

PROPERTIES OF PARTIALLY PURIFIED LIVER MICROSOMAL CYTOCHROME *P*-450: ACCEPTANCE OF TWO ELECTRONS DURING ANAEROBIC TITRATION*

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Received 15 October 1973

1. Introduction

A large variety of foreign compounds, including drugs, petroleum products and insecticides, as well as physiological substrates such as fatty acids and steroids, are attacked by molecular oxygen in the presence of NADPH, a reductase and liver microsomal cytochrome *P*-450 (*P*-450_{LM}) as the oxygenating catalyst. Although much has been learned about the hydroxylation reactions catalyzed by liver microsomes, characterization of the components of the enzyme system and elucidation of the reaction mechanism have been hampered by the instability of *P*-450_{LM}, which forms an altered, inactive hemoprotein (*P*-420) when removed from the membrane [1]. Several years ago, this laboratory [2, 3] reported the resolution of this enzyme system from rabbit and rat liver microsomes into three components which, when combined, reconstituted hydroxylation activity toward drugs, fatty acids and hydrocarbons [4–7]. These components were identified as a solubilized form of cytochrome *P*-450, a solubilized form of NADPH-cytochrome *P*-450 reductase, and phosphatidylcholine.

The present paper provides that partially purified *P*-450_{LM} [8, 9] accepts two electrons from dithionite

under anaerobic conditions. This unexpected finding suggests the presence of an electron acceptor distinct from the iron atom of the hemoprotein. It may be noted that the liver microsomal enzyme system apparently does not contain a non-heme iron protein, in contrast to the putidaredoxin-requiring, camphor-specific system of *Pseudomonas putida* [10, 11] or the adrenodoxin-requiring, steroid-specific system of adrenal cortical mitochondria [12–14]. The catalytically active *P*-450_{LM} preparation used in the present study was free of non-heme iron and contained other known electron acceptors (cytochrome *b*₅ and flavins) at levels much too low to account for the results obtained.

2. Materials and methods

P-450_{LM} from phenobarbital-induced rabbits was solubilized with cholate and partially purified by a procedure briefly described elsewhere [9, 15]. The fraction precipitating with polyethylene glycol 6000 between 10% and 13% (w/v) was resolubilized with cholate and then treated with ammonium sulfate. The fraction precipitating between 37% and 42% saturation was dissolved in 0.05 M Tris-chloride buffer, pH 7.4, containing 20% glycerol and 10⁻³ M EDTA, and dialyzed for 18 hr against 40 vol of the same buffer mixture. Cytochrome *P*-450 in the resulting clear solution was determined from the CO difference spectrum [16], cytochrome *b*₅ by enzymatic reduction with NADH as the electron donor [16] in the presence of detergent-solubilized NADH-cytochrome *b*₅ reductase [17], and total heme by the pyridine hemochrome method [16] with hemoglobin and myoglobin as standards. Total

* Presented in part at Symposium on Reaction Mechanisms of Cytochrome *P*-450 and Related Enzymes, International Union of Biochemistry, Stockholm, July (1973). This investigation was supported by Grant GB-30419X from the National Science Foundation and Grant AM-10339 from the United States Public Health Service.

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iron and copper were estimated by atomic absorption spectrophotometry and FMN and FAD by the procedure of King [18]. Dilauroylglyceryl-3-phosphorylcholine was synthesized by a published procedure [19] and sonicated before use. Highly purified cytochrome $P-450_{cam}$ was kindly provided by Dr. I.C. Gunsalus, and horse heart cytochrome c was obtained from Sigma.

The anaerobic titrations were carried out in an apparatus similar to that described earlier [20]. The gas phase was carbon monoxide in which the oxygen content was reduced to less than 0.5 ppm by passage through a train containing BASF catalyst at 100°C. Glucose, glucose oxidase and catalase were included in the enzyme reaction mixture to remove traces of dissolved oxygen and peroxide. A freshly prepared dithionite solution was standardized against tetraacetylriboflavin [20] before and after each experiment. The extinction coefficient (reduced minus oxidized) in the presence of carbon monoxide was taken

as $90 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for $P-450_{LM}$ at 450 nm and $P-450_{cam}$ at 447 nm. Aliquots of dithionite were added at intervals of about 10 min.

