PURIFICATION AND OPTICAL STUDIES OF CYTOCHROME *P*-450 FROM BOVINE ADRENOCORTICAL MICROSOMES

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1. Introduction

The adrenal gland cortex, which produces steroid hormones, is known to contain a number of *P*-450 cytochromes which function in oxygen activation for the hydroxylation of steroids. Recently, two different kinds of mitochondrial *P*-450 cytochromes, catalyzing the cholesterol side-chain cleavage ($P\text{-}450_{scc}$) and the steroid 11β-hydroxylation ($P\text{-}450_{11\beta}$) have been highly purified from bovine adrenal cortex [1–3]. Investigations on the reaction mechanism of cytochrome *P*-450 in adrenal endoplasmic reticulum have been carried out using a microsomal particle or its extract [4–13]. However, so far as we know, no isolation of cytochrome *P*-450 from the microsomes has been achieved.

The present paper reports for the first time the purification of cytochrome *P*-450 from adrenocortical microsomes, and discusses some optical properties of this protein.

2. Materials and methods

Progesterone and pregnenolone were purchased from Nakarai Chemical Co. and 17α-hydroxyprogesterone was obtained from Fluka Ag. Chemicals. Emulgen 913 (a polyoxyethylene nonylphenyl ether) was obtained from Kao-Atlas Co. An ω-amino-*n*-octyl derivative of Sepharose 4B (Pharmacia) was prepared as in [14]. Microsomes from adrenal cortexes were isolated by differential centrifugation as in [15]. Protein was determined as in [16] using bovine serum albumin (Sigma) as a standard. Spectrophotometric measurements were performed using a Union SM-401 split beam recording spectrophotometer equipped with a spectral data processer (model SM-450).

3. Results and discussion

Potassium phosphate buffers, pH 7.2, containing 100 μM EDTA, 100 μM dithiothreitol and 20% (v/v) glycerol were used throughout: they will be referred to simply as 50 mM or 100 mM buffer. Purification procedures were carried out at 5°C. The microsomal pellets (900 mg) were suspended (to 15 mg protein/ml) in 100 mM buffer containing sodium cholate (3 mg/mg protein). After stirring for 1 h, the mixture was centrifuged at 100 000 × *g* for 1 h. The supernatant solution was dialyzed overnight against 2 liters 50 mM buffer containing 0.4% (w/v) sodium cholate. The dialyzed solution was applied to a column (1.9 × 20 cm) of ω-amino-*n*-octyl Sepharose 4B as an affinity adsorbent which had been equilibrated with the dialysis buffer. After washing the column with 125 ml 100 mM buffer containing 0.4% cholate, the cytochrome *P*-450 was eluted at 16 ml/h with 100 mM buffer containing 0.4% cholate and 0.08% (v/v) Emulgen 913. The amount of the cytochrome in various fractions was estimated from the difference spectra induced by either carbon monoxide or steroid binding. Fractions containing the cytochrome were pooled, dialyzed overnight against 50 mM buffer containing 0.4% cholate and applied to the affinity column (1.5 × 6 cm) previously equilibrated with the same buffer. After washing the column with 50 ml 100 mM

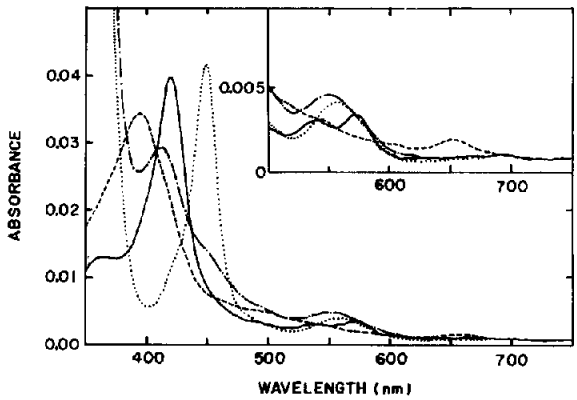

Fig.1. Absorption spectra of cytochrome *P*-450 from adrenocortical microsomes. The buffer system was 100 mM potassium phosphate buffer, pH 7.2, containing 100 μM EDTA, 100 μM dithiothreitol and 20% glycerol. Oxidized (————); dithionite-reduced (– · – · – ·); dithionite-reduced CO complexed (·····); and steroid-complexed in the presence of 10 μM 17α-hydroxyprogesterone (- - - - - - - -).

buffer containing 0.08% Emulgen, the cytochrome was eluted with 100 mM buffer containing 0.16% Emulgen. Finally, the cytochrome was dialyzed overnight against 100 mM buffer and used as the purified preparation. The specific content of the cytochrome *P*-450 preparation was 14 nmol/mg of protein.

