A NEW SPECTRAL INTERMEDIATE ASSOCIATED WITH CYTOCHROME P-450 FUNCTION IN LIVER MICROSOMES*

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SUMMARY: The spectrophotometric examination of pigment reduction during the steady state of drug oxidative metabolism, as catalyzed by hepatic microsomes, has revealed the presence of a new spectral intermediate presumed associated with an oxygenated form of reduced cytochrome P-450. This new spectral intermediate has absorption maxima at about 440 and 590 mμ in the difference spectrum. The magnitude of this intermediate is dependent on the presence of a hydroxylatable substrate, the oxygen concentration, and the use of TPNH as the source of reducing equivalents. Further, changes in the extent of the TPNH-dependent reduction of cytochrome b_5 during the steady state indicates that cytochrome b_5 serves as a donor of electrons to the oxygenated form of reduced cytochrome P-450.

The mechanism of "oxygen activation" by cytochrome P-450 during hepatic microsomal mixed-function oxidation reactions has been the subject of considerable speculation but few experimental observations. The unusual spectral properties of reduced cytochrome P-450 relative to oxidized cytochrome P-450 (1) have greatly hindered the observation of changes in oxidation and reduction of this pigment, in particular when examining turbid microsomal suspensions by difference spectrophotometry. The isolation and purification of a cytochrome P-450 from Pseudomonas putida by Katagiri et al. (2) now provides a system in which changes in absolute spectra can be readily measured for comparison with

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difference spectra obtained using hepatic microsomes. The presence of an oxygenated species of reduced cytochrome P-450 of P. putida is described by Ishimura et al. in the accompanying communication (3). A study of the pigment changes of hepatic microsomes revealed the presence of a new spectral species distinct from those changes observed during the transition of oxidized to reduced cytochrome b_5 or oxidized to reduced cytochrome P-450. The present communication summarizes the initial results obtained using a repetitive scanning spectrophotometric method to examine pigment reduction during the steady state of oxidative drug metabolism. The presence of an oxygenated form of reduced cytochrome P-450 as well as an alteration in the extent of reduction of cytochrome b_5 suggest a reaction mechanism consistent with a sequential transfer of two electrons to cytochrome P-450.

METHODS

Male albino Holtzman rats (50-60 g) received daily intraperitoneal injections of phenobarbital (40 mg/kg) for 4 days. Rats were fasted for 24 hours prior to sacrifice by decapitation. Livers were perfused in situ with cold 0.15 M NaCl and were homogenized in 0.25 M sucrose. Microsomes were isolated by differential centrifugation of the homogenate by minor modification of the method described previously (4). The microsomal pellet was washed once with 0.15 M KCl to remove traces of contaminating hemoglobin before resuspending to a protein concentration of 30 mg/ml in a buffer mixture containing 50 mM Tris-chloride, pH 7.4, and 0.25 M sucrose. Protein was determined by the biuret reaction (5). Spectral changes were measured with an Aminco-Chance dual wavelength/split beam recording spectrophotometer which had been modified for repetitive scanning. Sodium isocitrate and isocitrate dehydrogenase (pig heart, Type IV) were purchased from Sigma Chemical Company. TPNH was obtained from P.L. Biochemicals, Inc.

RESULTS

The addition of DPNH or TPNH to an aerobic suspension of rat liver microsomes results in a spectral change ascribable to the reduction of cytochrome

