CYTOCHROME P-450 OF LIVER MICROSOMES--ONE PIGMENT OR MANY*

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The hypothesis that liver microsomes contain two or more different hemoproteins functional in hydroxylation reactions has been proposed to explain the variability of enzymatic activities (see Conney, 1967) observed with different substrates of the mixed function oxidase reaction using microsomes from animals treated with various inducing agents. This hypothesis gains support from experiments showing two spectral species detectable when reduced cytochrome P-450 interacts with ethylisocyanide (Imai and Sato, 1966; Sladek and Mannering, 1966) or with carbon monoxide (Alvares, Schilling, Levin, and Kuntzmann, 1967). On the basis of the pH dependency of the ethylisocyanide reaction with reduced cytochrome P-450, Sladek and Mannering (1966) concluded that a new hemoprotein was formed which was interconvertible with the normally present pigment. In addition Schenkman, Remmer, and Estabrook (1967) and Imai and Sato (1967) have shown two types of spectral changes which occur when various substrates of mixed function oxidation are added to liver microsomes.

These studies pose the question whether a single cytochrome P-450 exists in two or more spectrally distinguishable forms, or whether two or more

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hemoproteins of the type of cytochrome P-450 are present in microsomes, which would be induced by various agents and could be specific for substrates metabolized via hydroxylation reactions. The present paper reports the absorption spectral properties of two forms of cytochrome P-450 in rabbit liver microsomes inducible preferentially either by phenobarbital (PB) or 3-methylcholanthrene (3-MC).

Methods

Liver microsomes were prepared from male albino rabbits (1.3 to 1.9 kg) by homogenization of the dissected liver in 0.25 M sucrose and differential centrifugation as previously described (Schenkman, Remmer, and Estabrook, 1967). The microsomal pellet was suspended in 150 mM KCl and resedimented to remove traces of hemoglobin. The washed microsomes were then suspended in a buffer mixture containing 50 mM tris chloride, pH 7.4, and 0.25 M sucrose. Four groups of animals were employed for these studies: (a) Phenobarbital treated animals. Phenobarbital solution (40 mg per ml dissolved in 150 mM NaCl) was injected intraperitonally daily at a concentration of 50 mg per kg body weight for seven days. The animals were sacrificed 25 hrs after the last injection of phenobarbital. (b) Saline controls. A volume of 150 mM NaCl comparable to that injected in group (a) was injected following the same time course as for group (a). (c) 3-MC treated animals. 3-methylcholanthrene suspension (12 mg per ml of Mazola corn oil) was injected intraperotinally at a concentration of 12 mg per kg body weight at 12 hr, 24 hr, and 36 hrs. The rabbits were sacrificed 40 hrs after the last injection of 3-MC. (d) Corn oil controls. A volume of Mazola corn oil comparable to that injected in group (c) was injected following the same time course as for group (c).

Spectral changes were measured with an Aminco-Chance wavelength scanning spectrophotometer as indicated in the figure legend. The concentration of cytochrome b_5 and protoheme was determined as the pyridine hemochromogen as described by Omura and Sato (1964). Recently Kinoshita and Horie (1967) have described a variety of ways of obtaining spectrophotometrically the absolute

absorption spectrum of oxidized and reduced cytochrome P-450. Using the principle of balancing the contribution of cytochrome b_5 to the absorption spectrum, by recording the spectral difference of pigments associated with microsomes from induced animals minus those from control animals, it is possible to determine the spectral properties of those pigments induced in liver microsomes from PB or 3-MC treated animals.