The absorption spectra of the purified cytochrome are depicted in fig.1. The cytochrome was isolated as the low spin ferric form having A_{max} at 360, 418, 538 and 571 nm. Upon reduction with sodium dithionite, the Soret maximum shifted to 414 nm and one broad absorption band was seen at 550 nm. The CO spectrum in the dithionite reduced form gave A_{max} at 450 nm and 557 nm. No peaks were observed around 420 nm, indicating that the preparation was free from cytochrome *P*-420. Cytochrome *P*-420 appeared only when the cytochrome was exposed to lyotropic salts such as KSCN and KI at the concentration of 0.75 M or kept at 47°C for 10 min. When 10 μM 17α-hydroxyprogesterone was added to the cytochrome, the Soret band shifted to lower wavelength, 396 nm, and a peak attributable to a high spin charge-transfer band appeared around 650 nm. When the cytochrome was treated with 10 μM progesterone at 25°C, the Soret band was not completely shifted to 396 nm. The conversion to the high spin form was considerably affected by increasing temperature. These phenomena may be attributable to the spin state equilibrium [17]. Pregnenolone did not induce any observable change in the spectrum of the cytochrome. The addition to the cytochrome of the substrate for the mitochondrial steroid hydroxylase such as cholesterol or deoxycorticosterone did not produce the high spin-type spectrum.

The difference spectra induced by addition of 17α-hydroxyprogesterone or progesterone to the cytochrome are illustrated in fig.2. All of the spectra show an A_{390} max, an A_{423} min and an isosbestic point at 407 nm. The magnitude of the difference between the maximum and the minimum can be assumed to be proportional to the concentration of the cytochrome-steroid complex. The total concentrations of cytochrome *P*-450, $[P\text{-}450_T]$, and the steroid added, $[S_T]$, are related with dissociation constant, K_d, as follows:

$$\frac{A_\infty}{A_\infty - A_{ob}} = \frac{1}{K_d}\left(\frac{A_\infty}{A_{ob}}[S_T] - [P\text{-}450_T]\right) \qquad (1)$$

where A_{ob} is the $\Delta A_{390-423}$ absorption difference produced at a given concentration of the steroid and A_∞ is that observed at a sufficient concentration of the steroid. When $A_\infty/(A_\infty - A_{ob})$ is plotted versus $(A_\infty/A_{ob})[S_T]$ as shown in fig.3, $1/K_d$ can be estimated from the slope and the intercept at the axis of $(A_\infty/A_{ob})[S_T]$ shows $[P\text{-}450_T]$. The values of K_d were found to be 0.10 μM for 17α-hydroxypro-

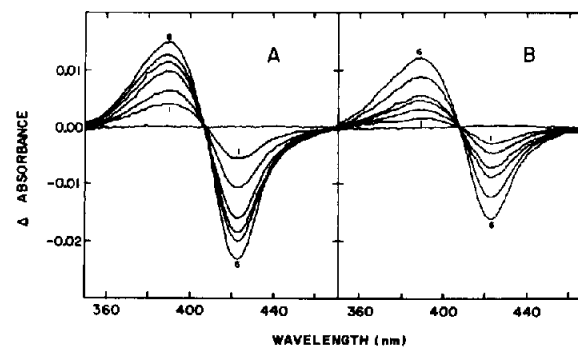

Fig.2. Titration of cytochrome *P*-450 with 17α-hydroxyprogesterone (A) or with progesterone (B). Curves 1–6 show difference spectra of cytochrome *P*-450 (0.35 μM) after addition of 0.1, 0.2, 0.4, 0.6, 1.6 and 10 μM of the steroid, respectively. For the buffer system, see fig.1 legend.

