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Biochemistry

Hepatic mitochondrial cytochrome *P*-450: Isolation and functional characterization

(heme protein/liver mitochondria/bile acid/cholestanetriol 26-hydroxylase)

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ABSTRACT **A CO-binding heme protein was solubilized and partially purified from the inner membrane fraction of rat liver mitochondria by a modification of a method [Imai, Y. & Sato, R. (1974) *Biochem. Biophys. Res. Commun.* 60, 8-14] developed to purify cytochrome *P*-450 from liver microsomes. The partially purified preparation contained protoheme and its spectral properties are characteristic of the heme proteins of the cytochrome *P*-450 family. The isolated cytochrome *P*-450 preparation could reconstitute a CO-sensitive, NADPH-dependent 26-hydroxylation activity for 5β-cholestane-3α,7α,-12α-triol when supplemented with NADPH-adrenodoxin reductase and adrenodoxin, both purified from bovine adrenocortical mitochondria. Unlike a cytochrome *P*-450 purified from liver microsomes of drug-untreated rats, however, the liver mitochondrial cytochrome *P*-450 could not catalyze NADPH-dependent benzphetamine *N*-demethylation in the presence of adrenodoxin reductase and adrenodoxin or function with the purified microsomal NADPH-cytochrome *c* reductase plus Emulgen 913 as an electron-donating system. It is concluded that the rat liver inner mitochondrial membrane houses a species of cytochrome *P*-450 functional in 5β-cholestane-3α,7α,12α-triol 26-hydroxylation.**

The 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol (cholestanetriol), a step involved in cholesterol catabolism, is catalyzed by both mitochondria (1-5) and microsomes (3, 6, 7) from rat liver, and available evidence indicates that the microsomal 26-hydroxylase activity is dependent on cytochrome *P*-450 (6, 7). The possibility therefore exists that a cytochrome *P*-450-like heme protein also is functional in the mitochondrial 26-hydroxylase system, which has been shown to be localized in the inner membrane-matrix region (4). Further, it has been shown that the liver mitochondrial 26-hydroxylase activity is sensitive to CO (3-5) and to phenylisocyanide (4). More recently, Okuda *et al.* (8) have demonstrated that the photochemical action spectrum for reversal of CO inhibition of rat liver mitochondrial cholestanetriol 26-hydroxylase, measured with isocitrate as electron donor, exhibits a maximum at 450 nm. In this communication, we report that a CO-binding heme protein, spectrally identifiable as a cytochrome *P*-450, can be isolated partially purified from the inner membrane of rat liver mitochondria. A CO-sensitive, NADPH-dependent cholestanetriol 26-hydroxylase activity can be reconstituted by combining the isolated heme protein with NADPH-adrenodoxin reductase and adrenodoxin, both purified from bovine adrenocortical mitochondria. The reconstituted system is, however, inactive in benzphetamine *N*-demethylation, a reaction that is actively catalyzed by the microsomal cytochrome *P*-450-containing mono-oxygenase system.

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MATERIALS AND METHODS

NADPH-adrenodoxin reductase (9) and adrenodoxin (10) purified from bovine adrenocortical mitochondria were kindly supplied by M. Katagiri of Kanazawa University. Liver microsomal cytochrome *P*-450 was purified to a specific content of about 12 nmol/mg of protein from drug-untreated rats by the method of Imai and Sato (11). Detergent-solubilized NADPH-cytochrome *c* reductase purified from rabbit liver microsomes (12) was a generous gift from H. Satake. 5β-[^{3}H]-Cholestane-3α,7α,12α-triol ([^{3}H]cholestanetriol) was prepared as described previously (2).

