OXIDATION-REDUCTION MECHANISMS OF CYTOCHROME P-450

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Received June 3, 1971

SUMMARY: The reduction of oxidized P-450 by dithionite gives a hyperbolic curve on semilog paper as does the formation of the CO complex of reduced P-450. Reduction of the oxidized P-450-phenylisocyanide complex is pseudo first order. The NADPH dependent oxygen consumption of microsomes is inhibited by cyanide and is correlated with the extent of modification in the ESR spectrum of cytochrome P-450. These results suggest that the reduction of the oxidized P-450 is a two-step reaction via an intermediate state in which the oxygen consumption by microsomes in the presence of NADPH is mainly due to the oxidase action of P-450. We therefore propose a bifunctional feature of the P-450 cytochrome, hydroxylase and oxidase activities.

According to Mason's classification of oxidases (1), the liver microsomal b type cytochrome, P-450, is a hydroxylase of the external mixed function oxidase class having an NADPH dependent electron transfer system as electron donor. The physicochemical properties and the physiological function of P-450 have been extensively studied. In 1968, Miyake, Gaylor, and Mason (2) showed that microsomal Fe_X is a manifestation of P-450 and established that P-450 is a low spin protoheme protein in its oxidized state and probably a high spin ferrohemoprotein in its reduced state. Changes in the spin state accompanied by oxidation-reduction of P-450 seems unusual in that electron transferring hemoproteins, such as cytochrome b_5 (3) and cytochrome c (4), are low spin in both the oxidized and reduced states, while hemoproteins which react with oxygen or hydrogen peroxide, such as hemoglobin (5) and horse raddish peroxidase (6), are high spin in both redox states. Therefore, it is of interest to study the changes in spin state of P-450 during the oxidation-reduction process.

Besides hydroxylase reactions in microsomes, it is well known that microsomes consume oxygen upon the addition of NADPH even in the absence of substrates which are hydroxylated by P-450. Staudinger et al. (7) showed that there are two different terminal oxidases having different affinities

for oxygen in the microsomal NADPH dependent oxidase system and suggested that one of them is P-450. Gigon, Gram, and Gillet (8) demonstrated that reduction of P-450 in microsomes by NADPH was biphasic, indicating the presence of two different types of P-450 in microsomes, and also presented a reaction mechanism in which hydrogen peroxide is the product of the reaction of reduced P-450 with oxygen. A similar biphasic reduction of P-450 by NADPH was also reported by Strobel et al. (9). However, the details of the oxidase reaction and its physiological role involving P-450 are still unclear.

The present communication concerns the NADPH dependent oxidase reaction in microsomes and the mechanism of oxidation-reduction of P-450 in this oxidase system.

MATERIALS AND METHODS: Microsomes were prepared from phenobarbital induced rabbit liver by the procedure of Mason et al. (10). Submicrosomal particles containing P-450 were prepared by the procedure of Miyake et al. (2). Phenylisocyanide was synthesized by the method of Prager et al. (11). P-450 was determined according to the method of Omura and Sato (12), or from absorbancy of the absolute spectrum of oxidized P-450 at 415 mμ (2).

Reduction of P-450 and its phenylisocyanide complex by dithionite were followed by stopped flow techniques using a Hitachi rapid scan spectrophotometer RSP-2. P-450 preparations were dissolved in 0.1 M sodium phosphate buffer, pH 7.5, containing 10 % glycerol, and dithionite (20 mg/ml) was also dissolved in the same buffer. The solutions were mixed by air pressure to initiate the reaction using a Hitachi RSP-2 rapid flow generator. Both solutions were also bubbled with CO gas sufficient for measurement of formation of the CO complex of reduced P-450.

Oxygen consumption of microsomes by NADPH was measured by a polarographic method, and ESR spectra were taken using a Varian ESR spectrometer V-4500 equipped with 100 kc field modulation and a variable temperature accessory.

