SOLUBLE CYTOCHROME P.450 FROM BOVINE ADRENOCORTICAL MITOCHONDRIA*

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SUMMARY

A method is given for solubilizing at least one species of cytochrome P.450 from bovine adrenocortical mitochondria. The soluble P.450 supports side-chain cleavage of cholesterol but not 11β-hydroxylation, thus clearly separating these two activities for the first time. Soluble P.450 shows a substrate-induced difference spectrum with both cholesterol and 11-deoxycorticosterone; these difference spectra are opposite in type (peak at 420 nm) to spectra observed when these substrates are added to crude P.450. It is suggested that there are at least two cytochromes P.450 in bovine adrenocortical mitochondria with distinct enzymatic activities namely, side-chain cleavage and 11β-hydroxylation.

The biosynthesis of adrenal steroids involves at least three mitochondrial enzyme systems, each responsible for hydroxylation of the steroid ring at specific carbon atoms, namely C_{11}, C_{18} and the side-chain of cholesterol. These hydroxylation reactions require TPNH which reduces cytochrome P.450 via an electron transport system consisting of a flavoprotein and non-heme iron (Omura *et al.*, 1966). Reduced cytochrome P.450 combines with oxygen and with the steroid substrate to bring about hydroxylation. It remains to determine how the specificity of each hydroxylation is achieved (e.g. C_{11} as opposed to the cholesterol side-chain etc.). Among a number of plausible alternatives it could be suggested that there are several mitochondrial cytochromes P.450 or alternatively there may be one cytochrome which serves several protein (hydroxylase)

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enzymes. These alternatives will be difficult to resolve until cytochrome P.450 is obtained in soluble form. This communication describes the solubilization and properties of cytochrome P.450 from bovine adrenal cortex.

EXPERIMENTAL PROCEDURE

Cytochrome P.450 was prepared according to Mitani and Horie (1969) up to and including their step 8. The middle supernate was treated with $(NH_4)_2SO_4$ to 60% saturation at pH 7.0. The precipitate (P_1) was suspended in 0.05 M potassium phosphate (pH 7.6) containing 10 mM cysteine and 1 mM EDTA. After dialysis for 3 hours against the same buffer, the suspension was centrifuged at 105,000 x g for 60 minutes to give a precipitate (P_2) and a supernate (S_2). Before use in these studies, P_2 was suspended in potassium phosphate (0.1 M, pH 7.0) containing 0.1 mM dithioerythritol. S_2 was precipitated with $(NH_4)_2SO_4$ in two fractions: 0-35% sat. (called S_2-35) and 35-80%(called S_2-80). S_2-35 was suspended in and dialysed over night against potassium phosphate (0.1 M pH 7.0) containing dithioerythritol (0.1 mM); the dialysed P.450 was used in these experiments. S_2-80 was dissolved in and dialysed against the same buffer overnight. The solution of S_2-80 was brownish red and clear to the naked eye; it remained clear for at least 7 days on standing at 0° and also after centrifugation at 105,000 x g for 120 minutes.

S_2-80 was applied to calcium phosphate gel from which P.450 was eluted by potassium phosphate 0.3 M containing EDTA 1 mM and dithioerythritol 0.1 mM pH 7.6. The eluate (soluble P.450) was dialysed against potassium phosphate (0.1 M pH 7.0) containing 0.1 mM dithioerythritol before use in the present studies; this preparation is called soluble P.450.

Side-chain cleavage (cholesterol-^3H $\longrightarrow$ pregnenolone-^3H)

and 11β-hydroxylation (11-deoxycorticosterone-^{14}C i.e. DOC-^{14}C → corticosterone-^{14}C) were measured as described elsewhere (Young et al., 1970). Amounts of P.450 were calculated on the basis of heme content according to the method of Omura (1967).

RESULTS

Substrate-Induced Difference Spectra: Table 1 shows that while P_2 and S_2-35 show a positive spectral shift at 390 nm (type I) with DOC, soluble P.450 shows a positive shift at 420 nm (type II) wi this substrate. Moreover the soluble P.450 shows a small peak at

TABLE 1

SUBSTRATE-INDUCED DIFFERENCE SPECTRA FOR P.450 FRACTIONS

Substrate	Spectral Difference (ΔA/μmole P.450)	
	390 - 420 (nm)	420 - 390 (nm)
Soluble P.450:		
Cholesterol	0	+ 0.005
DOC	0	+ 0.022
20α-OH Cholesterol	0	+ 0.145
Pregnenolone	0	+ 0.091
Corticosterone	0	+ 0.018
S_2-35:		
Cholesterol	0	0
DOC	+ 0.043	0
20α-OH Cholesterol	0	+ 0.190
Pregnenolone	0	+ 0.092
Corticosterone	0	+ 0.003
P_2:		
Cholesterol	0	0
DOC	+ 0.050	0
20α-OH Cholesterol	0	+ 0.079
Pregnenolone	0	+ 0.045
Corticosterone	0	0

Substrate difference spectra were measured by adding steroid substrates dissolved in propylene glycol to cytochrome P.450. Final concentration of substrates was 5 μM. Spectra were read after standing for 30 minutes.

