SPECTRAL INTERMEDIATES IN THE REACTION OF OXYGEN WITH PURIFIED LIVER MICROSOMAL CYTOCHROME P-450

F. Peter Guengerich,† David P. Ballou, and Minor J. Coon

Department of Biological Chemistry
Medical School, The University of Michigan
Ann Arbor, Michigan 48109

Received March 25,1976

Summary: Stopped flow spectrophotometry has shown the occurrence of two distinct spectral intermediates in the reaction of oxygen with the reduced form of highly purified cytochrome P-450 from liver microsomes. As indicated by difference spectra, Complex I (with maxima at 430 and 450 nm) is rapidly formed and then decays to form Complex II (with a broad maximum at 440 nm), which resembles the intermediate seen in steady state experiments. In the reaction sequence, $P\text{-}450_{LM}^{red.} \xrightarrow{O_2}$ Complex I $\longrightarrow$ Complex II $\longrightarrow P\text{-}450_{LM}^{ox.}$, the last step is rate-limiting. The rate of that step is inadequate to account for the known turnover number of the enzyme in benzphetamine hydroxylation unless NADPH-cytochrome P-450 reductase or cytochrome b_5 is added. The latter protein does not appear to function as an electron carrier in this process.

In 1971 Estabrook *et al.* (1) found a new spectral intermediate in liver microsomal suspensions under steady state conditions which they attributed to an oxygenated form of reduced cytochrome P-450. They also observed changes in the extent of NADPH-dependent cytochrome b_5 reduction which indicated that this hemeprotein may serve as an electron donor to the oxygenated form of cytochrome P-450. Gunsalus *et al.* (2) and Ishimura *et al.* (3) also found such a spectral intermediate in the reaction of oxygen with the reduced form of highly purified cytochrome $P\text{-}450_{cam}$ from *Pseudomonas putida*. Rösen and Stier (4) provided further evidence from flash photolysis experiments for the occurrence of the oxyferro complex in liver microsomes and showed that O_2 and CO are bound competitively to the cytochrome.

The mixed function oxidase of liver microsomal membranes was resolved in this laboratory into three components[1] ($P\text{-}450_{LM}$, NADPH-cytochrome P-450 reductase, and phosphatidylcholine) (5-8), and the purification and characterization of the reductase (9) and of multiple forms of $P\text{-}450_{LM}$ (10-12) have been reported more recently. The present paper describes rapid reaction and steady state spectral studies which demonstrate the occurrence of two distinct intermediates in the reaction of $P\text{-}450_{LM}^{red.}$ with molecular oxygen. The cytochrome preparations used

†Postdoctoral Fellow, United States Public Health Service. Present address, Department of Biochemistry, Vanderbilt University, Nashville, Tennessee.

[1]The following abbreviations are used: $P\text{-}450_{LM}$, liver microsomal cytochrome P-450; $P\text{-}450_{LM}^{ox.}$ and $P\text{-}450_{LM}^{red.}$, oxidized and reduced forms of the cytochrome; and dilauroyl-GPC, dilauroylglyceryl-3-phosphorylcholine.

appeared to be homogeneous by gel electrophoresis and were free of other known microsomal electron carriers, thereby permitting a study of the effect of added cytochrome b_5 on the kinetics.

Materials and Methods: Reaction mixtures were made highly anaerobic by procedures reported previously (13,14), except that glucose, glucose oxidase, and catalase were omitted. Rapid reaction studies were carried out with a stopped flow spectrophotometer designed for anaerobic work and having a light path of 2 cm. A Nova minicomputer system similar to that described by Wampler and DeSa (15) was used for control of the stopped flow spectrophotometer and for data collection. Steady state spectra were recorded with an Aminco-Chance DW-2 spectrophotometer. P-450LM was purified from phenobarbital-induced rabbit liver microsomes as described earlier (10-12). This form of the cytochrome, which is designated by its electrophoretic mobility as P-450LM$_2$ (12), migrates as a single polypeptide band of molecular weight 50,000 when analyzed by SDS-polyacrylamide gel electrophoresis. Apparently due to loss of heme during purification, the holoenzyme content of the preparations varies. Those used in the present study had a content of about 12 nmol per mg of protein. NADPH-cytochrome P-450 reductase was purified from phenobarbital-induced rabbit liver microsomes by a modification of procedures reported previously (9,10) and had a specific activity of 20 μmol cytochrome c reduced per min per mg of protein. Benzphetamine and desmethylbenzphetamine were generously provided by Dr. P. W. McConnell of the Upjohn Co., and dilauroyl-GPC was obtained from Serdary Research Laboratories.

