Possible Higher Valence States of Cytochrome P-450 During Oxidative Reactions

Anver D. Rahimtula, Peter J. O'Brien, Eugene G. Hrycay, Julian A. Peterson and Ronald W. Estabrook

Department of Biochemistry
Memorial University of Newfoundland
St. Johns, Newfoundland, Canada
and
Department of Biochemistry
The University of Texas Health Science Center
Dallas, Texas

Received July 29,1974

The addition of the organic hydroperoxide, cumene hydroperoxide, to liver microsomes results in the appearance of a transient spectral change associated with cytochrome P-450. In addition, unique electron paramagnetic resonance signals are observed with liver microsomal cytochrome P-450 comparable to signals obtained when peroxides interact with metmyoglobin. It is suggested that higher valence states of cytochrome P-450 may function during the activation of oxygen for the hydroxylation of a variety of xenobiotics.

The mechanism of oxygen activation required for substrate hydroxylation reactions catalyzed by cytochrome P-450 has eluded definition. A cyclic reaction sequence describing the interaction of various substrates, electrons, and oxygen with cytochrome P-450 has been proposed (1-5) and the details for some of these individual steps elucidated. However, the chemical nature of the oxygenated form of reduced cytochrome P-450, as well as the subsequent steps associated with the insertion of one atom of molecular oxygen into the substrate molecule, remain unknown. Recently, Hrycay and O'Brien (6,7) have described a peroxidative function of cytochrome P-450 by demonstrating its ability to catalyze the oxidation of a variety of electron donors such as NADH, NADPH, and TMPD in the presence of organic hydroperoxides. Further Kadlubar et al (8) have described the ability of similar organic hydroperoxides to support the oxidative metabolism of a variety of N-methyl compounds as catalyzed by liver microsomes.

The present study was carried out to examine the influence of the organic hydroperoxide, cumene hydroperoxide (CHP), on the electron paramagnetic

resonance spectral properties of liver microsomal cytochrome P-450 and to relate these results to optical absorbance changes. The results obtained suggest the presence of oxygen complexes of cytochrome P-450, comparable to the proposed higher valence states for the heme iron of metmyoglobin (9-13), as intermediates in the molecular function of cytochrome P-450 during drug and other xenobiotic metabolic reactions.

METHODS

Microsomes were prepared by differential centrifugation of homogenates of livers of rabbits that had been pretreated with phenobarbital (PB) as described previously (14). The isolated microsomal fraction was diluted in 0.1 M potassium phosphate buffer (pH 7.4) as described in the figure legends and examined spectrophotometrically using an Aminco DW-2 dual wavelength/split beam scanning spectrophotometer or with a Varian E-4 EPR spectrometer with the sample cooled to liquid nitrogen or helium temperatures for measurement of changes in the electron paramagnetic resonance characteristics of cytochrome P-450. Cumene hydroperoxide was obtained from Matheson, Coleman and Bell and prepared by dilution with water to a concentration of 30 mM which was used as a working solution. NADH, NADPH, and metmyoglobin were purchased from the Sigma Chemical Company.

RESULTS

The addition of cumene hydroperoxide (CHP) to a suspension of liver microsomes prepared from phenobarbital-pretreated rabbits results in the appearance of a transient spectral species as illustrated in Figure 1. After addition of CHP a rather intense absorbance change at about 440 nm in the difference spectrum appears concomitant with a loss of absorbance at about 420 nm. Repetitive scanning of the sample shows a progressive loss of the absorbance at 440 nm as the CHP added is transformed to cumenol (15). After completion of the reaction a second addition of CHP restores the spectral change at 440 nm. These spectrophotometrically observable cycles can be repeated depending upon the concentration of CHP added. The use of high con-

