SEPARATION OF TWO FORMS OF CYTOCHROME *P*450 ADRENAL CORTEX MITOCHONDRIA

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The separation of two distinct forms of cytochrome *P*450 from adrenal cortex mitochondria has been achieved by the following steps; (1) lyophilisation (2) iso-octane extraction, (3) $(NH_4)_2SO_4$ fractionation in the presence of sodium cholate. The fraction precipitating between 25–35 percent $(NH_4)_2SO_4$ gave a difference spectrum with 11-deoxycorticosterone (11-DOC) but not with 20α-hydroxycholesterol (20α-HOC). This fraction showed high 11β-hydroxylase activity but low activity for side chain cleavage of cholesterol (S.C.C.). The fraction precipitating between 45–60 percent $(NH_4)_2SO_4$ gave a difference spectrum with 20α-HOC but not with 11-DOC and exhibited high S.C.C. activity but low 11β-hydroxylase activity. The absorption spectrum of the 45–60 percent fraction indicated a preponderance of high spin hemoprotein (λ_{max} 395 nm).

1. Introduction

In adrenal cortex mitochondria, cytochrome *P*450 functions as the terminal oxidase for several hydroxylation reactions such as the conversion of cholesterol to pregnenolone [1], (side chain cleavage; S.C.C.) and the 11β-hydroxylation of specific steroids such as 11-deoxycorticosterone (11-DOC) [2].

Recently high and low spin states of cytochrome *P*450 have been inferred from distinct absorption maxima [4], particularly in the Soret region (395 nm, high spin: 415 nm low spin) [3, 4]. The difference spectrum produced by the binding of 20α-hydroxycholesterol (20α-HOC) to oxidised cytochrome *P*450 (λ_{max} 420 nm, 535, 570; λ_{min} 385 nm) was the exact reverse of that produced by 11-DOC [5], while the EPR spectrum of low spin *P*450 was shown to increase when 20α-HOC was added to the suspension and to diminish upon addition of 11-DOC [6]. One interpretation of these facts would be that binding of 20α-HOC to cytochrome *P*450 increases the proportion of low spin *P*450 while binding of 11-DOC increases the proportion of high spin *P*450. Furthermore, the additive effects of 20α-HOC and 11-DOC upon cytochrome *P*450 [5] and the selective titration of high and low spin forms by *n*-octylamine have indicated two independent *P*450 proteins [7]. Both the octylamine difference spectra and the ratio of the two types of steroid difference spectra have been used to quantitate the relative proportion of these two forms [8]. The latter method of estimation has been used extensively in this paper.

2. Methods

Bovine adrenal mitochondria, which were prepared as previously described [1], were sonicated four times in distilled water [Dawe Soniprobe, Power, 6] for 30 sec each time and then lyophilised. The lyophilised protein was suspended in 1.0 M phosphate buffer (25 mg protein/ml), and stirred rapidly with an equal volume of iso-octane. After 30 min stirring and then centrifugation (105,000 *g*, 30 min), three phases separated; 1 yellow iso-octane supernatant, a brown solid interlayer and a red-yellow lower phase. The supernatant was removed and the interlayer and lower phase were homogenized together and re-extracted with iso-octane. After three iso-octane extractions the homogenate of the lower two layers was dialysed against distilled water for 6 hr and then centrifuged (105,000 *g*, 60 min). The pellet was lyophilised and

suspended in 0.1 M phosphate buffer (20 mg protein/ml) and sodium cholate was added (0.5 mg cholate/mg protein). Protein was precipitated at 15, 25, 35, and 60 percent $(NH_4)_2SO_4$ and recovered by centrifugation (10,000 *g*, 15 min). The precipitated protein was suspended in 0.1 M phosphate buffer containing 20 percent glycerol and the difference spectra were taken after the addition of 4 volumes of 0.1 M phosphate buffer (table 1). Glycerol solutions (20%) were dialysed against distilled water and the hydroxylase activities and difference spectra were measured on these samples (table 2).

