RAT LIVER MITOCHONDRIAL CYTOCHROME *P*-450 ACTIVE IN A RECONSTITUTED STEROID HYDROXYLATION REACTION

Jan I. PEDERSEN

Institute for Nutrition Research, School of Medicine, University of Oslo, Blindern, Oslo 3, Norway

Received 20 October 1977

1. Introduction

Evidence has been presented for the location in liver mitochondria of a steroid 26-hydroxylase involved in the formation of bile acids [1—3]. This hydroxylation reaction is inhibited by carbon monoxide [1,2] and the inhibition can be reversed by light of 450 nm [4]. The enzyme is thus most likely a cytochrome *P*-450. So far no direct evidence has been presented for the existence of cytochrome *P*-450 in liver mitochondria.

This communication reports the identification in liver mitochondria of a cytochrome *P*-450. It is shown that this enzyme, in the presence of a previously isolated ferredoxin [5] and a liver mitochondrial NADPH-ferredoxin reductase will catalyze the conversion of cholesterol into a more polar product, tentatively identified as 26-hydroxycholesterol.

2. Materials and methods

Rat liver mitochondria were prepared by standard procedure in ice cold 0.25 M sucrose containing 1 mM EGTA and 15 mM Hepes buffer, pH 7.4. The mitochondrial pellet was resuspended 3 times and if not immediately processed frozen and stored in liquid nitrogen.

Cytochrome *P*-450 was solubilized from the mitochondria by a modification of the procedure [6] as described [5]. The concentrations of cytochromes *P*-450 and *P*-420 in the soluble preparation were calculated from the CO difference spectrum of the reduced sample [7].

The content of cytochrome *P*-450 in intact mitochondria was determined as described in the legend to fig.1. This method will reveal the reduced-CO minus the oxidized-CO difference spectrum of the cytochrome. A molar extinction difference between 450 nm and 490 nm of 104 mM^{-1} cm^{-1} was used for calculation [8].

Bovine liver ferredoxin was purified as described [5].

The isolation and purification of a mitochondrial NADPH-ferredoxin reductase will be published elsewhere (Pedersen, J. I. and Godager, H., in preparation).

The optical spectra were recorded at room temperature on a Cary 118 spectrophotometer.

The reconstitution of steroid hydroxylase activity was essayed in an incubation medium that contained in 1.5 ml 0.1 M Mops buffer, pH 7.4: 1 μmol glucose-6-phosphate, 0.5 unit glucose-6-phosphate dehydrogenase, 1 μmol $MgCl_2$, and approx. 200 000 cpm [1α,2α(*n*)-^{3}H]cholesterol in 10 μl acetone (spec. act. 43 Ci/mmol). No unlabelled cholesterol was added, and based on the gas chromatographic determination of the endogenous cholesterol in the soluble cytochrome *P*-450 preparation (13 nmol/nmol *P*-450) the specific activity in the incubation mixture was estimated to be approx. 100 000 cpm/nmol cholesterol. The amounts of enzymes added are given in table 2. The reaction was started by the addition of 50 nmol NADP and continued for 30 min at 30°C under oxygen. The extraction procedure was as described [5]. Aliquots of the extracts were injected into a Spectra Physics high performance liquid chromatographic instrument fitted with a Rheodyne injector and a Spherisorb 5 μm silica column (0.3 X 25

cm). The samples were eluted with 5% isopropanol in *n*-hexane at a flow rate of 0.4 ml/min. One fraction/min was collected in counting vials. The solvent was evaporated, counting solution was added and the samples counted in a Tri-Carb liquid scintillation counter. Some incubation extracts were also subjected to thin-layer chromatography [1]. The solvent was benzene:ethyl acetate (1:1). Zones containing radioactivity were scraped directly into counting vials and counted as above.

Protein was determined by the Lowry method [9].

26-hydroxycholesterol was the generous gift of Dr I. Björkhem, Huddinge Hospital, Sweden. 25-Hydroxycholesterol was purchased from Steraloids Inc., Wilton, USA.

All other chemicals and biochemicals were standard commercial high purity materials.

3. Results

3.1. *Detection of cytochrome* P-*450 in intact rat liver mitochondria*

The detection of cytochrome *P*-450 in intact mitochondria by the typical CO difference spectrum is difficult due to interference by the CO complex of reduced cytochrome oxidase. Using a procedure similar to the one described [10] it is possible to overcome this difficulty and a CO complex of reduced cytochrome *P*-450 can be detected in rat liver mitochondria (fig.1).