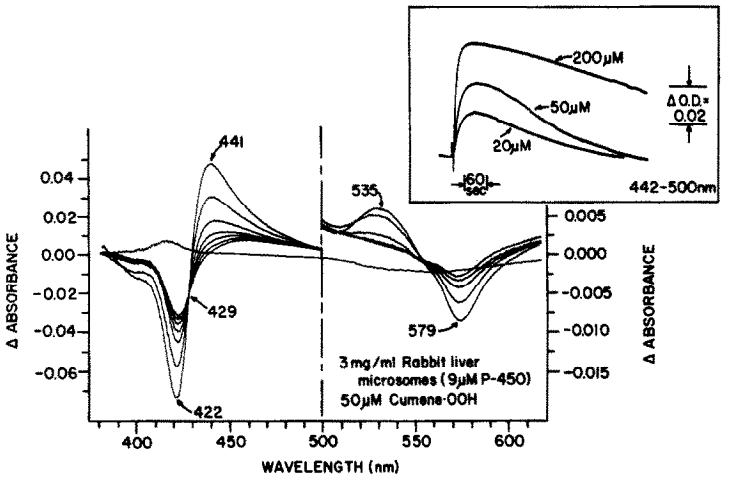

Figure 1: Repetitive scanning of the difference spectra obtained on the addition of CHP to a suspension of liver microsomes at 5°C. A 6 ml sample of microsomes (3 mg protein/ml: 9 μM cyt. P-450) was divided equally into two cuvettes. After recording the baseline of equal light absorption, 50 μM CHP (final conc.) was added to the sample cuvette and the change with time of the difference spectrum was determined by repetitive scanning at a speed of 2 nm/sec. The scans from 380 to 500 nm and from 500 nm to 620 nm were recorded in two separate experiments. Insert Kinetics of formation and decay of the peak at 442 nm at different CHP concentrations (20 μM, 50 μM, 200 μM).

centrations of CHP (greater than 1 mM) causes a partial destruction of cytochrome P-450 as measured by the progressive loss of the CO-reactive form of the reduced pigment. Comparable spectral changes are not observed when an equivalent concentration of H_2O_2 is added to a microsomal suspension.

Spectral measurements of the duration of this cyclic absorbance change observed after addition of CHP indicate that (a) there is a direct dependence on the concentration of CHP added to the suspension within the range of 5 μM to 0.5 mM final concentration as shown in the insert of Figure 1; (b) approximately 20 μM CHP is required to cause a half-maximal initial absorbance change at 440 nm; (c) the addition of NADH, NADPH, and/or substrates for cytochrome P-450 catalyzed oxidation reactions such as hexobarbital, aminopyrine, and cyclohexane decreases the $t_{1/2}$ for the duration of the cycle and decreases the magnitude of the initial absorbance change observed at 440 nm; (d) the absorbance change is not altered by anaerobiosis in the presence or absence of CO and (e) the $t_{1/2}$ for the reaction is increased

and the amount of cytochrome P-450 destroyed is decreased when the reaction is carried out at lower temperatures [5°C].

When samples of liver microsomes were examined by EPR spectroscopy, the presence of CHP results in a decrease in the magnitude of the low spin form of ferric cytochrome P-450 and the appearance of a unique EPR signal at

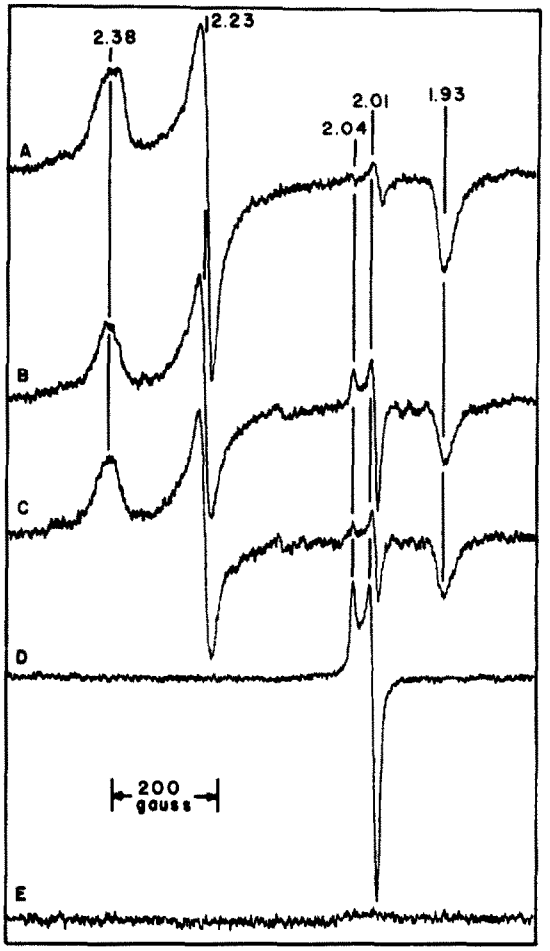

Figure 2: The EPR spectra were obtained with a Varian E4 EPR spectrometer equipped with a variable temperature accessory. The spectra were recorded with a PDP-11 minicomputer and the baseline (curve E) was subtracted from each spectrum. The appropriate instrument parameters were: gain, 3.2×10^3; modulation amplitude, 12.5 G; modulation frequency, 100 KHz; time constant, 0.3 sec; scan time, 4 min; microwave power, 50 mW; microwave frequency 9.138 GHz; temperature, - 170°C. Curves A to C all contained liver microsomes from phenobarbital pretreated rabbits (cytochrome P-450 50 μM). Curve A, no additions; Curve B, with CHP, 1.5 mM; Curve C, cyclohexane, 46 mM followed by CHP, 1.5 mM; Curve D, metmyoglobin, 50 μM with CHP, 1.5 mM (gain for this curve was 2.0×10^3). The samples were frozen 15 sec after addition of CHP to the reaction mixture.