Steady State Experiments: The spectral changes observed when purified P-450LM was reduced enzymatically in the presence of an NADPH-generating system and then exposed to oxygen are shown in Fig. 1. The difference spectrum of the steady state oxyferro complex formed when the solution of P-450$_{LM}^{red.}$ was exposed to oxygen has maxima at about 442, 560, and 588 nm. The spectrum is clearly different from that of P-450$_{LM}^{red.}$ and resembles that described by Estabrook et al. (1) for microsomal suspensions with correction for the contribution by microsomal cytochrome b_5.

As shown in Table I, the components known to be required for substrate hydroxylation in the reconstituted system (10) are also necessary for formation of the oxyferro complex. Phospholipid and benzphetamine are presumably required for optimal formation of the oxygenated complex because of their ability to stimulate the rate of electron transfer to P-450LM (8,17), but an additional role of these components cannot be ruled out at this time. The results show that an oxygenated complex is formed with various substrates or with desmethylbenzphetamine present, and to a small but significant extent without added substrate or product.

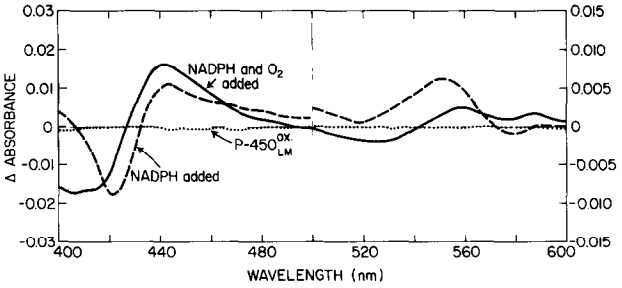

Fig. 1. Steady-state difference spectra of purified P-450LM upon reduction and oxygenation. A reaction mixture containing P-450LM$_2$ (1.3 μM; 0.11 mg protein per ml), NADPH-cytochrome P-450 reductase (25 μg protein per ml), dilauroyl-GPC (30 μM), benzphetamine (1.0 mM), potassium HEPES buffer (pH 7.7; 0.05 M), $MgCl_2$ (15 mM), glucose 6-phosphate (1.0 mM), and glucose 6-phosphate dehydrogenase (1.0 μg per ml) in a final volume of 2.5 ml was equilibrated with N_2 under anaerobic conditions. The temperature was 20°. The baseline was recorded against an identical reaction mixture in an Aminco DW-2 spectrophotometer. NADPH (50 μM) was added anaerobically to the sample, and the resulting reduced minus oxidized difference spectrum was recorded. The reaction mixture containing reduced P-450LM was then aerated, and the resulting reduced, oxygenated minus oxidized difference spectrum was recorded. The spectrum of the oxygenated complex as shown was constant for about 5 min at 20° and then gradually changed due to product formation from benzphetamine as observed by others with microsomes (16).

Table I

Requirements for formation of oxygenated P-450LM

System	$\Delta A_{442-412}$
Complete	0.031
No P-450LM	0
No NADPH-cytochrome P-450 reductase	0
No dilauroyl-GPC	0.013
No benzphetamine	0.005
" " ; desmethylbenzphetamine added	0.009
" " ; cyclohexane added	0.033
" " ; p-nitroanisole added	0.025[a]
" " ; hexobarbital added	0.034[a]

The complete reaction mixture was as in Fig. I. The magnitude of the reduced, oxygenated minus oxidized spectrum of P-450LM was determined under various conditions, as indicated. Benzphetamine was replaced by other compounds (in both cuvettes) at the following concentrations: desmethylbenzphetamine (1.0 mM), *p*-nitroanisole (0.25 mM), cyclohexane (10 mM), or hexobarbital (2.0 mM).

[a]In these experiments $\Delta A_{447-412}$ was determined because the spectral characteristics of the resulting oxyferro complex were slightly different.