The experiment described in fig.1 is based on the following:

In the reference cuvette the terminal part of the mitochondrial respiratory chain is completely reduced by succinate. Reversal of electron flow is inhibited by rotenone. Reduction of NADP by endogenous substrates via the energy-dependent transdehydrogenase is inhibited by maintaining low energy condition in the presence of valinomycin. In the sample cuvette maximal reducing and high energy conditions are ensured by succinate and malate in the presence of ATP. After anaerobiosis and CO addition to both mitochondrial suspensions a reduced cytochrome *P*-450 species becomes detectable in the sample cuvette by the A_{450} max (spectrum A).

Upon subsequent addition of dithionite to the sample an insignificantly small further reduction is seen (fig.1, spectrum B). This shows that all reducible mitochondrial pigments are completely reduced by the substrates added.

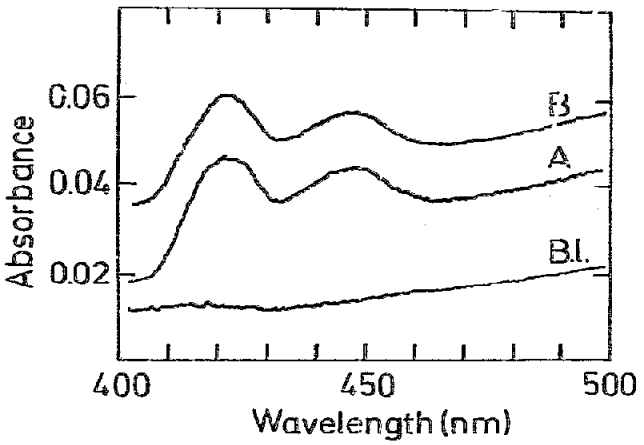

Fig.1. Completely-reduced minus partly-reduced difference spectra of carbon monoxide treated intact rat liver mitochondria. Rat liver mitochondria were suspended at a concentration of 1.16 mg protein/ml in a medium containing 0.11 M KCl, 30 mM Hepes buffer, pH 7.4, 1 mM EDTA, 2 mM $MgCl_2$ and 10 mM succinate. The suspension was equally divided into a sample and a reference cuvette and nitrogen was bubbled through both. After anaerobiosis 5 mM malate and 1 mM ATP were added to the sample cuvette and 5 μM rotenone and 4 μM valinomycin to the reference cuvette. The baseline was repeatedly recorded until it became stable as shown in the figure (B.l.). CO was then bubbled through both cuvettes and the difference spectrum (A) was recorded. After a A_{450} max was attained 0.5 mM dithionite (dissolved in anaerobic 1 M Tris base) was added to the sample cuvette and the difference spectrum (B) was recorded.

The amount of cytochrome *P*-450 was calculated from fig.1 to be 0.1 nmol/mg mitochondrial protein (0.12 nmol/mg protein when based on the reduction by dithionite).

The CO-binding pigment with an A_{420} max (fig.1) has not yet been identified. No hemoglobin was detected in the preparation. It might, however, represent contaminating methemoglobin, that becomes more reduced by NADH in the sample than in the reference cuvette [11].

3.2. *Solubilization of cytochrome* P-*450 from rat liver mitochondria*

By the solubilization procedure described [5] it

Table 1
Content of cytochrome *P*-450 in rat liver mitochondria

	Total protein (mg)	Specific content cyt. *P*-450 (nmol/mg protein)	Total amount cyt. *P*-450 (nmol)
Mitochondria	831	0.10	83.1
Soluble preparation	93.4	0.16	14.9

was possible to solubilize cytochrome *P*-450 from rat liver mitochondria with a specific content of 0.16 nmol/mg protein (table 1). This corresponds to a purification factor of 1.6 and a yield of 18%. The reduced-CO difference spectrum of this preparation is shown in fig.2. The peak at 420 nm shows that a small fraction (at most 25%) of the total CO-binding heme is accounted for as cytochrome *P*-420.

3.3. *The cytochrome P-450 reductase components of rat liver mitochondria*

Reduction of cytochrome *P*-450 by NADPH in adrenal mitochondrial [12] or chick kidney mitochondrial [13] hydroxylase systems involves a ferredoxin type iron-sulfur protein in addition to a NADPH-ferredoxin reductase (a FAD containing flavoprotein) [12,14]. It was assumed that the liver mitochondrial cytochrome *P*-450 described here also depended on two similar components for enzymatic activity.

A mitochondrial ferredoxin has already been isolated and partly purified from bovine liver and shown to be active in a reconstituted hydroxylation reaction [5]. A similar ferredoxin could also be isolated from rat liver mitochondria (Pedersen, J. I., unpublished). Although enzymatically active, this component was rather impure and the bovine liver ferredoxin was therefore used in the reconstitution experiments reported below.