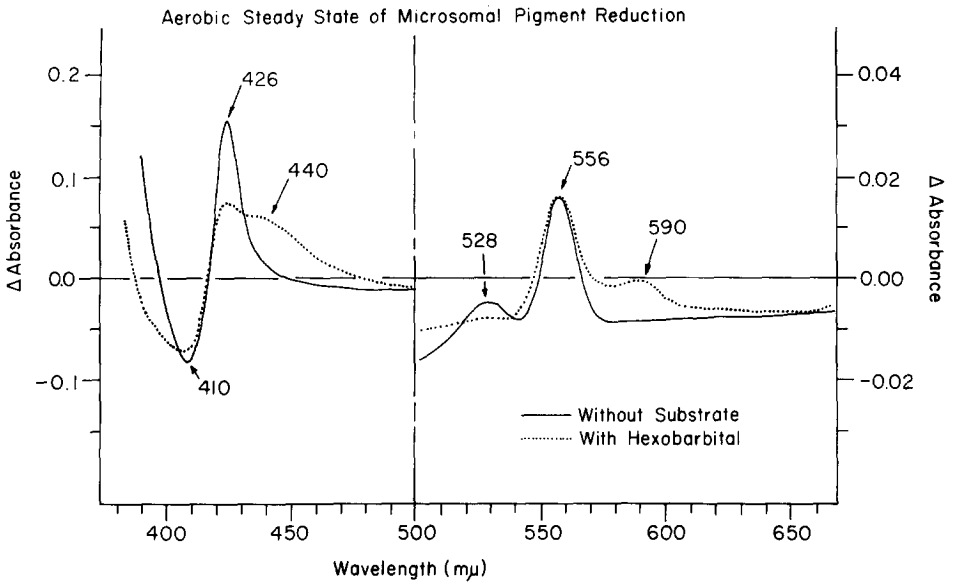

FIGURE 1: Difference Spectra of Hepatic Microsomes During the Aerobic Steady State in the Absence and Presence of Hexobarbital.

Liver Microsomes from phenobarbital-treated rats were diluted to a protein concentration of 2 mg/ml in a buffer mixture containing 50 mM Tris-chloride buffer, pH 7.4, with 150 mM KCl, 10 mM $MgCl_2$, 10 mM nicotinamide, 7 mM sodium isocitrate, and excess isocitrate dehydrogenase. When included in this buffer mixture, the concentration of hexobarbital was 2 mM. This suspension was divided equally between two cuvettes and a baseline of equal light absorbance was recorded. TPNH (200 μM) was then added to the sample cuvette and the difference spectrum was immediately recorded. The solid line represents the difference spectra obtained in the absence of substrate, and the dotted line represents the difference spectra obtained in the presence of 2 mM hexobarbital.

$\underline{b}_5$. When comparable experiments are carried out in the presence of substrates such as hexobarbital, ethylmorphine, aminopyrine, etc. which can undergo enzymatic hydroxylation, the spectral changes observed during the aerobic steady state reveal the presence of additional absorption bands not associated with the reduction of cytochrome $\underline{b}_5$. As shown in Figure 1, the difference spectrum is modified by the presence of hexobarbital with the appearance of new absorption bands at about 440 mμ and 590 mμ. In addition, there is a marked decrease in the spectral contribution of reduced cytochrome $\underline{b}_5$ at 426 mμ and 528 mμ. These spectral changes were dependent on (1) the presence of

TPNH, (2) the presence of oxygen, and (3) the presence of a Type 1 hydroxylatable substrate. It was observed that a Type 2 class of substrate, such as aniline, did not result in the formation of the additional spectral species during the steady state.

To ascertain the pattern of spectral transitions occurring during the aerobic steady state of drug metabolism as catalyzed by liver microsomes, the Aminco-Chance wavelength scanning recording spectrophotometer was modified to permit the simultaneous repetitive scanning of the spectral changes as well as individual recordings of each spectrum. The results of one series of