A source of NADPH-*P*450 reductase containing adrenodoxin and flavoprotein was prepared from mitochondria and assayed as described by Omura et al. [9]. For both S.C.C. and 11β-hydroxylase assays the incubation comprised: *P*450 preparation, 1 ml; NADPH-*P*450 reductase, 0.5 ml; steroid solution, 10 μl and then an NADPH generating system consisting of NADP (7.5 mg/ml), glucose-6-phosphate, (25 mg/ml), 0.5 ml, and glucose-6-phosphate dehydrogenase (0.5 unit in 0.05 ml). Deoxycorticosterone (150 μg) was added in ethanol (10 μl) and cholesterol (100 μg and 0.1 μCi ^{14}C-cholesterol) was added in acetone (10 μl). Incubations were conducted at 37° for 20 min; and stopped in the case of the 11β-hydroxylase assay by shaking the reaction mixture with methylene chloride (15 ml) and in the case of the S.C.C. assay by shaking with methanol (10 ml). Corticosterone formation was assayed fluorimetrically [10], while S.C.C. activity (pregnenolone formation) was estimated by radioactive assay after a separation from cholesterol had been achieved by thin layer chromatography [1].

Steroid difference spectra were obtained by adding ethanol solutions of the respective steroids into the sample cuvette while an equal quantity of ethanol was added to the reference cuvette. *P*450 and *P*420 were determined spectrally using millimolar extinction coefficients reported by Omura and Sato [11]. Protein estimations were performed by the Biuret method.

3. Discussion

In table 1 the difference spectra produced by 11-DOC and 20α-HOC have been used to estimate respectively the amounts of low and high spin forms of *P*450. Clearly most of the low spin *P*450 has precipitated below 35 percent $(NH_4)_2SO_4$ while high spin *P*450 precipitated between 35 and 60 percent. This critical concentration of $(NH_4)_2SO_4$ varied according to preparations, in the range 30–40 percent.

Table 1
Separation of 2 forms of *P*450 by ammonium sulphate fractionation. Distribution among the fractions.

Fraction	Protein	Difference Spectra ΔA				Recovery of *P*450	
(%) $(NH_4)_2SO_4$	(mg/ml)	11-DOC*	20α-HOC*	Red CO 450 nm	Red CO 420 nm	nmoles	(%)
Iso-octane particles	18	0.49	0.57	1.2		204	100
0–15	13	0.05	<0.01	0.15	0.65	8	4
15–25	11	0.29	<0.03	0.32	0.35	17.5	8.7
25–35	12	0.66	0.10	0.64	0.13	35	17.5
35–60	10	<0.03**	0.67	0.88	0	58	29
				Total recovery of *P*450 =			59%

* Concentration 20 μM, ΔA (390–420 nm) for 11-DOC and (420–390 nm) for 20α-HOC.

** Small Type II difference spectrum (λ_{max} 420 nm) from binding of 11-DOC to high spin *P*450. Limit estimated for contribution of binding to low spin *P*450.

Table 2
Separation of 11β-hydroxylase and side chain cleavage enzymes†.

Fraction	Difference Spectra ΔA*			11β-hydroxylase	S.C.C.
$(NH_4)_2SO_4$ (%)	11-DOC	20α-HOC	Red CO 450 nm	(nmoles/min/ nmole *P*450)	conversion (%/nmole *P*450)
25–34	0.030	<0.01	0.078	4.4	1
34–45	0.020	0.050	0.090	3.1	15
45–60	<0.01**	0.20	0.24	0.4	6

* Concentration 20 μM, ΔA (390–420 nm) for 11-DOC, (420–390 nm) for 20α-HOC.
** See table 1.
† Preparations described in table 2 differ from those in table 1 due to 12 hr dialysis to remove glycerol and sodium cholate.