3. Results

The $P-450_{LM}$ preparation used in the present work had only low levels of cytochrome b_5 and flavins and contained no measurable non-heme iron, as indicated in table 1. The titration by dithionite was carried out in an atmosphere of CO under strictly anaerobic conditions, which are necessary because of the ready autoxidizability of $P-450_{LM}$. As shown in fig. 1, the Soret band at 416 nm disappeared with the formation of the expected peak at 450 nm; several isosbestic points are evident in the titration curves. The effect of the reduction on the α and β bands is shown in the figure in a 5-fold expanded scale. Following a brief lag period, apparently due to the reaction of dithionite with the

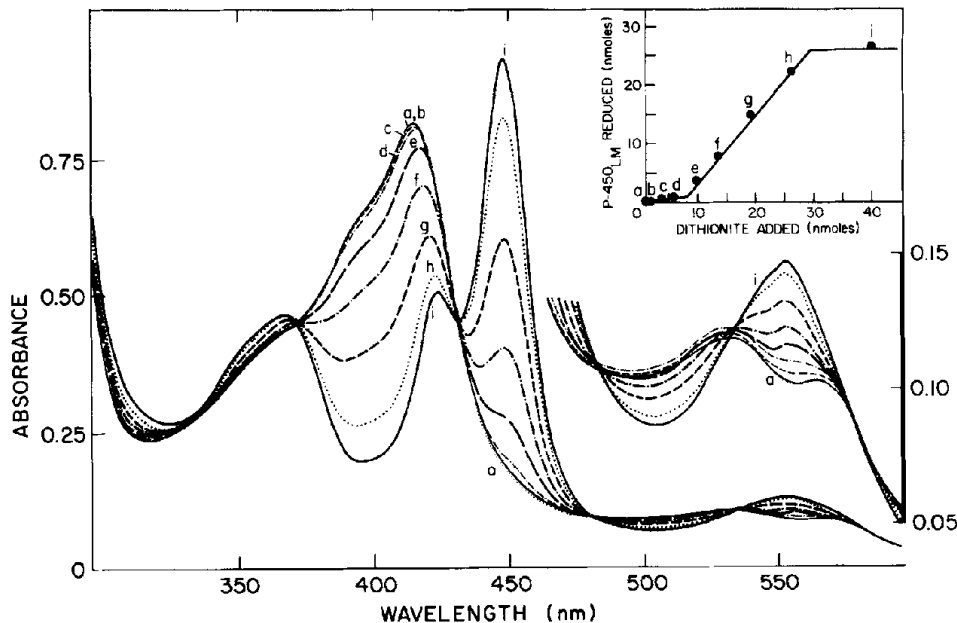


Fig. 1. Anaerobic titration of partially purified $P-450_{LM}$ at 25°C. The reaction mixture contained the $P-450$ preparation described in table 1 (24.8 nmoles; 4.1 mg of protein), potassium phosphate buffer, pH 7.4 (0.1 M), Tris-chloride buffer, pH 7.4 (1.0 mM), glycerol (26% v/v), EDTA (0.2 mM), benzphetamine (0.7 mM), phosphatidylcholine (0.1 mg), glucose (0.07 M), glucose oxidase (0.03 unit) and catalase (0.03 unit) in a final volume of 3.0 ml. A correction for the small volume of dithionite added (0.12 ml, total) was made in determining electron uptake but not in the spectra shown. Under the conditions employed the presence of benzphetamine (or hexobarbital) cause no significant shift of the Soret band.

Table 1
Analysis of partially purified $P-450_{LM}$ used for anaerobic titration.

Component	Concentration (nmoles per mg protein)
Cytochrome $P-450$	6.0
Cytochrome $P-420$	0
Cytochrome b_5	0.1
Heme	6.4
Iron	6.4
Copper	Trace
FMN	0.19
FAD	0.28

known traces of other acceptors present, cytochrome $P-450$ reduction was found to be proportional to the amount of dithionite added. The results indicate that 1.1 nmoles of dithionite were consumed per nmole of cytochrome $P-450$ converted to the reduced CO complex. Since dithionite is a two-electron donor, it is evident that slightly over two electrons were taken up for each molecule of $P-450_{LM}$ reduced. When the solution was then bubbled with oxygen, the original spectrum of oxidized $P-450_{LM}$ appeared, but no evidence was obtained at either 5°C or 25°C , in the presence of

hexobarbital or benzphetamine for the spectral changes attributed by Estabrook et al. [21] to an oxygenated complex.

Cytochrome $P-450_{cam}$ was then titrated under similar conditions with the results given in fig. 2. The disappearance of the oxidized heme-camphor complex at 391 nm was accompanied by the formation of the reduced CO complex at 447 nm, and the data obtained indicate that $P-450_{cam}$ is a one-electron acceptor. Peterson [22] has stated the same conclusion, but his published data show that two electrons are accepted per molecule of heme reduced by dithionite; Gunsalus et al. [23] have reported that only one electron is donated to $P-450_{cam}$ by NADH. The expected oxygenated complex [24, 25] was observed in our experiments when the reduced bacterial pigment was exposed to air.