about g = 2.0 (Figure 2). The presence of the EPR signal at about g = 2.0 is optimally observed shortly (about 15 seconds) after addition of CHP and at a ratio of CHP to cytochrome P-450 of approximately 20 to 1 or greater. The magnitude of the EPR signal at about g = 2.0 was depressed when NADH, NADPH, or a substrate of cytochrome P-450 (cyclohexane) was added simultaneously with CHP and the reaction quenched in liquid nitrogen 15 seconds later. Comparable changes in the EPR spectrum of the low spin form of oxidized cytochrome P-450 were not observed when H_2O_2 was added to a suspension of microsomes.

The unique nature of the EPR signal at about g = 2.0 observed when CHP reacts with microsomal cytochrome P-450 is similar to the signal observed when H_2O_2 is added to metmyoglobin or cytochrome c peroxidase (12,13). It was suggested that this EPR signal is associated with higher valence states of the heme iron, such as the ferryl (+4) state. Addition of CHP or H_2O_2 to metmyoglobin results in the formation of an EPR spectrum (Figure 2) which is very similar to that observed with liver microsomal cytochrome P-450. However, there exists a marked difference in the temperature dependence of these EPR signals since the one observed at about g = 2.0 with microsomal cytochrome P-450 is significantly altered at the temperature of liquid helium while the shape of the signal observed with metmyoglobin is essentially unaffected. The possibility that the g = 2.0 signals observed with microsomal cytochrome P-450 might arise from a non-specific reaction of the organic hydroperoxide with denatured cytochrome P-450 (i.e. P-420) was excluded when it was observed that P-420 failed to form a comparable complex as detected by EPR spectroscopy.

DISCUSSION

Yamazaki et al (16) have proposed a general scheme whereby five redox states exist in the oxidative function of hemoproteins. This scheme was developed principally to describe results obtained with peroxidase and catalase, although it does include a consideration of the earlier experimental

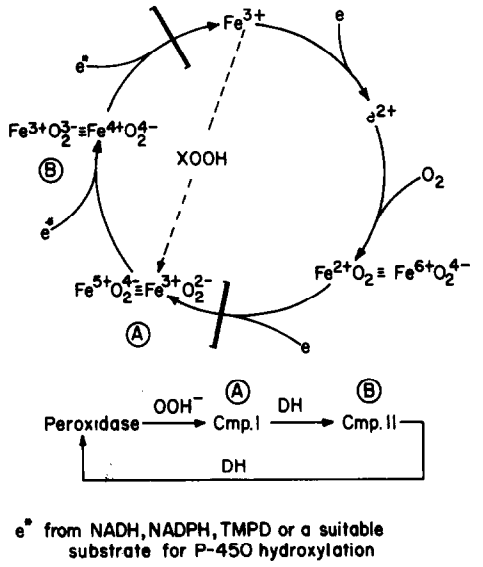

Figure 3: Proposed scheme for the cyclic reduction and oxidation transitions of cytochrome P-450 during hydroxylation reactions indicating the proposed site of interaction of organic hydroperoxides and the higher valence states of heme iron. The relationship to the mechanism of peroxidase function is indicated.

---

observations by George and Irvine (9,10,11) who studied the interaction of various peroxides with metmyoglobin. This general mechanism is expanded here to include possible intermediate steps in the function of cytochrome P-450 as shown in Figure 3 (for clarity this scheme has been simplified by not including a consideration of the role of the substrate complex of cytochrome P-450, although it is recognized that this is a necessary extension of the proposed mechanism).

The ternary complex of reduced cytochrome P-450 with substrate and oxygen is equivalent to Compound III of peroxidase as proposed earlier by Ullrich and Staudinger (17). Various valence states for the heme iron and oxygen in this complex can be written ranging from $Fe^{+2} \cdot O_2$ to $Fe^{+6} \cdot O_2^{4-}$ depending on the distribution of electrons. The one electron reduction of this complex would lead to an iron atom which can be considered to be in the perferryl or 5+ state comparable to Compound I of peroxidase (16). Again the pattern of electron distribution between the iron atom, substrate, and the oxygen can result in different