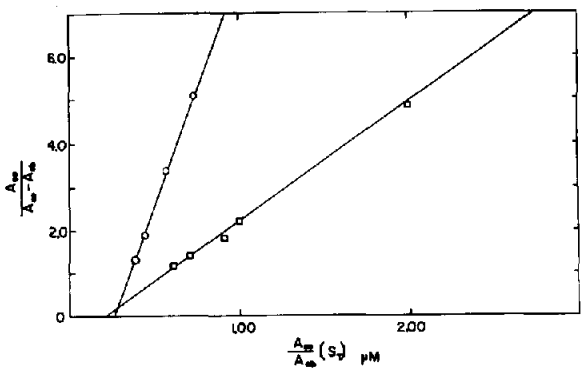

Fig.3. Determination of the dissociation constant of cytochrome *P*-450 for the steroid. Cytochrome *P*-450 was titrated with 17α-hydroxyprogesterone or with progesterone as shown in fig.2 and the dissociation constant determined from eq. (1). (—○—) for 17α-hydroxyprogesterone; (—□—) for progesterone.

gesterone and 0.35 μM for progesterone at 25°C. The total concentration of cytochrome *P*-450 in the assay system was found to be about 0.25 μM, which agreed fairly well with the value estimated by a reduced carbon monoxide difference spectrum [18].

It is important to examine whether 17α-hydroxyprogesterone and progesterone interact with a single species of cytochrome *P*-450 or with the separate species. If the former were case, the following relations can be derived for the solution containing the both steroids in excess:

$$\frac{K_d}{K_d'} = \frac{[P\text{-}450\text{–}S'][S_T]}{[P\text{-}450\text{–}S][S_T']} \quad (2)$$

$$[P\text{-}450_T] = [P\text{-}450\text{–}S] + [P\text{-}450\text{–}S'] \quad (3)$$

where K_d and K_d' are the dissociation constants for 17α-hydroxyprogesterone and progesterone, respectively. $[S_T]$ and $[S_T']$ are the total concentrations of 17α-hydroxyprogesterone and progesterone, respectively. $[P\text{-}450\text{–}S]$ and $[P\text{-}450\text{–}S']$ are the concentrations of cytochrome *P*-450 complexes with 17α-hydroxyprogesterone and with progesterone, respectively, which are much less than $[S_T]$ or $[S_T']$. The observed change (A_{ob}) in the difference spectrum is assumed to be the sum of the spectral change caused by 17α-hydroxyprogesterone and that by progesterone in the presence of the both steroids:

$$A_{ob} = \frac{[P\text{-}450\text{–}S]}{[P\text{-}450_T]} A_\infty + \frac{[P\text{-}450\text{–}S']}{[P\text{-}450_T]} A_\infty' \quad (4)$$

where A_∞ or A_∞' is the difference in absorption produced when 17α-hydroxyprogesterone or progesterone is added individually to the solution to the saturation level. By rearranging eq. (2), eq. (3) and eq. (4), the following relation can be obtained:

$$A_{ob} = A_\infty + \frac{A_\infty' - A_\infty}{1 + \frac{K_d'[S_T]}{K_d[S_T']}} \quad (5)$$

As shown in fig.4, when the steroid solutions containing the various ratio of 17α-hydroxyprogesterone to progesterone were added to the solution containing the cytochrome at 25°C, the plots of the absorption difference induced by steroid binding showed fairly good agreement with the theoretical curve which was calculated by using eq. (5) and the dissociation constants of 17α-hydroxyprogesterone and progesterone.

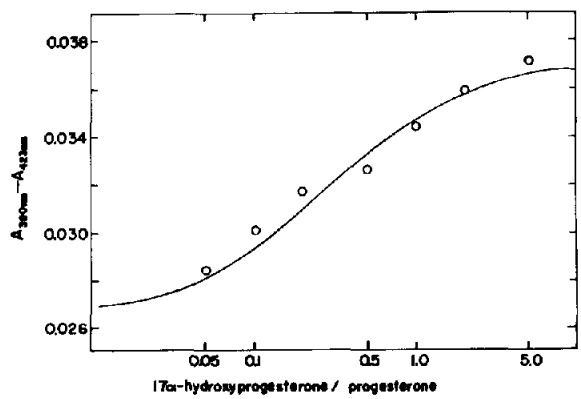

Fig.4. Competition of 17α-hydroxyprogesterone with progesterone for binding to cytochrome *P*-450. The absorption differences ($\Delta A_{390-420}$) of difference spectra of cytochrome *P*-450 (0.35 μM) induced by addition of the both steroids to the total concentration of 100 μM were plotted for the various ratio of 17α-hydroxyprogesterone to progesterone. For the buffer system, see fig.1 legend. The solid line shows the theoretical curve.

In the case that 17α-hydroxyprogesterone and progesterone bind to separate cytochrome *P*-450 species, A_{ob} is expected to be constant in the presence of the sufficient concentrations of the both steroids, even if their ratio was altered. The results suggest that 17α-hydroxyprogesterone and progesterone compete with each other for binding to the same species of cytochrome *P*-450.

Acknowledgements

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