The inner membrane-matrix fraction was prepared by digitonin treatment of liver mitochondria isolated from male rats (Wistar strain) as described previously (4). This fraction was suspended in 0.2 M potassium phosphate buffer at pH 7.4 and sonicated at 0° in a Branson sonifier for a total period of 5 min with 30-s intervals at 20 kHz and 50 W output. The supernate of the sonicate, after centrifugation at 8500 × *g* for 10 min, was centrifuged again at 105,000 × *g* for 1 hr. The resultant pellet was used as the inner membrane fraction. The NADPH-cytochrome *c* reductase activity, a marker of microsomes, of this preparation indicated that contamination by microsomes was considerably less than 1% on protein basis.

Protein was determined by the method of Lowry *et al.* (13) with bovine serum albumin as the standard. Cytochrome *P*-450 was estimated from the CO difference spectrum as described by Omura and Sato (14). Alkaline pyridine hemochrome was formed and determined essentially as described previously (14). Spectrophotometric measurements were conducted in the high sensivity range by using a Hitachi 356 dual-wavelength spectrophotometer.

The standard reaction mixture for reconstitution of the mitochondrial cholestanetriol 26-hydroxylase system contained, in a final volume of 0.5 ml, an appropriate amount of liver mitochondrial cytochrome *P*-450, 0.1 unit (9) of bovine adrenodoxin reductase, 5 nmol of adrenodoxin, 1.2 μmol of $MgCl_2$, 20 μmol of potassium phosphate buffer (pH 7.0), 0.5 μmol of NADPH, and 30 nmol of [^{3}H]cholestanetriol dissolved in 10 μl of acetone. The reaction was initiated by adding [^{3}H]cholestanetriol after preincubation at 30° for 5 min and terminated by adding 50 μl of 0.1 M HCl. The hydroxylated product, 5β-cholestane-3α,7α,12α,26-tetrol (cholestanetriol-26-OH), was separated by thin-layer chromatography and determined from its radioactivity as described previously (4). CO inhibition of the reconstituted 26-hydroxylase system was studied in a closed vessel under a gas phase consisting of 25% O_2 and 75%

Abbreviations: cholestanetriol, 5β-cholestane-3α,7α,12α-triol; cholestanetriol-26-OH, 5β-cholestane-3α,7α,12α,26-tetrol.

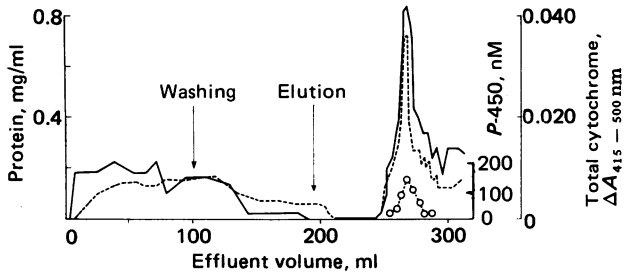

FIG. 1. Elution profile of ω-amino-*n*-octyl Sepharose 4B column chromatography of the solubilized supernate obtained from the inner membrane fraction of rat liver mitochondria. The washing and elution were conducted with the equilibration medium containing 0.5% (instead of 0.8%) sodium cholate and with the same medium containing 0.4% sodium cholate and 0.08% Emulgen 913, respectively. - - - -, Protein; ——, "total cytochrome" ($\Delta A_{415-500\ nm}$); O···O, cytochrome *P*-450.

CO (by volume). In control experiments CO was replaced by argon gas.

Benzphetamine *N*-demethylase activity was studied in both the microsomal and mitochondrial types of reconstituted systems. The microsomal system contained an appropriate amount of either microsomal or mitochondrial cytochrome *P*-450, 0.2 unit (12) of detergent-solubilized NADPH–cytochrome *c* reductase, 0.005% Emulgen 913, 0.2 μmol of NADPH, 1 μmol of benzphetamine, and 0.1 nmol of potassium phosphate buffer (pH 7.25) in a final volume of 1.0 ml. Emulgen 913 used in this reconstitution has been shown to substitute for phosphatidylcholine effectively (15). In the mitochondrial system, both Emulgen and NADPH–cytochrome *c* reductase were replaced by 0.1 unit of adrenodoxin reductase and 20 nmol of adrenodoxin. In both systems, the reaction was started by the addition of benzphetamine and the benzphetamine-dependent NADPH oxidation was followed spectrophotometrically as described by Lu *et al.* (16).