formal representations of this complex as discussed by Ullrich and Staudinger (17) when describing the formation of a proposed "oxene complex" of cytochrome P-450. Of greatest interest is the ability to observe the presence of a free radical type signal with cytochrome P-450 after addition of cumene hydroperoxide comparable to that observed when H_2O_2 or cumene hydroperoxide is added to metmyoglobin (Figure 2). Yonetani and Schleyer (13) and King and Winfield (12) have described the EPR spectra of transient free radicals formed during the reaction of metmyoglobin and cytochrome c peroxidase with H_2O_2. These authors conclude that this free radical species is associated with the ferryl (4+) form of the iron and may be equivalent to the perferryl (5+) heme of Compound I (Complex ES) of peroxidase, i.e. it has undergone a 2-electron equivalent oxidation. The mechanism described in Figure 3 suggests that with cytochrome P-450, the substrate to be hydroxylated or other sources of reducing equivalents derived from NADH, NADPH, or TMPD can be utilized to discharge this proposed higher valence state intermediate of cytochrome P-450 in a manner analogous to that proposed for peroxidase catalyzed reactions. It must be emphasized that the reaction of cytochrome P-450 described here does not occur with H_2O_2 but only with organic hydroperoxides. The presence of possible higher valence states of cytochrome P-450 has been proposed by a number of authors (1-7,18) although no experimental evidence has been presented to support any of these hypotheses. The present study describing a unique optical spectral species, as well as the existence of EPR detectable free radical signals upon the addition of CHP to microsomal cytochrome P-450, lends credence to the possible existence of such intermediates and emphasizes the similarity of the mechanism of function of cytochrome P-450 to that proposed for some peroxidase catalyzed reactions.

Acknowledgements: These studies have been supported in part by USPHS grants GM-19036 and GM-16488, a Robert A. Welch Research Foundation Grant (I-405), and a grant from the National Research Council of Canada (A4918).

REFERENCES

1. Estabrook, R.W., Hildebrandt, A., Remmer, H., Schenkman, J.B., Rosenthal. O., and Cooper, D.Y. (1968) Colloquium der Gesselschaft fur Biologische Chemie, Vol. 19, pp. 142-177 (B. Hess and Hj. Staudinger, editors) Springer Verlag, New York.

2. Peterson, J.A., Ishimura, Y., Baron, J. and Estabrook, R.W. (1973) Oxidases and Related Redox Systems, Vol. 2, pp. 565-581 (T.E. King, H.S. Mason, and M. Morrison, editors) Univ. Park Press, Baltimore.

3. Gunsalus, I.C., Tyson, C.A. and Lipscomb, J.D. (1973) Oxidases and Related Redox Systems, Vol. 2, pp. 583-603 (T.E. King, H.S. Mason, and M. Morrison, editors) Univ. Park Press, Baltimore.

4. Schleyer, H., Cooper, D.Y. and Rosenthal, O., Oxidases and Related Redox Systems, Vol. 2, pp. 469-492 (T.E. King, H.S. Mason, and M. Morrison, editors) Univ. Park Press, Baltimore.

5. Coon, M.J., Autor, A.P., Boyer, R.F., Lode, E.T. and Strobel, H.W. (1973) Oxidases and Related Redox Systems, Vol. 2 pp. 529-553 (T.E. King, H.S. Mason, and M. Morrison, editors) Univ. Park Press, Baltimore.

6. Hrycay, E.G. and O'Brien, P.J. (1972) Arch. Biochem. Biophys. 153, 480-494.

7. Hrycay, E.G. and O'Brien, P.J. (1974) Arch. Biochem. Biophys. 160, 230-245.

8. Kadlubar, F.F., Morton, K.C. and Ziegler, D.M. (1973) Biochem. Biophys. Res. Comm. 54, 1255-1261.

9. George, P. and Irvine, D.H. (1952) Biochem. J. 52, 511-517.

10. George, P. and Irvine, D.H. (1953) Biochem. J. 55, 230-236.

11. George, P. and Irvine, D.H. (1955) Biochem. J. 60, 596-604.

12. King, N.K. and Winfield, M.P. (1963) J. Biol. Chem. 238, 1520-1528.

13. Yonetani, T. and Schleyer, H. (1967) J. Biol. Chem. 242, 1974-1979.

14. Remmer, H., Greim, H., Schenkman, J. and Estabrook, R.W. (1967) Methods in Enzymology, Vol. X, pp. 703-708 (R. W. Estabrook and M. Pullman, editors) Academic Press, New York.

15. Hrycay, E.G., O'Brien, P.J., Van Lier, J.E. and Kan, G. (1972) Arch. Biochem. Biophys. 153, 495-501.

16. Yamazaki, I., Nakajima, R., Miyoshi, K., Makino, R. and Tamura, M. (1973) Oxidases and Related Redox Systems, Vol. 1, pp. 407-418. (T.E. King, H.S. Mason, and M. Morrison, editors) Univ. Park Press, Baltimore.

17. Ullrich, V. and Staudinger, Hj. (1966) Biological and Chemical Aspects of Oxygenases, pp. 235-246 (. Bloch and O. Hayaishi, editors) Maruzen Company Ltd. Tokyo.

18. Sih, C.J. (1969) Science 163, 1297-1300.