The results of a series of such titrations are given in table 2. Whereas cytochrome c and cytochrome $P-450_{cam}$ accept a single electron from dithionite, the liver microsomal cytochrome $P-450$ preparation clearly requires two electrons for reduction. For optimal stability of the partially purified $P-450_{LM}$, both glycerol and phosphatidylcholine must be present, but these components have no effect on electron uptake by the hemoprotein.

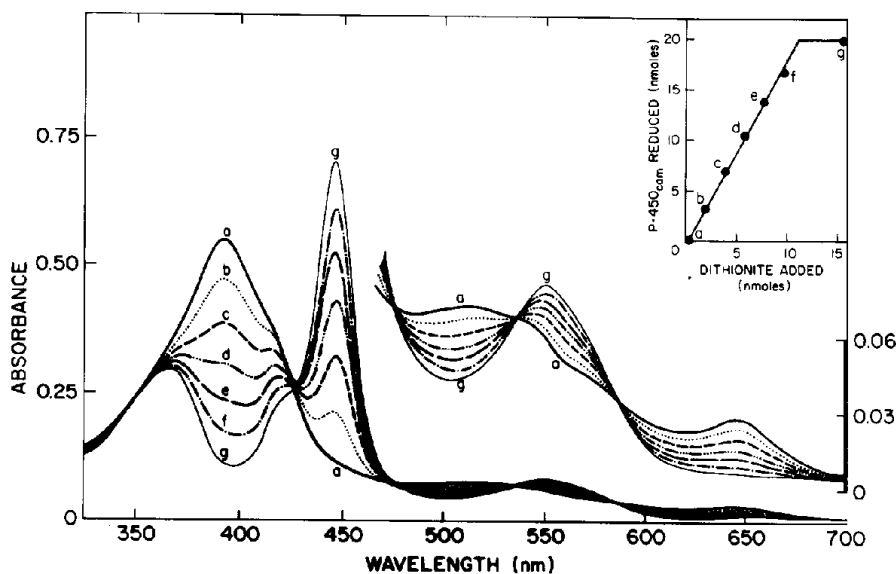


Fig. 2. Anaerobic titration of $P-450_{cam}$. The reaction mixture contained the bacterial $P-450$ (20.6 nmoles; 0.6 mg of protein), phosphate buffer, pH 7.0 (0.1 M), glycerol (30% v/v), glucose (0.07 M), glucose oxidase (0.03 unit) and catalase (0.03 unit) in a final volume of 3.1 ml. The solution was saturated with camphor. A correction was made for the small volume of dithionite added (0.06 ml, total) in determining electron uptake but not in the spectra shown.

Table 2

Electron uptake by various hemoproteins upon titration with dithionite under anaerobic conditions.

Hemoprotein titrated	Electrons consumed per molecule	
	Lag phase	Linear phase
Cytochrome <i>c</i>	0	1.0
<i>P</i> -450 _{cam}	≤ 0.1	1.1
<i>P</i> -450 _{cam} (phosphatidylcholine and glycerol present)	≤ 0.1	1.1
<i>P</i> -450 _{LM} (glycerol present)	0.5	≈ 2.0
<i>P</i> -450 _{LM} (phosphatidylcholine and glycerol present) ^a	0.5	2.0 ± 0.2

^a Similar results were obtained when hexobarbital was substituted for benzphetamine or when substrate was omitted.

4. Discussion

The detergent-solubilized, partially purified *P*-450_{LM} has an apparent mol.wt. of 300 000, as judged by gel exclusion chromatography on a column of Sepharose 6B; when treated with sodium dodecyl sulfate and mercaptoethanol and subjected to polyacrylamide gel electrophoresis, it appears to consist almost entirely of polypeptide chains in the 51 000–56 000 mol.wt. range [8]. Based on the known extinction coefficient of the reduced CO complex, it appears that our best preparations (having about 9 nmoles of *P*-450_{LM} per mg of protein) are about 50% pure, or, possibly, that the 300 000 mol.wt. aggregate may be a functional unit in which half of the polypeptide chains are hemoproteins and the other half serve some other function.

The results obtained clearly indicate the presence of an electron acceptor distinct from the iron atom of the hemoprotein and suggest that the two acceptors may have similar oxidation-reduction potentials. Whether the additional electron acceptor is a component of the cytochrome *P*-450 molecule or of a separate polypeptide chain and whether the second group titrated by dithionite is functional in catalytic hydroxylation are questions which remain to be answered. It may be noted that a readily reducible disulfide bond joining two molecules of cytochrome *P*-450 would account for the observed electron uptake.

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