RESULTS

Partial Purification of Liver Mitochondrial Cytochrome *P*-450. All the manipulations were conducted at 0–4°. Potassium phosphate buffers (pH 7.25) containing 20% (vol/vol) glycerol were used throughout; they are referred to simply as 100 mM buffer, etc. The solubilization and purification procedure employed was an adaptation of the method described by Imai and Sato (11) for cytochrome *P*-450 of phenobarbital-induced rabbit liver microsomes.

The mitochondrial inner membrane fraction (150 mg of protein) was subjected to solubilization treatment as described by Imai and Sato (11), except that the sodium cholate concentration in the solubilization medium was increased from 0.6 to 0.8% (wt/vol). This treatment resulted in the recovery of about one-third of the membranous protein together with a *b*-type cytochrome, detectable by the dithionite-reduced minus oxidized difference spectrum, in the solubilized supernate, the CO difference spectrum of which exhibitied a very faint peak around 450 nm, indicating the presence of a CO-binding protein. However, the level detected was too low to permit accurate estimation. The supernate was then subjected to chromatography on an ω-amino-*n*-octyl Sepharose 4B column equilibrated with the solubilization medium containing 0.5% sodium cholate, essentially as described by Imai and Sato (11). After extensive washing with the equilibration medium, a spectrally identifiable cytochrome *P*-450 could be eluted with 100 mM buffer containing 1 mM dithiothreitol, 0.4% sodium cholate, and 1 mM Emulgen 913, as recorded in the chromatogram of Fig. 1. The

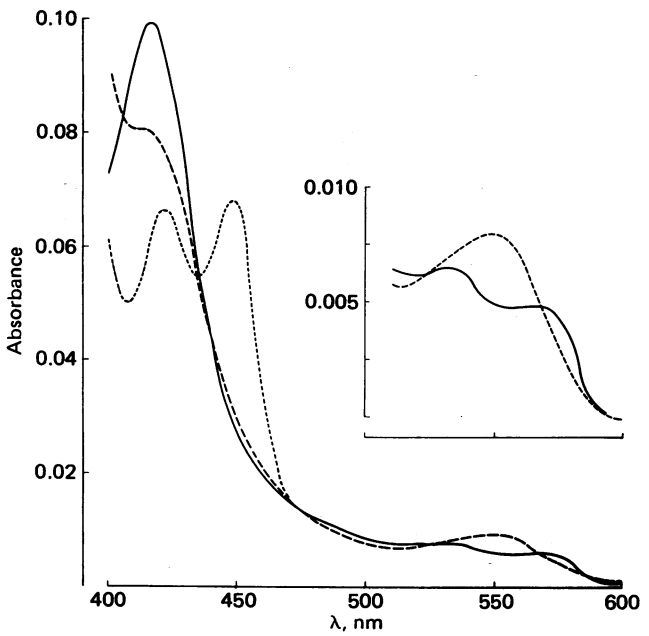

FIG. 2. Absorption spectra of partially purified mitochondrial cytochrome *P*-450. The spectra were measured with a sample containing approximately 0.52 μM cytochrome *P*-450 (0.85 mg of protein per ml) in 150 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol and 1.25 mM Emulgen 913. ——, Oxidized form; – – –, dithionite-reduced form; - - - -, CO complex of dithionite-reduced form. *Inset* shows visible region with expanded vertical scale.