RESULTS: The reduction of oxidized P-450 was measured from the decrease in absorbancy of oxidized P-450 at 415 mμ. As shown in Fig. 1, logarithmic plots of the decrease in absorbancy against time (curve A) were hyperbolic. Accordingly, the result indicates that P-450 reduction by dithionite is not a single unimolecular first order type reaction. This same type of process was also observed for the formation of the CO complex of reduced P-450 by reduction of oxidized P-450 with dithionite in the presence of CO. The increase in absorbancy of the CO complex at 450 mμ was not linear in logarithmic plots of the changes in absorbancy against time (curve B in Fig. 1). These observations suggest that at least two reactions are involved in these reactions. The validity of these results was confirmed from the following experiments. The reduction of 2,6-dichlorophenol indophenol by ascorbate in the presence of 10 % glycerol gave a straight line in logarithmic plots of

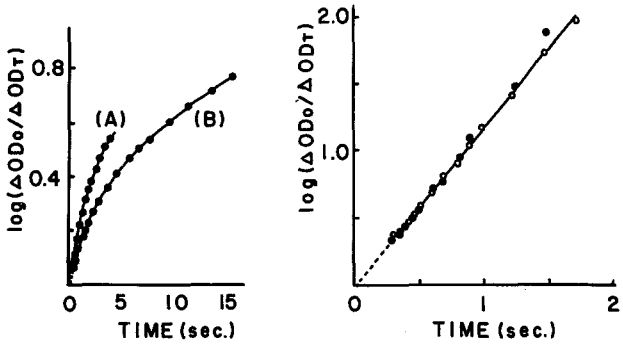

Fig. 1 (A) Reduction of oxidized P-450 by dithionite. ΔOD_0 represents difference in absorbancy between fully oxidized and fully reduced P-450, and ΔOD_T represents difference in absorbancy between the reaction mixture in the reduction process and fully reduced P-450. The changes in absorbancy at 415 mμ were measured. (B) Reduction of oxidized P-450 by dithionite in the presence of CO. ΔOD_0 represents difference in absorbancy between fully oxidized P-450 and the CO complex of fully reduced P-450, and ΔOD_T represents difference in absorbancy between the CO complex of fully reduced P-450 and the solution during the reaction. The changes in absorbancy at 450 mμ were measured.

Fig. 2 Reduction of the phenylisocyanide complex of oxidized P-450 by dithionite. ΔOD_0 represents difference in absorbancy between the complex of fully oxidized and reduced P-450, and ΔOD_T represents difference in absorbancy between the complex of fully reduced P-450 and the solution in the reduction process. The changes in absorbancy at 430 mμ (solid circles) and 455 mμ (open circles) were measured. The phenylisocyanide complex of oxidized P-450 was prepared as described previously (14).

the changes in absorbancy at 600 mμ against time, though the rate of the reaction was slower than that in the absence of glycerol. Under the same conditions employed for the reduction of P-450, phenosafranine was reduced completely within 200 msec. Therefore, oxygen in the reaction mixture is exhausted within the same order of time.

Formation of the phenylisocyanide complex of reduced P-450 by the reduction of the oxidized P-450 complex with dithionite was measured from the changes in absorbancy at 430 mμ and at 455 mμ. As shown in Fig. 2, logarithmic plots of the changes in absorbancy against time at respective wavelengths showed straight lines.

Since it has been demonstrated that P-450 forms a complex at relatively high concentrations of cyanide (13, 14, 15), the effect of cyanide on the NADPH dependent oxygen consumption of microsomes and on the ESR signal of P-450 were investigated. As seen in Fig. 3, oxygen consumption was inhibited at a relatively high concentration of cyanide, and the decrease in the rate