In the supernatant of the rat liver mitochondrial sonicate a NADPH-ferredoxin reductase could be detected by its ability to reduce cytochrome *c* with NADPH in the presence of adrenal ferredoxin. This reductase has been purified and shown to be a flavoprotein with properties rather similar to the adrenal ferredoxin reductase (Pedersen, J. I. and Godager, H., in preparation).

3.4. *Reconstitution of a liver mitochondrial steroid hydroxylation system*

That the three isolated enzyme components function in a steroid hydroxylation system is demonstrated by the reconstitution experiment shown in

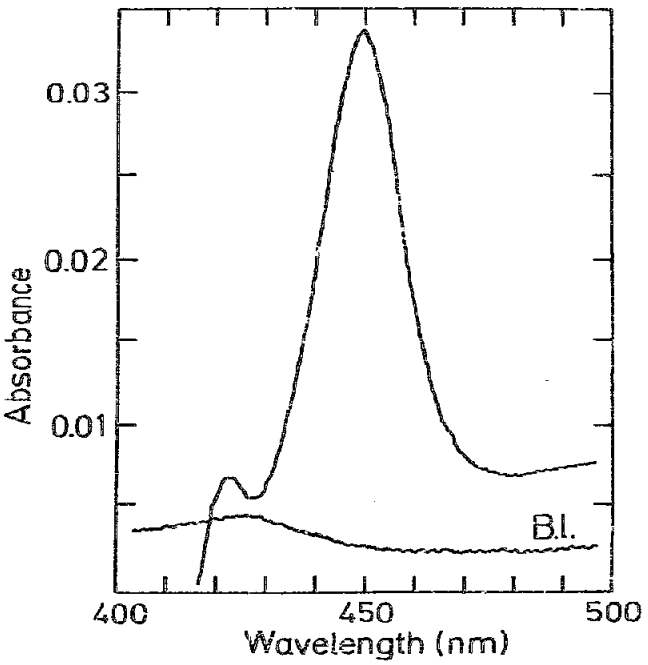

Fig.2. Difference spectrum of the carbon monoxide complex of reduced cytochrome *P*-450 solubilized from rat liver mitochondria. A cytochrome *P*-450 preparation containing 1.8 mg protein/ml in 0.1 M potassium phosphate buffer, pH 7.4, 25% glycerol and 1 mM dithiothreitol was equally divided into a sample and a reference cuvette. The baseline (B.l.) was recorded after bubbling of nitrogen and addition of 0.6 mM dithionite to both cuvettes. After bubbling CO through the sample for 60 s the difference spectrum was recorded.

Table 2
Hydroxylation of cholesterol by soluble enzyme components from liver mitochondria

Components present in incubation medium	Incubation number			
	1	2	3	4
Rat liver mitochondrial cytochrome *P*-450 (nmol)	0.162	0.162	0.162	0.162
Bovine liver mitochondrial ferredoxin (nmol)	–	–	0.18	0.18
Rat liver mitochondrial NADPH-ferredoxin reductase (units)[a]	–	5.12	–	5.12
Product formation (%)	0	0	3.2	14.1

[a] One activity unit is defined as the amount of enzyme catalyzing the reduction of 1 nmol cytochrome *c*/min by NADPH in the presence of excess adrenodoxin under the conditions described [5]

For incubation conditions see Materials and methods

table 2. It is seen that liver mitochondrial cytochrome *P*-450 in the presence of ferredoxin, ferredoxin reductase and NADPH will catalyze the conversion of cholesterol into a more polar product (fig.3). On thin-layer chromatography the product was found to co-migrate with 25-hydroxycholesterol and 26-hydroxy-cholesterol (not shown). Since rat liver mitochondria catalyze the hydroxylation of cholesterol almost exclusively in the 26 position [3] it is tentatively suggested that the product of the reaction represents 26-hydroxycholesterol.

4. Discussion

By a highly efficient solubilization procedure it has been possible to isolate a cytochrome *P*-450 from rat liver mitochondria. The presence of such a heme protein in these mitochondria was antecipated by previous findings of a steroid hydroxylation reaction that was inhibited by CO [1,2] and the reversal of the inhibition by light at 450 nm [4].

It can be argued that the cytochrome *P*-450 described in this report represents a microsomal contaminant. There are two strong arguments against such a view. First, the CO-sensitive pigment detected in the mitochondrial preparation was almost completely reduced by mitochondrial substrates (fig.1).