Without extraction by iso-octane the majority of *P*450 was precipitated by 37 percent $(NH_4)_2SO_4$, with the two forms remaining unseparated, as was found in the preparation of Mitani and Horie [12].

On most fractions the weak binding of 11-DOC to high spin *P*450 produces a small contribution to the difference spectrum of the type produced by 20α-HOC and thus causes an underestimation of low spin *P*450. However, the difference spectra obtained by addition of 11-DOC to the 60 percent $(NH_4)_2SO_4$ fractions (table 1 and 2) were of the 20α-HOC type and were of a size predicted from the binding constant (0.15 mM) [7] and concentration of 11-DOC. Thus binding of 11-DOC to low spin *P*450 in the 60 percent fractions was estimated to be very small and less than the limiting values quoted in tables 1 and 2. The recovery of each of the two separated forms of *P*450 was about the same, while in absence of glycerol, low spin *P*450 was much less stable than high spin *P*450.

Since cholate (0.4 mg/ml protein) and glycerol (4%) partially inhibited 11β-hydroxylase and S.C.C. activities, the preparations had to be dialysed to remove these inhibitors. In table 2 the steroid difference spectra which were obtained after dialysis are compared with the hydroxylase activities. The *P*450 fraction (24–34%) which exclusively bound 11-DOC, exhibited high 11β-hydroxylase activity but low S.C.C. activity. Conversely the *P*450 fraction (45–60%), which bound 20α-HOC, exhibited high S.C.C. activity but low 11β-hydroxylase activity. Thus the hydroxylase activities of the high and low spin *P*450 were clearly separated (11β-hydroxylation 10×, S.C.C. ×) while the intermediate fraction exhibited activities for both reactions. The activities have been expressed in terms of *P*450 content, rather than protein content, as the former was considered more relevant to the separation.

The conversion of cholesterol to pregnenolone achieved by the high spin *P*450 fraction would be equivalent to an activity of 0.9 nmole/nmole *P*450/

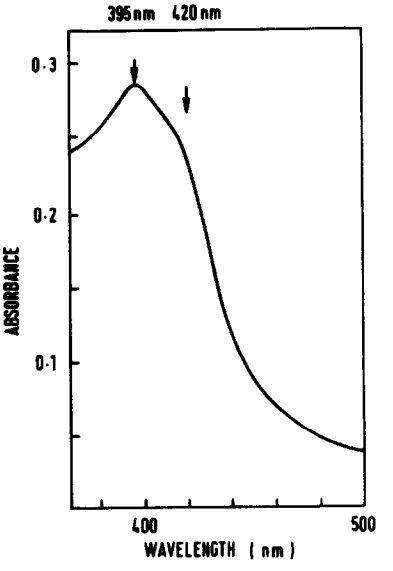

Fig. 1. The absorption spectrum of the 35–60 percent $(NH_4)_2SO_4$ fraction in the Soret region. The precipitated protein (3 mg/ml) from the 35–60 percent $(NH_4)_2SO_4$ fraction was dissolved in 0.1 M potassium phosphate buffer (pH 7.4).

min without a contribution from endogenous cholesterol. Preliminary measurements (by GLC, S.E. 30 column, 220°) of the total pregnenolone formation indicates this contribution to be small.

The absolute spectrum (fig. 1) of an optically clear solution of the 35–60 percent fraction indicated that the major Soret absorption maximum was at 395 nm which confirms the dominant high spin character of this *P*450 fraction. The weak shoulder at 420 nm may be due to contaminant haemoprotein or could indicate that this form of *P*450 has a minor contribution of low spin *P*450.

This separation suggests that the majority of 11β-hydroxylase activity in adrenal cortex mitochondria is associated with a low spin form of cytochrome *P*450 while a distinct high spin form of cytochrome *P*450 is responsible for most of the S.C.C. activity. Further work on the purification will determine the selectivity of each cytochrome *P*450.

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