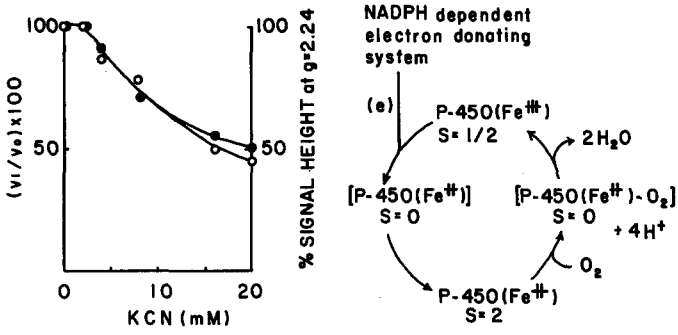

Fig. 3 The effect of cyanide on the oxygen consumption of microsomes reduced by NADPH and on the ESR signal of P-450. For oxygaphy, microsomes (8 mg protein) were suspended in 0.1 M phosphate buffer, pH 7.5. Total volume of the reaction mixture was 2.5 ml. The reaction was initiated by the addition of 3 μmoles of NADPH, and the reaction was carried out at 25°. vo and vi represent the rate of oxygen consumption in the absence and in the presence of KCN, respectively. For ESR measurements, 0.3 ml of the solution of microsomes (55 mg P/ml) in 0.1 M phosphate buffer, pH 7.5, was used. Modulation amplitude, 15 gauss; microwave power, 10 db; microwave frequency, 9.15 GHz; magnetic field measured, 2500-3500 gauss; temperature, -160°. KCN was added to the P-450 solution after dissolving it in 0.1 M phosphate buffer, pH 7.5, and adjusted the pH to 7.5 with HCl. Open circles, oxygen consumption; Solid circles, ESR signal of native P-450 at g=2.24

Fig. 4 Proposed reaction mechanism of the NADPH dependent oxidase action of P-450.

of the oxygen consumption by cyanide corresponded with the decrease in the ESR signal of native P-450 accompanied by the concomitant formation of the ESR signal of the cyanide complex.

In the present case, catalase did not affect the oxygen consumption of rabbit liver microsomes by NADPH.

DISCUSSION: If it is assumed that there are two different species of P-450 in P-450 preparations, the curves in Fig. 1 seem to show that one is reduced roughly 10 times faster than the other from the analyses of the slopes. However, it is unlikely that there exists two species of P-450 having such a large difference in reactivity for dithionite. Furthermore, the plots in Fig. 1 did not show typical straight lines with different slopes, but rather hyperbolic curves. These observations suggest that the reduction of P-450 by dithionite involves two successive reactions, and that the intermediate form might be a low spin reduced P-450, though the spectrum of such an intermediate could not be detected. The rate of reaction of reduced P-450 with CO was so rapid that the curve B in Fig. 1 must reflect the reduction of P-450 involved in the reaction. In contrast to the reduction of P-450, formation of the phenylisocyanide complex of reduced P-450 seems to be a single step reaction, in which the complex of oxidized P-450 is directly reduced to the complex of reduced P-450. Therefore, the reduction of the phenylisocyanide complex of oxidized P-450 may be similar to that observed for the reduction of low spin electron transferring hemoproteins. It is still unclear, however, whether the complex of oxidized P-450 directly forms two species of the complex of reduced P-450, the 428 mμ and 455 mμ components (14), or whether rapid equilibrium is attained between these two components after the phenylisocyanide complex of oxidized P-450 is reduced.

With respect to oxygen consumption of rabbit liver microsomes reduced by NADPH, reactions such as (a) NADPH dependent oxidase action of P-450, (b) oxygen consumption via a cyanide sensitive factor, and (c) lipid peroxidation of microsomes must be considered. Among these possible reactions, hydroxylase