Results

As illustrated in Figure 1, phenobarbital treatment of rabbits results in

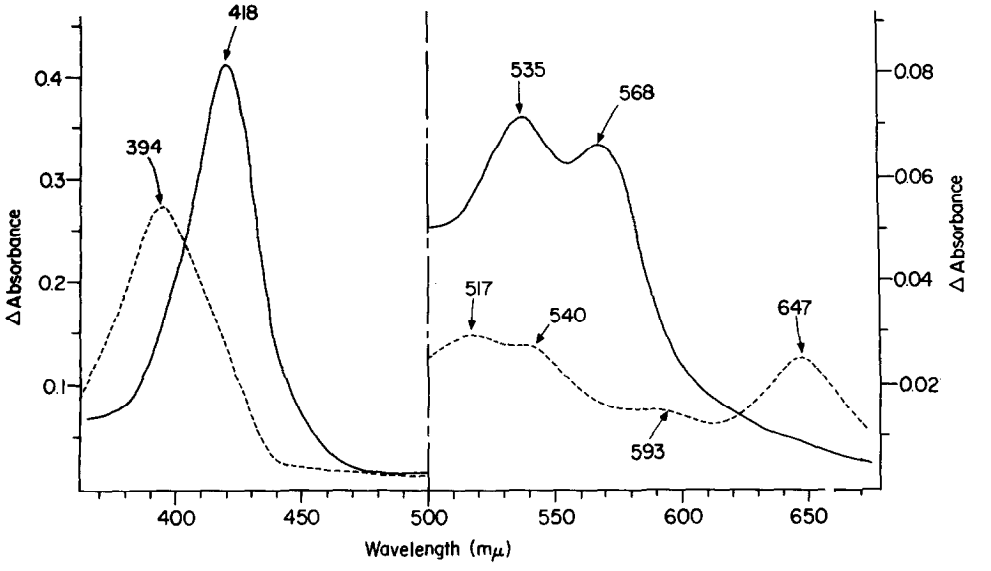

Figure 1. Comparison of the absorption spectrum of the oxidized hemoprotein of liver microsomes induced by treatment of rabbits with 3-methylcholanthrene or phenobarbital. Microsomes from livers of control, PB or 3-MC treated rabbits were examined spectrophotometrically to determine the content of cytochrome b_5 using the procedure described by Omura and Sato (1964). The spectrum of the additional pigment associated with microsomes from PB treated rabbits (solid line curve) was determined by recording the spectral difference between a cuvette containing liver microsomes from PB treated rabbits (2.2 mg protein per ml, 0.73 mμ moles cytochrome b_5 per mg, 2.92 mμ moles hemin per mg) minus a cuvette containing liver microsomes from a saline control rabbit (3.0 mg protein per ml, 0.51 mμ moles cytochrome b_5 per mg, 1.44 mμ moles hemin per mg). The additional pigment associated with microsomes from 3-MC treated rabbits (dashed line curve) was determined in a similar manner by recording the spectral difference between a cuvette containing liver microsomes from 3-MC treated rabbits (3.0 mg protein per ml, 0.79 mμ moles cytochrome b_5 per mg, 2.5 mμ moles hemin per mg) minus a cuvette containing liver microsomes from a corn oil control treated rabbit (3.0 mg protein per ml, 0.78 mμ moles cytochrome b_5 per mg, 1.6 mμ moles hemin per mg). Microsomes were diluted in 50 mM tris buffer, pH 7.5 containing 15 mM KCl to the protein concentrations indicated. Temperature, 25°.

the formation of a pigment with absorption maxima at 568, 534, and 418 mμ. In contrast, treatment of rabbits with 3-MC results in a formation of a pigment with absorption maxima at 647, 540, 517, and 394 mμ. These results are consistent with those reported by Sladeck and Mannering (1966) and by Alvares et al. (1967). Similar spectral studies of the reduced MC or PB induced pigments were carried out: absorption maxima at 553 and 423 mμ are observed with microsomes from phenobarbital treated animals, while a broad absorption maximum at 552 mμ associated with a Soret absorption band at 409 mμ was observed with microsomes from 3-MC treated animals. Determination of the spectral properties of the CO complex of the reduced hemoproteins revealed slight differences in the location of the absorption maxima. A maximum for the absorption band of the pigment associated with microsomes from 3-MC induced animals was at about 446 mμ, while that for the PB treated animals was about 450 mμ.