amount of cytochrome *P*-450 obtained was very low; about 2 nmol of the cytochrome was isolated from about 1 g of mitochondrial protein. The combined cytochrome *P*-450-containing fractions were diluted 4-fold with 20% glycerol and applied to a hydroxyapatite column equilibrated with 25 mM buffer. After the column had been washed with 35 mM buffer containing 0.2% Emulgen 913, cytochrome *P*-450 was eluted with 150 mM buffer containing 0.2% Emulgen 913, resulting in a 5- to 7-fold purification. The eluate was then dialyzed for 40 hr against 100 volumes of 20 mM buffer, and the dialysate was applied to a CM-Sephadex C-50 column equilibrated with 20 mM buffer containing 0.2% Emulgen 913. A reddish-brown band that formed at the top of the column was carefully removed and resuspended in a small amount of 20 mM buffer containing Emulgen, then packed in a pencil-sized column and eluted with 300 mM buffer containing Emulgen. This step resulted in very effective (20- to 40-fold) concentration of cytochrome *P*-450.

The final preparation thus obtained contained 0.4–0.7 nmol of protoheme per mg of protein and gave at least three protein bands upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The preparation could be stored at −70° for at least 5 months without appreciable loss of the cholestanetriol-26-hydroxylase reconstituting activity.

Spectral Properties of Partially Purified Mitochondrial Cytochrome *P*-450. As shown in Fig. 2, the oxidized (untreated) form of the preparation showed an intense Soret absorption peak at 415 nm together with two minor peaks between 530 and 580 nm, whereas the dithionite-reduced form exhibited peaks at around 414 and 550 nm. On addition of CO to the reduced form, a Soret peak characteristic of heme proteins of the cytochrome *P*-450 family was seen at 448 nm, together with a peak at 424 nm of comparable intensity. The 424-nm peak seemed to be due partly to the presence of cytochrome *P*-420, a denatured form of the cytochrome (17). Fig. 3 shows the CO-dif-

Table 1. Reconstitution of cholestanetriol-26-hydroxylase activity from the mitochondrial cytochrome *P*-450, NADPH–adrenodoxin reductase, and adrenodoxin

Complete system	Complete system in 25% O_2/75% CO	Component omitted: Cytochrome *P*-450	Adrenodoxin reductase	Adrenodoxin	NADPH
21.1	—	0	0	4.3	0
19.1	7.6	—	—	—	—

Values are nmol of cholestanetriol-26-OH formed per min/nmol of cytochrome *P*-450. The composition of the complete system and assay method are described in *Materials and Methods*.

ference spectrum of the preparation, exhibiting a clear maximum at 450 nm; the significant shoulder around 420 nm again suggested the presence of cytochrome *P*-420. The absorption spectrum of the alkaline pyridine hemochrome derived from the preparation possessed a symmetrical α peak at 556 nm, indicating that the prosthetic group of heme protein was iron protoporphyrin IX. The increment of molar extinction coefficient between 450 and 490 nm in the CO difference spectrum was tentatively estimated as 78 cm^{-1} mM^{-1} on the basis of protoheme content. This value is similar to, but somewhat lower than, that reported for hepatic microsomal cytochrome *P*-420 (91 cm^{-1} mM^{-1}) (14). When the dithionite-reduced sample was allowed to stand at room temperature, there was a gradual conversion to cytochrome *P*-420 showing a Soret peak at 419 nm in the CO-difference spectrum, as in the case of microsomal cytochrome *P*-450. All these observations indicated that the heme protein contained in the partially purified preparation was an authentic cytochrome *P*-450.

Reconstitution of Cholestanetriol 26-Hydroxylase Activity. As shown in Table 1, a cholestanetriol-26-hydroxylase activity could be reconstituted when the rat liver mitochondrial cytochrome *P*-450 preparation was mixed with adrenodoxin and NADPH–adrenodoxin reductase, both purified from bovine adrenocortical mitochondria. Omission of the cytochrome, adrenodoxin reductase, or NADPH from the system resulted in complete loss of activity, whereas omission of adrenodoxin left observable activity, though greatly reduced. It was, therefore, suggested that a small amount of adrenodoxin-like iron–sulfur protein might be present in the partially purified cytochrome *P*-450 preparation. Further, the reconstituted 26-hydroxylase system was sensitive to CO (62% inhibition in 25% O_2/75% CO in the dark), eliminating the possibility that a component other than cytochrome *P*-450 in the partially purified preparation was responsible for the observed activity. Half-maximal activity of the reconstituted 26-hydroxylase was

