ENZYMATIC AND SPECTRAL PROPERTIES OF SOLUBILIZED CYTOCHROME P-450 FROM BOVINE ADRENOCORTICAL MITOCHONDRIA

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SUMMARY

A solubilized preparation of cytochrome P-450 from bovine adrenocortical mitochondria is capable of reconstituting 11β-hydroxylase and side-chain cleavage activities (the latter with two substrates : cholesterol and cholesterol SO_4). Cholesterol SO_4 induces a type I spectrum with P-450 and this substrate appears to compete with cholesterol in producing this spectrum but not with 11-deoxycorticosterone (the substrate for 11β-hydroxylation). The process of solubilization selectively retains side-chain cleavage at the expense of 11β-hydroxylation. These observations suggest that more than one cytochrome P-450 or one P-450 with more than one active center, exists in adrenocortical mitochondria.

Mitochondria of steroid-forming cells possess, in addition to the classical cytochrome system of electron transport, a system which provides electrons for oxygen activation associated with steroid hydroxylation reactions:

TPNH → Diaphorase → Non-heme Iron → Cytochrome P-450 → Steroid
Steroid OH ↙

In the adrenal cortex purified diaphorase and non-heme iron together with cytochrome P-450 reconstitute an enzyme system capable of hydroxylating steroids at the 11β (Omura et al., 1966) and 18 (Greengard et al., 1967) positions as well as converting cholesterol to pregnenolone and cholesterol sulfate to pregnenolone sulfate (the so-called side-chain cleavage reactions) (Young and

Hall, 1968); the side-chain cleavage reactions are believed to require hydroxylation of C_{20} and C_{22} before the C_{20}- C_{22} bond is cleaved. These reactions all require TPNH and molecular oxygen so that they are believed to be typical monoxygenases. Among the many problems associated with these monoxygenase systems is the question of whether one cytochrome P-450 serves to activate oxygen for all the hydroxylations of which the system is capable and if so how specificity of the site to be hydroxylated is secured in the cell.

A method for solubilizing cytochrome P-450 from bovine adrenocortical mitochondria was recently reported by Mitani and and Horie (1969). It was decided to examine the activity of cytochrome P-450 prepared by this method with respect to specific activity of the various hydroxylations and the nature of the substrate-induced difference spectra produced by the substrates for these hydroxylation reactions.

EXPERIMENTAL PROCEDURE

Cytochrome P-450 was prepared according to Mitani and Horie (1969) and TPNH-diaphorase and non-heme iron by the methods of Omura et al. (1966) and of Kimura and Suzuki (1967) respectively. Conditions for incubation and methods for measuring side-chain cleavage of cholesterol and cholesterol sulfate and for measuring endogenous cholesterol by gas chromatography are given in detail elsewhere (Young and Hall, 1969). The same paper describes the source and purification of chemicals. Protein was determined by the method of Lowry. Conversion of 11-deoxycorticosterone (DOC) to corticosterone (11β-hydroxylation) was measured by incubating the enzyme system with 11-deoxycorticosterone-4-^{14}C. Corticosterone-^{14}C was isolated by two systems of thin layer chromatography on Silica gel G: (I) chloroform/ethanol/water, 92:8:0.5 and (II)

SUBSTRATE-INDUCED DIFFERENCE SPECTRA AND ENZYMATIC ACTIVITY

OF THREE PREPARATIONS OF CYTOCHROME P-450

Preparation and Step	Enzymatic Activity (nmoles product/mg protein)			Ratio	Endogenous Cholesterol	Substrate-induced Spectra			
	11β-OH	SCC+	SCC-SO_4+	(11β=1.0)	(nmoles/mg protein)	DOC	CHOL.	CHOL-SO_4	Pregnenolone
I : Step 15	51.5	86.0	<0.2	1 : 1.7 : <0.004	34.6	N.T.*	-*	-*	Type 2
II Step 6	2.2	0.7	1.2	1 : 0.3 : 0.5					
II Step 15	4.0	6.1	18.3	1 : 1.5 : 4.6	11.8	Type 1	Type 1	Type 1	Type 2
III Step 6	10.1	0.9	0.8	1 : 0.09 : 0.08					
III Step 15	14.4	7.7	16.6	1 : 0.5 : 1.2	31.0	Type 1	-*	-*	Type 2

The table summarises the characteristics of three preparations of solubilized cytochrome P-450. Enzymatic activity was measured as described in the text. Steps in the purification of cytochrome P-450 are numbered according to Mitani and Horie (1969). Step 6 refers to the pellet obtained by centrifugation following the first exposure of the preparation to cholate. Step 15 is the final cytochrome P-450. 11β-OH : 11β-hydroxylation.