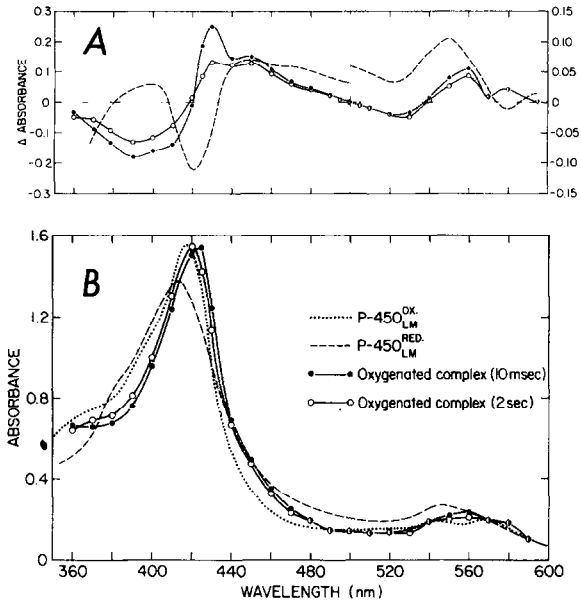

Fig. 2A. Difference spectra of complexes resulting from reaction of P-450$_{LM}^{red.}$ with oxygen as determined by stopped flow technique. One syringe contained P-450$_{LM}^{ox.}$ (13.5 μM; 1.0 mg protein per ml), dilauroyl-GPC (0.22 mM), benzphetamine (1.0 mM), Tris-acetate buffer (pH 7.5; 0.06 M), EDTA (10 mM), proflavin sulfate (0.7 μM), and methyviologen (0.2 μM) under anaerobic nitrogen; the mixture was photoreduced at 12°. The other syringe contained an aerobic solution of benzphetamine, Tris buffer, and EDTA at the same concentrations as in the first syringe. The reaction was initiated by mixing equal volumes (ca. 0.1 ml each) of the two reaction mixtures at 12°. Data were obtained from the kinetic traces at various wavelengths at the time intervals indicated and are presented as reduced minus oxidized and reduced, oxygenated minus oxidized difference spectra.

Fig. 2B. Absolute spectra of oxidized and reduced P-450LM and oxygenated complexes of the latter. The spectra of the oxygenated complexes were derived from the same experiments as in Fig. 2A. The spectra of the oxidized and reduced forms were determined with 13.5 μM P-450LM in a spectrophotometer having a 1-cm light path.

Rapid Reaction Studies: The reaction of P-450$_{LM}^{red.}$ with oxygen in the presence of benzphetamine, as studied by stopped flow techniques, showed biphasic kinetics. The kinetics were studied in detail as a function of wavelength, and evidence was obtained for two distinct intermediates, as indicated by the difference spectra in Fig. 2A. For comparison, the reduced difference spectrum is also included in the figure. The first oxygenated complex, with maxima at 430 and 450 nm, was formed in the dead time of the stopped flow equipment (2 msec) and was largely unchanged at 10 msec. At longer times a second

Table II

Rate constants for individual reactions:

$$P\text{-}450_{LM}^{red.} \xrightarrow{k_1} \text{Complex I} \xrightarrow{k_2} \text{Complex II} \xrightarrow{k_3} P\text{-}450_{LM}^{ox.}$$

Substrate	Rate constants (min^{-1})		
	k_1	k_2	k_3
None	$\geq$ 60,000	210	12
Benzphetamine	$\geq$ 60,000	270	3
Cyclohexane	$\geq$ 60,000	215	7
p-Nitroanisole	$\geq$ 60,000	205	27

complex was formed with a broad maximum centered at 440 nm. Since these reactions showed no dependence on the oxygen concentration over the range from 25 to 500 μM, it appears that the oxygenation of $P\text{-}450_{LM}^{red.}$ is complete in the dead time of the instrument. The spectrum of Complex II is similar to that seen in the steady state experiments, allowing for the differences in experimental conditions, particularly the absence of reductase in the rapid reaction experiments. The absolute spectra in the stopped flow studies are shown in Fig. 2B; the Soret maxima of $P\text{-}450_{LM}^{ox.}$, $P\text{-}450_{LM}^{red.}$, Complex I, and Complex II are at about 418, 414, 423, and 421 nm, respectively.