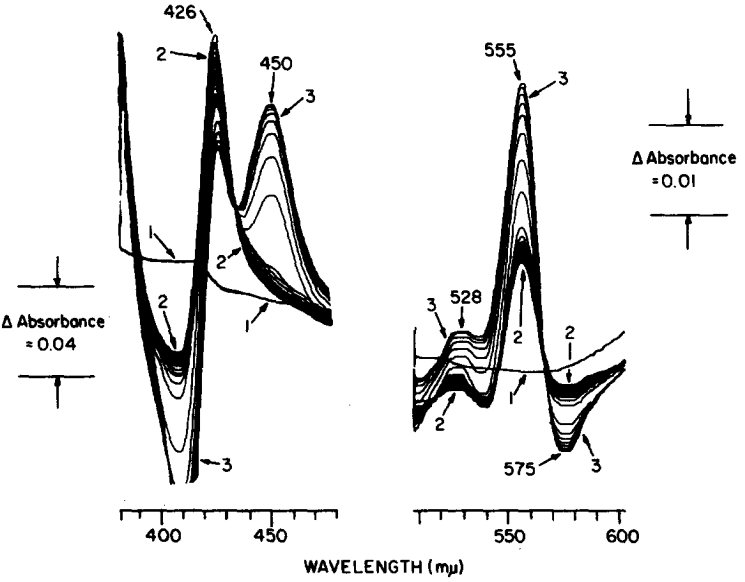

FIGURE 2: Repetitive Scanning of Difference Spectra of Hepatic Microsomes in the Absence of Substrate.

Liver Microsomes were diluted to a protein concentration of 2 mg/ml with the same buffer mixture as described in Figure 1. After dividing the diluted microsomal suspension equally between two cuvettes, a baseline (1) of equal light absorbance was recorded. TPNH (200 μM final concentration) was then added to the sample cuvette and the difference spectra were continually recorded using an Aminco-Chance spectrophotometer modified for repetitive scanning. The curves labeled (2) represent the spectral changes in the aerobic steady state. The curves labeled (3) were obtained after anaerobiosis. The time of each scan of the spectrum was 20 seconds.

experiments are illustrated in Figures 2 and 3. For the experiment illustrated in Figure 2, rat liver microsomes were diluted in a buffer mixture in the absence of a hydroxylatable substrate. After establishing a baseline of equal light absorbance for the contents of the two cuvettes, TPNH was then added to the contents of one cuvette and the aerobic steady state showing the reduction of cytochrome $\underline{b}_5$ was recorded. After about eight minutes a further spectral change was observed associated with the attainment of anaerobiosis. The appearance in the anaerobic system of an absorbance band at 450 mμ is associated with the formation of a small amount of the CO-complex of reduced cytochrome P-450. This CO is generated (6) during heme breakdown concomitant with microsomal electron transport reactions. In addition, it is observed that the magnitude of the Soret band of reduced cytochrome $\underline{b}_5$ is decreased on attaining anaerobiosis due to the negative spectral contribution of reduced cytochrome P-450 and its CO derivative in the difference spectrum. The reduction of

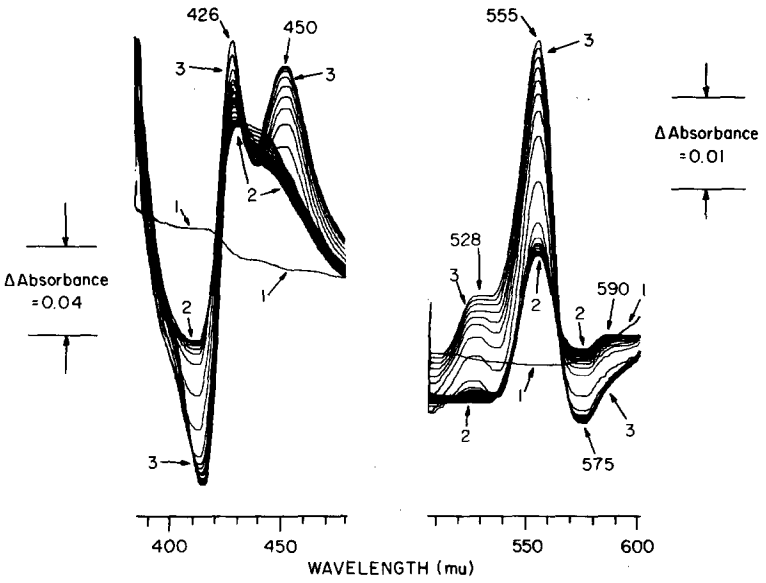

FIGURE 3: Repetitive Scanning of Difference Spectra of Hepatic Microsomes in the Presence of Hexobarbital.