reactions which are dependent on a cyanide sensitive factor (16, 17) are inhibited by a few millimolar of cyanide. However, both the rate of oxygen consumption of microsomes reduced by NADPH and the ESR spectrum of native P-450 were not influenced by such a low concentration of cyanide (Fig. 3). In addition, oxygen consumption of microsomes reduced by NADH was only about one tenth of that in the presence of NADPH. Lipid peroxidation of rabbit liver microsomes was very slight even in the presence of NADPH, as previously reported by Gram and Fouts (18). Therefore, the oxygen consumption by NADPH may be mainly due to NADPH dependent oxidase action of P-450, and the oxidase reaction may involve the transfer four reducing equivalents to oxygen producing $2H_2O$, as indicated from the fact that catalase does not affect the oxygen consumption. From these results, the mechanism of reaction of the NADPH dependent oxidase action of P-450 is proposed in Fig. 4. The proposed mechanism differs from that of hydroxylase reactions of P-450, especially in the spin states of the reduction step. In the present case, low spin oxidized P-450 is reduced to low spin reduced P-450. It has been proposed that in hydroxylase reactions low spin oxidized P-450 becomes high spin upon complex formation with substrate, and is then reduced to high spin reduced P-450 (19-21). Elucidation of the significance of the differences in the reaction mechanism described above and the physiological role of the oxidase action of P-450, however, await further investigation.

REFERENCES

1. H. S. Mason, Ann. Rev. Biochem., 34, 595 (1965).
2. Y. Miyake, J. L. Gaylor, and H. S. Mason, J. Biol. Chem., 243, 5788 (1968).
3. R. Bois-poltoratsky, and A. Ehrenberg, European J. Biochem., 2, 361 (1967).
4. H. Theorell, J. Am. Chem. Soc., 63, 1820 (1941).
5. C. D. Coryell, F. Stitt, and L. Pauling, J. Am. Chem. Soc., 59, 633 (1937).
6. H. Theorell, Ark. Kemi. Min. Geol., 16A, No 3 (1942).
7. Hj. Staudinger, B. Kerekjarto, V. Ullrich, and Z. Zubrzycki, Oxidases and Related Redox Systems, eds. T. E. King, H. S. Mason, and M. Morrison, John Wiley and Sons, Inc., New York, 1965, p.815.
8. P.L. Gigon, T. E. Gram, and J. R. Gillete, Mol. Pharmacol., 5, 109 (1969).
9. H. W. Strobel, A. Y. H. Lu, J. Heidema, and M. J. Coon, J. Biol. Chem., 245, 4851 (1970).
10. H. S. Mason, T. Yamano, J. C. North, Y. Hashimoto, and P. Sakagishi, Oxidases and Related Redox Systems, eds. T. E. King, H. S. Mason, and M. Morrison, John Wiley and Sons, Inc., New York, 1965,p.879.

11. B. Prager, P. Jacobson, P. Schmidt, and D. Stern, Beilsteins Handbuch der Organischen Chemie, Band XII, p. 191, Julius Springer, Berlin, 1929.
12. T. Omura, and R. Sato, J. Biol. Chem, 239, 2370 (1964).
13. H. Nishibayashi, and R. Sato, Seikagaku, 37, 630 (1965).
14. Y. Miyake, K. Mori, and T. Yamano, Arch. Biochem. Biophys., 133, 318 (1969).
15. C. R. E. Jefcoate, and J. L. Gaylor, Biochemistry, 9, 3816 (1970).
16. N. Oshino, and R. Sato, 7th Intern. Congr. Biochem. (Tokyo), p.725, (1967).
17. J. L. Gaylor, and H. S. Mason, J. Biol. Chem., 243, 4966 (1968).
18. T. E. Gram, and J. R. Fouts, Arch. Biochem. Biophys., 114, 331 (1966).
19. A. Hildebrandt, H. Remmer, and R. W. Estabrook, Biochem. Biophys. Res. Communs., 30, 607 (1968).
20. R. Tsai, C. A. Yu, I. C. Gunsalus, J. Peisach, W. Blumberg, W. H. Orme-Johnson, and H. Beinert, Proc. Nat. Acad. Sci. USA, 66, 1157 (1970).
21. J. A. Whysner, J. Ramseyer, and B. W. Harding, J. Biol. Chem., 245, 5441 (1970).