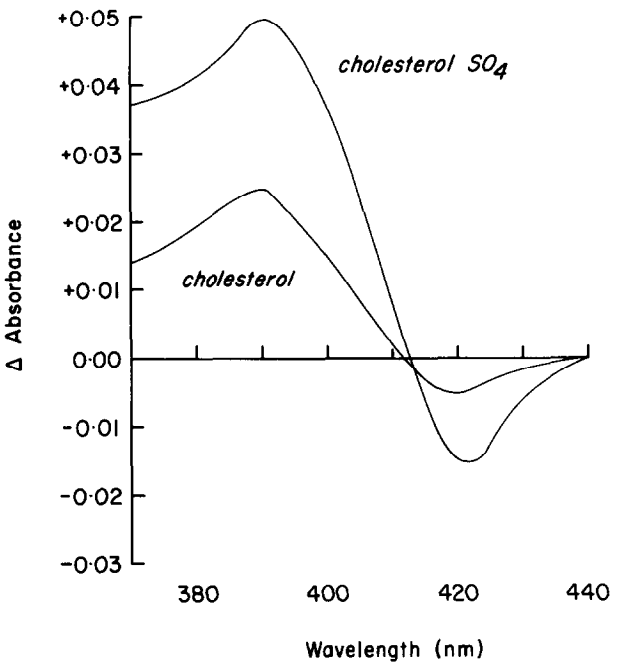
* N.T. : not tested; - : indicates that no difference spectrum was observed with concentrations of substrate up to 66 μM.
\+ SCC : side-chain cleavage of cholesterol; SCC-SO_4 : side-chain cleavage of cholesterol SO_4.

ethylacetate/chloroform, 90:10; corticosterone-^{14}C was measured by liquid scintillation spectrometry under conditions published elsewhere (Means and Hall, 1967). Spectroscopy was performed with a Gilford recording spectrophotometer.

Cytochrome P-450 was suspended in phosphate buffer (100mM, pH 7.0) (1-2 mg protein per ml) and steroid substrates were suspended in propylene glycol (0.1 ml) at final concentrations of 66 μM or less.

RESULTS

Figure 1 shows substrate-induced difference spectra for cholesterol and pyridinium cholesterol sulfate. Both spectra are typical of the so-called Type 1 with a peak at 390 and a trough at 420 nm. Preparation II (endogenous cholesterol 11.8 nmoles/mg protein) was used for these studies; exogenous cholesterol was added to give a final concentration of 66 μM. Preparations I and III (with high levels of endogenous cholesterol), showed difference spectra with neither cholesterol nor cholesterol SO_4 but all three preparations gave characteristic Type II spectrum with pregnenolone and preparation II showed Type 1 spectrum with DOC (preparation I was not tested with DOC). The table shows the absolute and relative (11β-hydroxylation as 1.0) specific activities of three preparations of cytochrome P-450 with respect to 11β-hydroxylation and side-chain cleavage of cholesterol and cholesterol sulfate. Preparations II and III were examined at various steps during the preparation; values are shown for step 6 (after first addition of cholate) and step 15 the final preparation; values for intervening steps showed progressive changes in specific activities consistent with the change from initial to final



values shown, indicating that the changes are not artificitious. It is clear that the procedure used selectively retains side-chain cleavage at the expense of 11β-hydroxylation.

DISCUSSION

The present observations are reported, in preliminary form, for four reasons. Firstly to indicate that solubilized P-450 is enzymatically active towards the same substrates as cruder particulate preparations. Evidently, solubilization has removed neither specific protein or heat-stable factors proposed as agents responsible for conferring specificity on a single cytochrome P-450, nor non-specific lipid components essential for the activity of P-450 (Lu and Coon, 1968). Secondly we have observed that cholesterol SO_4 induces a difference spectrum in confirmation of our earlier finding that this substance can act as substrate for an enzyme system which

includes P-450 (Hall and Young, 1969). It is of interest that cholesterol SO_4 produces the same type of difference spectrum as cholesterol and DOC (type 1) (Mitani and Horie, 1969). Our results further suggest that cholesterol and cholesterol SO_4 may compete for one site on P-450, because difference spectra were not observed with these two substrates when levels of endogenous cholesterol were high (preparations I and III), whereas DOC appears to induce a difference spectrum inspite of high levels of endogenous cholesterol (preparation III).

Thirdly the relative specific activities of the three enzymatic activities show that purification has enriched side-chain cleavage activity to a considerably greater extent than 11β-hydroxylation. Moreover activities towards cholesterol and cholesterol SO_4 show important differences between the three preparations : preparation I was extremely active with cholesterol but not with the sulfate; preparations II and III were more active with the sulfate than with the free sterol by factors of 1.5 and 4 respectively. These findings confirm extensive experience with the enzymatic activities of less purified particulate P-450; certain individual preparations were observed to be inactive with one substrate but active with others.

Such observations are consistent with the existence of separate cytochromes P-450 for DOC and side-chain cleavage as demonstrated by induced spectra and relative specific activities. It is however impossible to exclude the possibility of two sites on one P-450 but in this case the site for DOC must be preferentially inactivated during preparation so that this site loses activity relative to the side-chain cleavage site. The position with respect to side-chain cleavage is less

clear since in producing spectra, the two substrates appear to compete for one site, yet purification influences the two activities differently. Further speculation is unwarranted at this point.

Fourthly, attention is called to the importance of considering endogenous cholesterol when difference spectra are measured. Clearly no difference spectrum can be expected when endogenous levels of cholesterol approach saturation; such preparations are sometimes encountered and levels of endogenous cholesterol are extremely variable from one preparation to another.

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