When the balance of heme, as determined by the pyridine-hemochromagen method, was evaluated, it was established that after induction with 3-MC the spectral form of the reduced hemoprotein CO complex at 446 mμ had an extinction coefficient of about 220 mM^{-1} cm^{-1}. The extinction coefficient of the CO compound of the reduced hemoprotein of microsomes from PB treated animals had an extinction coefficient of about 50 mM^{-1} cm^{-1}. The previously reported extinction coefficient of about 91 mM^{-1} cm^{-1} reported by Omura and Sato (1964) apparently represents a composite of these two extinction coefficients, since liver microsomes, even from control animals, contain a mixture of these two forms of cytochrome (P-450 and P-446). These differences in extinction coefficients will require a re-evaluation of the extent of cytochrome induction of liver microsomes as reported by Orrenius and Ernster (1964), Remmer and Merker (1965), etc.

Of interest was the question whether these two spectrally distinguishable pigments represented two hemoproteins or a single hemoprotein interconvertible between two spectral species. The absorption spectrum of the pigment induced by phenobarbital treatment has the spectral characteristics (George, Bettle-

stone, and Griffith, 1961) of a low spin hemoprotein such as alkaline methemoglobin, whereas the absorption spectrum of the pigment induced by 3-MC has spectral properties similar to a high spin hemoprotein such as acid methemoglobin. Examination of the two types of microsomes by electron paramagnetic resonance spectroscopy revealed a relatively weak absorbance at about g = 6 indicative of high spin hemoproteins. Significant differences were observed (Hildebrandt and Schleyer, unpublished observations) in the location of the first derivative maxima at about g = 1.9, 2.2 and 2.4 for microsomes from 3-MC and PB treated animals. These differences were comparable to those reported previously by Cammer, Schenkman, and Estabrook (1966) for changes observed during aniline or hexobarbital binding by cytochrome P-450 of microsomes.

Spectrophotometric studies were therefore undertaken to determine the influence of various substrates such as aniline, hexobarbital, etc., on the pigments induced by PB or 3-MC. It was observed that addition of aniline to microsomes from 3-MC treated animals resulted in loss of absorbance at 647, 517, and 394 mμ concomitant with the appearance of absorption bands at about 568, 534, and 418 mμ. Indeed, the spectral properties of the additional pigment of microsomes from 3-MC treated animals after treatment with aniline was very similar to that of microsomes from PB treated animals. In a comparable experiment, addition of hexobarbital to microsomes from PB treated animals caused a partial conversion to the spectral type observed with 3-MC treated animals.

Conclusion

The present studies have demonstrated directly the spectral characteristics of two inconvertible forms of the microsomal mixed function oxidase called P-450 and P-446 which appear to be synthesized preferentially upon treatment of rabbits with the inducing agents phenobarbital or 3-methylcholanthrene. These results strengthen the hypothesis of a single cytochrome of liver microsomes capable of interacting with two different types of substrates causing a modification in the spectral properties of the oxidized and reduced hemoprotein as well as the CO derivative of the reduced hemoprotein.

REFERENCES

Alvares, A. P., Schilling, G., Levin, W., and Kuntzman, R., Biochem. Biophys. Res. Comm. 29, 521 (1967).

Cammer, W., Schenkman, J. B., and Estabrook, R. W., Biochem. Biophys. Res. Comm. 23, 264 (1966).

Conney, A. H., Pharmacol. Rev. 19, 317 (1967).

George, P., Bettlestone, J., and Griffith, J. S., in "Haematin Enzymes" (J. E. Falk, R. Lemberg, and R. K. Morton, eds.), Volume I, p. 105, Pergamon Press, London, 1961.

Imai, Y., and Sato, R., Biochem. Biophys. Res. Comm. 23, 5 (1966).

Imai, Y., and Sato, R., J. Biochem. 62, 239 (1967).

Kinoshita, T., and Horie, S., J. Biochem. 61, 26 (1967).

Omura, T., and Sato, R., J. Biol. Chem. 239, 2370 (1964).

Orrenius, S., and Ernster, L., Biochem. Biophys. Res. Comm. 16, 60 (1964).

Remmer, H., and Merker, H. J., Ann. N. Y. Acad. Sci. 123, 79 (1965).

Schenkman, J. B., Remmer, H., and Estabrook, R. W., Mol. Pharmacol. 3, 113 (1967).

Sladek, N. E., and Mannering, G. J., Biochem. Biophys. Res. Comm. 24, 668 (1966).