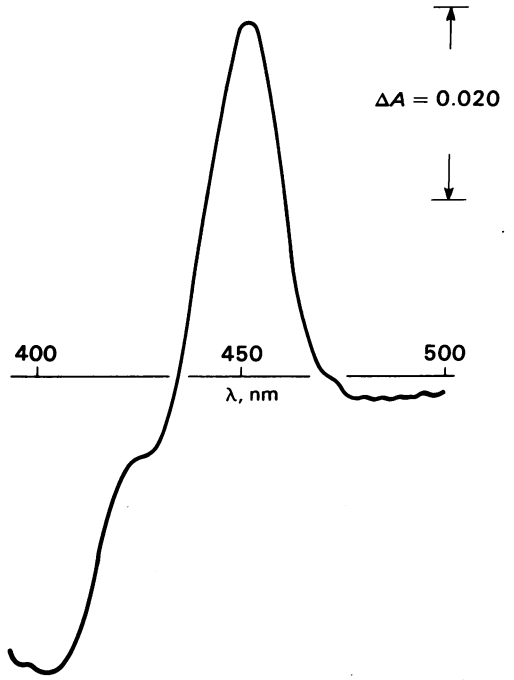

FIG. 3. CO difference spectrum of partially purified mitochondrial cytochrome *P*-450. The spectrum was measured with a sample containing approximately 0.45 μM cytochrome *P*-450 (0.80 mg of protein per ml) in 150 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol and 1.25 mM Emulgen 913.

obtained at a reductase concentration of 10–20 units per vessel in the presence of a saturating amount of adrenodoxin and at 3–6 μM adrenodoxin, respectively, when a sufficient amount of adrenodoxin reductase was present.

Functional Comparison with Microsomal Cytochrome *P*-450. The catalytic properties of the mitochondrial cytochrome *P*-450 were then compared with those of a cytochrome *P*-450 purified from liver microsomes of drug-untreated rats. As shown in Table 2, the microsomal cytochrome *P*-450 could reconstitute benzphetamine *N*-demethylase activity when combined with detergent-solubilized NADPH–cytochrome *c* reductase in the presence of Emulgen 913, in agreement with previous reports (15, 16). The microsomal cytochrome *P*-450 could also catalyze NADPH-dependent benzphetamine *N*-demethylation when supplemented with adrenodoxin and adrenodoxin reductase, indicating that both the hepatic microsomal (NADPH–cytochrome *c* reductase) and adrenocortical mitochondrial (adrenodoxin reductase and adrenodoxin) electron-donating systems could provide reducing equivalents

Table 2. Benzphetamine *N*-demethylase activities of the reconstituted microsomal and mitochondrial cytochrome *P*-450s

Electron-donating system employed	Cytochrome *P*-450 employed	*N*-Demethylase activity, nmol/min per nmol cytochrome *P*-450: Complete system		Component omitted: Cytochrome *P*-450	Adrenodoxin reductase	Adrenodoxin
Microsomal	Microsomal	—	2.4*			
	Mitochondrial	~0†	0			
Mitochondrial	Microsomal	—	5.6	0	0	<1.5
	Mitochondrial	~0†	—	—	—	—

Cytochrome *P*-450s were reconstituted with either the microsomal electron donating system (NADPH–cytochrome *c* reductase *plus* Emulgen 913) or the mitochondrial system (NADPH–adrenodoxin reductase and adrenodoxin).

* This value is lower than normal in similar reconstituted systems owing to the low activity preparation of NADPH–cytochrome *c* reductase used.

† Only 0.043 nmol of cytochrome *P*-450 (instead of 0.10 nmol) was used for reconstitution. The composition of the complete system and assay method are described in *Materials and Methods*.