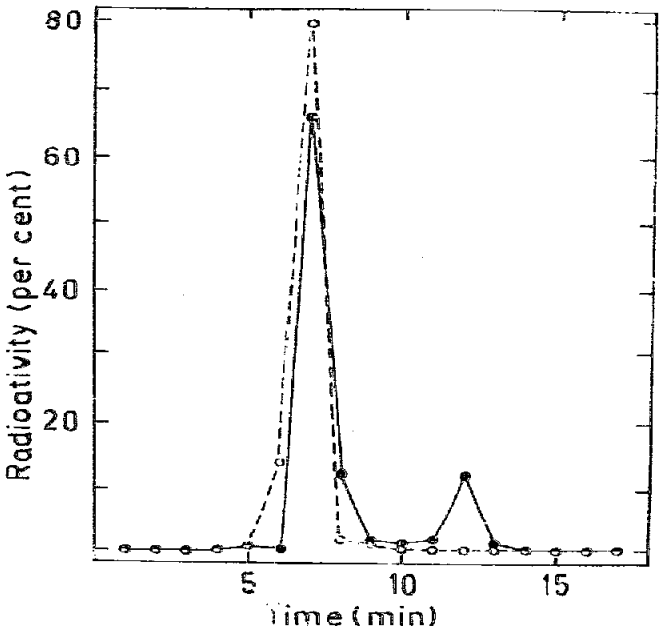

Fig.3. High performance liquid chromatography of the chloroform/methanol extract of the incubations containing the reconstituted steroid hydroxylase components. The incubation conditions as well as the extraction and chromatography are given in Materials and methods. The results shown correspond to incubation No. 4, table 2, i.e., the complete system (●——●), and incubation No. 2, i.e., without ferredoxin (o– – – –o).

Since no significant further reduction was seen upon addition of dithionite the microsomal contamination must be extremely small. The second and most important argument is the absolute dependence on ferredoxin for catalytic activity of this cytochrome (table 2, fig.3). No such dependence has ever been demonstrated for liver microsomal cytochrome *P*-450 [15]. It must therefore be concluded that liver cells contain two essentially different forms of cytochrome *P*-450 with different subcellular location and different mechanism of reduction.

During the preparation of this manuscript additional spectral evidence for the presence of cytochrome *P*-450 in liver mitochondria has been published [16]. The reported content, 0.15 nmol/mg mitochondrial protein, is in good agreement with the figures presented in this paper.

Acknowledgements

The skillful technical assistance of Guri Stuevold is appreciated. This work was supported by The Norwegian Research Council for Science and the Humanities.

References

[1] Björkhem, I. and Gustafsson, J. (1973) Eur. J. Biochem. 36, 201–212.
[2] Taniguchi, S., Hoshita, N. and Okuda, K. (1973) Eur. J. Biochem. 40, 607–617.
[3] Björkhem, I. and Gustafsson, J. (1974) J. Biol. Chem. 249, 2528–2535.
[4] Okuda, K., Weber, P. and Ullrich, V. (1977) Biochem. Biophys. Res. Commun. 74, 1071–1076.
[5] Pedersen, J. I., Oftebro, H. and Vänngård, T. (1977) Biochem. Biophys. Res. Commun. 76, 666–673.
[6] Mitani, F. and Horie, S. (1969) J. Biochem. (Japan) 65, 269–280.
[7] Omura, T. and Sato, R. (1967) Meth. Enzymol. 10, 556–561.
[8] Matsubara, T., Koike, M., Touchi, A., Tochino, Y. and Sugeno, K. (1976) Anal. Biochem. 75, 596–603.
[9] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[10] Ghazarian, J. G., Jefcoate, C. R., Knutson, J. C., Orme-Johnson, W. H. and DeLuca, H. F. (1974) J. Biol. Chem. 249, 3026–3033.
[11] Matsubara, T., Prough, R. A., Burke, M. D. and Estabrook, R. W. (1974) Cancer Res. 34, 2196–2203.
[12] Omura, T., Sanders, E., Estabrook, R. W., Cooper, D. Y. and Rosenthal, O. (1966) Arch. Biochem. Biophys. 117, 660–673.
[13] Pedersen, J. I., Ghazarian, J. G., Orme-Johnson, N. R. and DeLuca, H. F. (1976) J. Biol. Chem. 251, 3933–3941.
[14] Chu, J.-W. and Kimura, T. (1973) J. Biol. Chem. 248, 2089–2094.
[15] Lu, A. Y. H. and Levin, W. (1974) Biochim. Biophys. Acta 344, 205–240.
[16] Okuda, K., Ruf, H.-H. and Ullrich, V. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 689–694.