420 nm (type II) with cholesterol while P_2 and S_2-35 give no demonstrable difference spectrum with cholesterol. Both pregnenolone and 20α-hydroxycholesterol show well-marked positive spectral shifts at 420 nm (type II) with all three preparations; corticosterone shows a small type II difference spectrum with soluble P.450 and S_2-35 but not with P_2. These findings were confirmed with two additional preparations of P.450.

Enzyme Activity: It is clear from Table 2 that the soluble

TABLE 2

ENZYME ACTIVITY OF P.450 FRACTIONS

Fraction	Side-Chain Cleavage (% Conversion/μmole P.450)	11β-Hydroxylation (nmoles/μmole P.450/ 20 min)
Preparation 1		
P_2	0	0
S_2 - 35	0	0
Soluble P.450	45.1	0
Preparation 2		
P_2	0	0
S_2 - 35	0	0
Soluble P.450	26.2	0
P_2 + S_2 - 35	0	48.7

P.450 fractions were incubated with TPNH, non-heme iron, diaphorase and cholesterol-7α-^{3}H or DOC-^{14}C for 20 minutes at 37°. Side-chain cleavage and 11β-hydroxylation were measured as described elsewhere (Young et al., 1970). Zero means < 0.01% conversion of substrate for side-chain cleavage and <0.01 nmoles/μmole P.450/20 minutes for 11β-hydroxylation. The amounts of the various additions are given elsewhere (Young and Hall, 1971) except for P.450 of which 1-6 μmoles was added to each flask. Values for side-chain cleavage are expressed as % conversion because of the presence of variable amounts of endogenous cholesterol.

P.450 possesses side-chain cleavage activity but no 11β-hydroxylase activity ($<$ 0.01 nmoles product/μmole P.450/20 minutes). By contrast P_2 and S_2-35 show neither side-chain cleavage nor 11β-hydroxylase activities. However, P_2 plus S_2-35 showed 11β-hydroxylase but not side-chain cleavage activity (preparation No. 2). The observation that soluble P.450 is capable of supporting side-chain cleavage activity but not 11β-hydroxylation has been found with five separate enzyme preparations.

DISCUSSION

The present findings reveal three important features of cytochrome P.450 from bovine adrenocortical mitochondria. Firstly, at least one species of mitochondrial P.450 has been solubilised to the extent that the pale straw-coloured solution remains clear for at least 7 days and does not sediment during centrifugation at 105,000 x g for 2 hours. The best previous preparation (Mitani and Horie, 1969) only remains in suspension as long as cholic acid is present.

Secondly, soluble P.450 shows the following interesting properties: the cytochrome will support side-chain cleavage of cholesterol but not 11β-hydroxylation. The solution shows a spectral shift with cholesterol and with DOC, giving a peak at 420 nm (type II). The preparation of Mitani and Horie (1969) shows both side-chain cleavage and 11β-hydroxylase activities (Young *et al.*, 1970) and gives type I difference spectrum with both cholesterol and DOC. Although the absence of 11β-hydroxylase activity from the soluble preparation might mean either that one part or site of the enzyme has been damaged during purification or that some (?) protein hydroxylase factor, specifically needed for 11β-hydroxylation, has been removed, the

fact that P_2 plus S_2-35 are together capable of 11β-hydroxylation but not of side-chain cleavage, argues in favour of the existence of two distinct cytochromes P.450 - one for side-chain cleavage and one for 11β-hydroxylation. This is perhaps consistent with the fact that P_2 and S_2-35 always show type I difference spectrum with DOC but no difference spectrum with cholesterol.

Thirdly, the difference spectra of the soluble enzyme and those of P_2 and S_2-35, serve as a caution against extravagant interpretation of this empirical phenomenon. For example, DOC is reported to show a type I spectrum e.g. (Mitani and Horie, 1969); if no interaction occurs between our three fractions (soluble P.450, P_2 and S_2-35), an equimolar mixture of these fractions would probably show type I spectrum for DOC (A 390: +0.043 +0.050 -0.022). On the other hand, soluble P.450 shows a type II spectrum with DOC (Table 1).

The only one of our fractions to show a spectral shift with cholesterol is soluble P.450 which is the only fraction showing enzymatic activity with this substrate; the spectrum observed is type II (Table 1 and 2). Cholesterol has been reported to show a type I spectrum with crude P.450 (Mitani and Horie, Young et al., 1970). Furthermore 20α-hydroxycholesterol shows a type II spectrum with all three fractions, only one of which is capable of side-chain cleavage (Table 1).

These findings prepare the way for further characterisation of the P.450 associated with side-chain cleavage of cholesterol and encourage attempts to solubilise a second P.450 capable of supporting 11β-hydroxylation. Such endeavours are at present being pursued in this laboratory.

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