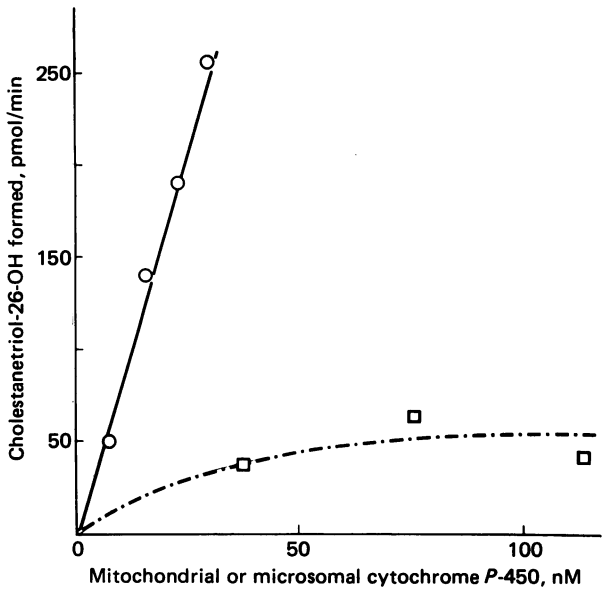

FIG. 4. Dependence of reconstituted cholestanetriol-26-hydroxylase activity on the amount of *P*-450 added. The reconstitution was carried out as described in *Materials and Methods*, except that the indicated amount of either mitochondrial (○) or microsomal (□) cytochrome *P*-450 was used.

to the microsomal cytochrome *P*-450 for the demethylation reaction. On the other hand, the mitochondrial cytochrome *P*-450 was completely inert toward benzphetamine when reconstituted with either the microsomal or the mitochondrial electron-donating system. In the presence of NADPH–cytochrome *c* reductase and Emulgen 913, the mitochondrial cytochrome *P*-450 was reducible by NADPH, as evidenced by the appearance of the characteristic CO-difference spectrum on introduction of CO. Nevertheless, the cytochrome was unable to demethylate benzphetamine.

Fig. 4 shows the dependence of the cholestanetriol-26-hydroxylase activity reconstituted with adrenodoxin reductase and adrenodoxin on the concentration of either the mitochondrial or microsomal cytochrome *P*-450 added. With the mitochondrial cytochrome *P*-450, the hydroxylase activity was linearly dependent on the amount of the heme protein added under the conditions employed. On the other hand, the activity reconstituted with the microsomal cytochrome *P*-450 was at most one-seventh of that obtained with the mitochondrial cytochrome *P*-450, as judged from the initial slopes. Moreover, the activity did not increase linearly as the amount of the heme protein was increased. The low 26-hydroxylase activity with the microsomal cytochrome *P*-450 could not be ascribed to the use of the adrenal mitochondrial components as an electron-donating system, because this system could effectively provide reducing equivalents for benzphetamine *N*-demethylation catalyzed by the microsomal cytochrome *P*-450 (Table 2). It was further found that the addition of cholestanetriol to the mitochondrial cytochrome *P*-450 induced a type I spectral change (data not shown). In contrast, no positive sign of cholestanetriol interaction with the microsomal cytochrome *P*-450 was obtained—at least by difference spectrophotometry.

DISCUSSION

The results presented above, together with the report that the photochemical action spectrum for CO inhibition of cholestanetriol-26-hydroxylation by rat liver mitochondria has a maximum at 450 nm (8), provide ample evidence supporting the occurrence in rat liver mitochondria of a cytochrome *P*-450 that is functional in cholestanetriol-26-hydroxylation. The recent observation by Ohashi and Omura (18) that bovine liver mitochondria contain a protein that is immunochemically indistinguishable from bovine adrenodoxin provides additional support to the above conclusion. It is very likely that the reducing equivalents required by the mitochondrial cytochrome *P*-450 for its function *in vivo* are supplied by way of an adrenodoxin reductase-like flavoprotein and an adrenodoxin-like iron–sulfur protein, as in the cases of adrenocortical mitochondria (19) and chick renal mitochondria (20). A chick renal mitochondrial ferredoxin active in the 25-hydroxyvitamin-D_3-1α-hydroxylase system has been reported (21). The present study has shown that the isolated rat liver mitochondrial cytochrome *P*-450 can reconstitute a cholestanetriol-26-hydroxylase activity when mixed with bovine adrenodoxin reductase and adrenodoxin (Table 1). 26-Hydroxylation of 5β-cholestane-3α,7α,-diol, a more hydrophobic analogue of cholestanetriol known to branch into the so-called chenodeoxycholate pathway, has recently been reconstituted from the mitochondrial cytochrome *P*-450 in a similar manner (unpublished results).