Rates of Reactions: The rate constants of the individual reactions are shown in Table II. The formation of Complex I is complete in the dead time of the instrument, as already indicated, whereas the conversion of Complex I to Complex II and the decomposition of Complex II, with the regeneration of $P\text{-}450_{LM}^{ox.}$, are considerably slower. The last step, which is rate-limiting in the overall reaction, involves the formation of hydrogen peroxide and also, when substrate is present, the formation of product (18). Other experiments showed that k_3 was increased to 90 min^{-1} when cytochrome b_5 was added at the same concentration as $P\text{-}450_{LM}$ prior to photoreduction. Similar experiments with NADPH-cytochrome P-450 reductase added in place of cytochrome b_5 were more difficult to interpret but provided indirect evidence that k_3 was increased to 23 min^{-1}. For comparison, the turnover number of the enzyme (moles of benzphetamine hydroxylated per mole of $P\text{-}450_{LM}$ per min) was also determined at 12° in the complete reconstituted system and found to be 24 min^{-1}. Cytochrome b_5 was not oxidized significantly in the time required for Complex II to form $P\text{-}450_{LM}^{ox.}$; thus, it appears that cytochrome b_5 may act as an effector, in addition to its well-known function as a microsomal electron carrier. Precedent for such a dual function for an elec-

tron carrier associated with cytochrome P-450 is provided by the studies of Lipscomb et al. (19) describing an effector role for putidaredoxin in the bacterial hydroxylation system.

Acknowledgements: This research was supported by Grants BMS71-01195 from the National Science Foundation and AM-10339 from the National Institutes of Health to M.J.C. and Grant GM-20877 from the National Institutes of Health to D.P.B.

REFERENCES

1. Estabrook, R. W., Hildebrandt, A. G., Baron, J., Netter, K. J., and Leibman, K. (1971) Biochem. Biophys. Res. Commun. 42, 132-139.
2. Gunsalus, I. C., Tyson, C. A., Tsai, R., and Lipscomb, J. D. (1971) Chem.-Biol. Interactions 4, 75-78.
3. Ishimura, Y., Ullrich, V., and Peterson, J. A. (1971) Biochem. Biophys. Res. Commun. 42, 140-146.
4. Rösen, P., and Stier, A. (1973) Biochem. Biophys. Res. Commun. 51, 603-611.
5. Lu, A. Y. H., and Coon, M. J. (1968) J. Biol. Chem. 243, 1331-1332.
6. Coon, M. J., and Lu, A. Y. H. (1969) in Gillette, J. R., et al., eds., Microsomes and Drug Oxidations, pp. 151-166, Academic Press, New York.
7. Lu, A. Y. H., Junk, K. W., and Coon, M. J. (1969) J. Biol. Chem. 244, 3714-3721.
8. Strobel, H. W., Lu, A. Y. H., Heidema, J., and Coon, M. J. (1970) J. Biol. Chem. 245, 4851-4854.
9. Vermilion, J. L., and Coon, M. J. (1974) Biochem. Biophys. Res. Commun. 60, 1315-1322.
10. van der Hoeven, T. A., and Coon, M. J. (1974) J. Biol. Chem. 249, 6302-6310.
11. van der Hoeven, T. A., Haugen, D. A., and Coon, M. J. (1974) Biochem. Biophys. Res. Commun. 60, 569-575.
12. Haugen, D. A., van der Hoeven, T. A., and Coon, M. J. (1975) J. Biol. Chem. 250, 3567-3570.
13. Ballou, D. P., Veeger, C., van der Hoeven, T. A., and Coon, M. J. (1974) FEBS Lett. 38, 337-340.
14. Guengerich, F. P., Ballou, D. P., and Coon, M. J. (1975) J. Biol. Chem. 250, 7405-7414.
15. Wampler, J. E., and DeSa, R. J. (1971) Appl. Spect. 25, 623-627.
16. Werringloer, J., and Estabrook, R. W. (1973) Life Sciences 13, 1319-1330.
17. Guengerich, F. P., and Coon, M. J. (1975) Fed. Proc. 34, 622.
18. Nordblom, G. D., and Coon, M. J. (1976) Fed. Proc. 35, 281.
19. Lipscomb, J. D., Sligar, S. G., Namtvedt, M. J., and Gunsalus, I. C. (1976) J. Biol. Chem. 251, 1116-1124.