Conditions were the same as described in Figure 2, except that hexobarbital (2 mM) was included in the buffer mixture.

cytochrome b_5 and cytochrome P-450 is more clearly delineated by evaluating the spectral changes at about 555 mμ and 575 mμ.

Of greater interest are the results shown in Figure 3 where a comparable series of experiments were carried out in the presence of the Type 1 substrate, hexobarbital. In this case the addition of TPNH, after establishment of a baseline of equal light absorbance in the two cuvettes, results in only a partial reduction of cytochrome b_5 and the appearance of the new spectral species with a broad maximum at about 440 mμ. Further, the appearance of a weak absorbance band at about 590 mμ and the quenching of the β absorbance band of cytochrome b_5 at 528 mμ are apparent. The stimulation of oxygen utilization by the presence of a substrate such as hexobarbital results in the establishment of anaerobiosis in a shorter time (after about five minutes) than the experiment described in Figure 2. Again, the spectral changes occurring on attaining anaerobiosis are reflected by the formation of an absorption band at 450 mμ and the marked intensification of absorbance at 555 mμ. The latter is associated with the further reduction of cytochrome b_5 as well as the reduction of cytochrome P-450.

DISCUSSION

The reduction-oxidation cycle (Figure 4) of cytochrome P-450 during substrate hydroxylation is a two electron process which involves the transient formation of an oxygenated intermediate of reduced cytochrome P-450. The difference spectrum of the new spectral species described here is very similar to the difference spectrum calculated from the studies of the oxygenated intermediate of reduced cytochrome P-450 observed (3) with the comparable pigment purified from *P. putida*. As detailed elsewhere (7), the transitions in the extent of reduction of cytochrome b_5, in particular where examined in the presence of both DPNH and TPNH, are consistent with its role as the donator of an electron to the oxygenated intermediate. The establishment of this new intermediate, together with the kinetic evaluation of its role in drug hydroxylation, should open new vistas for the understanding of factors

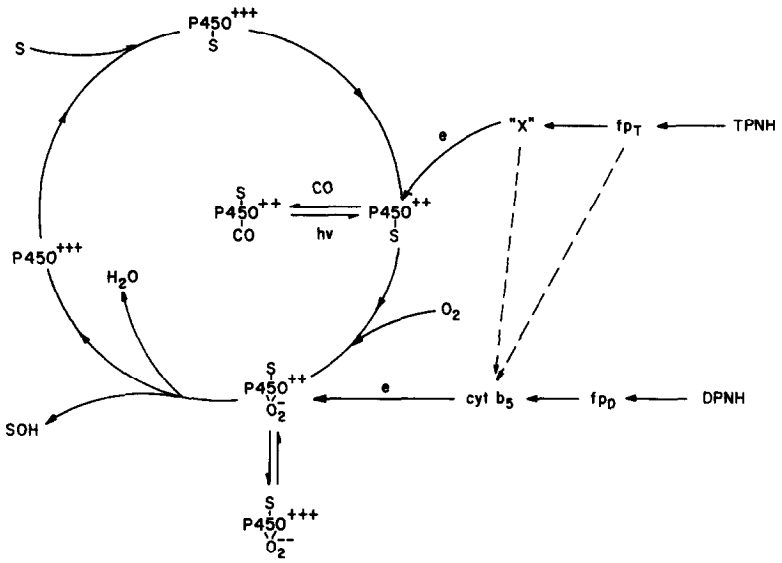

FIGURE 4: A Postulated Scheme of Microsomal Electron Transport Reactions Associated with Cytochrome P-450 Function during Hydroxylation Reactions.

The flavoproteins, TPNH-dehydrogenase and DPNH-dehydrogenase, are labeled f_{pT} and f_{pD}, respectively. The valence state of cytochrome P-450 is indicated by the plus charges associated with the iron (Fe), while substrate is designated as S.

controlling the function of the microsomal electron transport system in detoxification reactions.

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