Although no accurate estimation is possible, the content of cytochrome *P*-450 in rat liver mitochondria must be very small in view of the fact that only about 2 nmol of the cytochrome can be recovered in the aminooctyl column eluate from 1 g of mitochondrial protein. This very low level of cytochrome *P*-450 appears to explain our earlier failure to detect it in rat liver mitochondria by routine difference spectrophotometry, such as employed successfully by Ghazarian *et al.* (20). It is worth noting that the cytochrome *P*-450 isolated was preferentially solubilized from the inner mitochondrial membrane with 0.8% sodium cholate, leaving most of the respiratory redox components, such as cytochrome oxidase, still bound. Therefore, we cannot exclude the possibility of the occurrence of another form of cytochrome *P*-450 among unsolubilized components of the inner membrane.

The very low yield of cytochrome *P*-450 from the inner mitochondrial membrane raises the possibility that the isolated heme protein was derived from contaminating microsomes rather than from the inner membrane. However, the inner membrane fraction used as the starting material was contaminated by microsomes only to an insiginficant extent, as judged from the content of a microsomal marker, NADPH–cytochrome *c* reductase. Furthermore, the cytochrome isolated from the inner mitochondrial membrane was inactive in benzphetamine *N*-demethylation (Table 2), a reaction catalyzed by a cytochrome *P*-450 purified from liver microsomes of drug-untreated rats (Table 2) and also by two homogenous preparations of cytochrome *P*-450 purified from liver microsomes of phenobarbital- and β-naphthoflavone-pretreated rabbits (22). Although the possibility still remains that rat liver microsomes contain a species of cytochrome *P*-450 completely incapable of benzphetamine *N*-demethylation, it may be concluded that the cytochrome *P*-450 isolated in this study is mitochondrial in origin.

The isolated mitochondrial cytochrome *P*-450 also differs from that purified from liver microsomes derived from untreated rats in that the latter shows a much lower cholestanetriol-26-hydroxylase activity than the former in the reconstituted system containing adrenal components as the electron-donating system (Fig. 4). The activity with the microsomal cytochrome *P*-450 is not linearly dependent on the amount of the heme protein added. Moreover, cholestanetriol does not induce any spectral change in the microsomal cytochrome, though a clear type I difference spectrum is seen in the mito-

chondrial type. The significance of these findings is not clear at present, in view of the reports that cholestanetriol-26-hydroxylation is also catalyzed by a cytochrome *P*-450-linked system in rat liver microsomes (3, 6, 7). One possibility is that cholestanetriol-26-hydroxylation in rat liver microsomes is catalyzed by a minor cytochrome *P*-450 component that is removed during the purification of the main component by the method of Imai and Sato (11).

Finally, it should be noted that the quantitative data obtained, including the molar extinction coefficient and turnover numbers, are preliminary because they were derived by using limited amounts of only partially purified material. For instance, the turnover number attained by our reconstituted cholestanetriol-26-hydroxylase system (about 20 min^{-1}) is still much less than that for intact mitochondria (about 200 min^{-1}). It is likely that the turnover number can be improved considerably by further refinement of the reconstitution conditions. Definitive determination of these data and fundamental physicochemical characteristics of the cytochrome as well as improvement of purification procedure to isolate ample amounts of homogenous sample remain for future effort.

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