can be purified to at least near homogeneity. This substance has a high molecular weight (approximately 800,000) and is capable of converting cholesterol-^{14}C to pregnenolone-^{14}C. The P.450 possesses limited 11β-hydroxylase activity (<13% of side-chain cleavage) and no detectable 18-hydroxylase activity. Whether the 11β-hydroxylase activity is due to the presence of traces of a second enzyme molecule or whether it represents an inherent activity of the side-chain cleavage enzyme, cannot be determined at this time. However when the relative specific activities of the two enzyme activities are measured at various stages during the purification procedure from sonication of mitochondria to the final purification, the ratio: side-chain cleavage/11β-hydroxylation progressively increases to a final value of 30. This observation would favor the idea that the 11β-hydroxylase activity is due to traces of a contaminating enzyme.

As expected of such a large molecule, the adrenal P.450 studied here can dissociate into subunits under certain conditions. The nature of this dissociation will be reported elsewhere.

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