EPR MEASUREMENTS OF SUBSTRATE INTERACTION WITH CYTOCHROME P-450*

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Cytochrome P-450 has been characterized (1,2,3) as the oxygen activating enzyme for a number of mixed function oxidation reactions (e.g. steroid hydroxylations, N-and O-demethylations, ring hydroxylations, etc.). More recently, optically observed spectral changes associated with the interaction of various substrates with cytochrome P-450 have been described (4,5,6,7). These spectral changes have been shown (5) to be related to the affinity of the mixed function oxidase for its substrate. It was suggested (5) that these spectral changes resulted from a modification of the binding of a ligand with the heme iron of cytochrome P-450 and represented an enzyme-substrate complex prerequisite to the hydroxylation reaction. Mason and his colleagues (8,9) have described the presence in liver microsomes of a component, termed FeX, detectable by electron paramagnetic resonance (EPR) spectroscopy. The EPR spectrum of FeX resembles (9) that obtained for a number of low-spin ferrihemoproteins such as alkaline metmyoglobin (10, 12) and alkaline horseradish peroxidase (9). Recent studies of Mason et al. (8,9) have indicated that FeX may be related to cytochrome P-450, and have suggested that FeX represents the sulfide complex of the protoheme-protein.

In the present study the changes in the EPR characteristics of FeX during substrate interaction have been examined. Two types of modifications of the

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EPR spectrum were observed, suggesting a correlation with the two types of optically observed spectral changes previously described (5) for substrates interacting with cytochrome P-450.

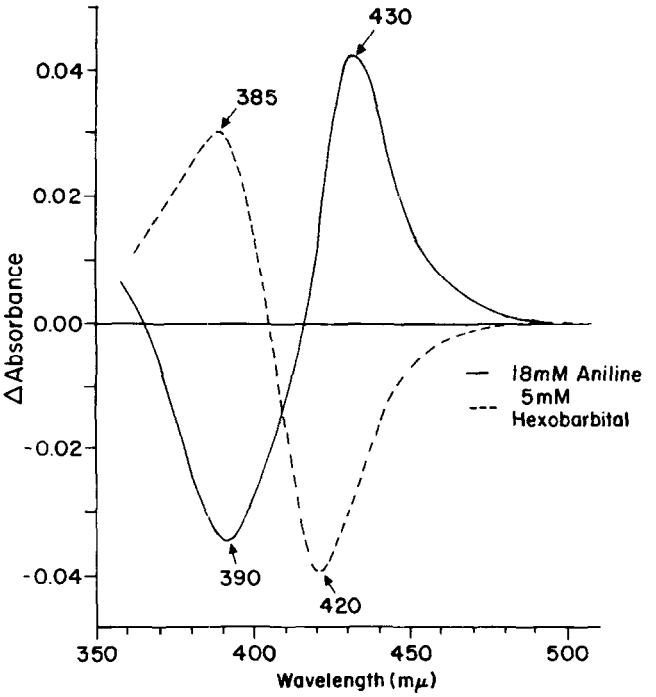

Figure 1. Difference spectra obtained by adding 18 mM aniline or 5 mM sodium hexobarbital to liver microsomes from rats pretreated by the daily injection of phenobarbital. Microsomes were suspended to 5 mg protein per ml in 0.1 M potassium phosphate buffer, pH 7.4, and equally divided into two cuvettes. After the baseline of equal light absorbance was recorded, aniline or hexobarbital was added to the experimental cuvette and the difference spectrum determined at room temperature with a wavelength scanning recording spectrophotometer.

A variety of mixed function oxidases has been examined, such as the enzyme systems for the C-21 hydroxylation of 17-hydroxyprogesterone by adrenal cortex microsomes, the 11β hydroxylation of deoxycorticosterone (DOC) by adrenal cortex mitochondria, and the O-demethylation of aminopyrine and the p-hydroxylation of aniline by liver microsomes. Narasimhulu et al. (4) have described the appearance of a spectral trough at 420 mμ on addition of 17-hydroxyprogesterone to adrenal microsomes. A similar spectral change was ob-

served when DOC was added to adrenal mitochondria. As recently described (5), a number of substrates such as aminopyrine, hexobarbital, etc., caused a similar spectral change when added to liver microsomes. In addition a second type of spectral change was observed (5) when substrates of the hepatic mixed function oxidase system such as aniline, acetanilide, nicotinamide, etc., were added to liver microsomes. These two types of spectral changes are illustrated in Figure 1.

Examination by EPR spectroscopy of three sources of mixed function oxidases (i.e. beef adrenal cortex microsomes and mitochondria and rat liver microsomes) revealed in each case EPR spectra similar to that described by Mason et al. (8,9) for FeX. The three components of the first derivative EPR

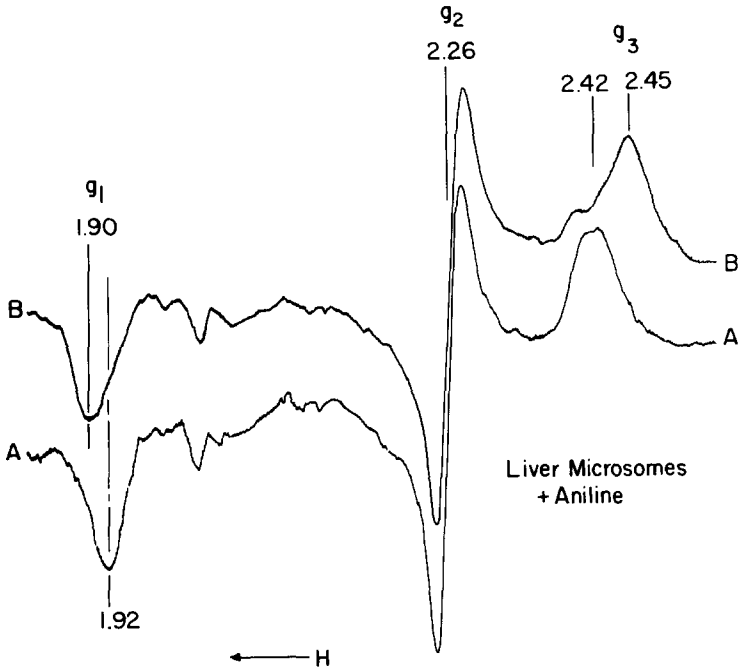

Figure 2. EPR spectra (first derivative) of rat liver microsomes in the presence and absence of aniline. Liver microsomes were obtained from rats pretreated by daily intraperitoneal injections of phenobarbital (5). The microsomes were suspended in 0.2 M tris buffer (pH 7.4) to a protein concentration of 38 mg/ml. A 1.0 ml aliquot of microsomal suspension was placed in a 3 mm (O.D.) quartz EPR tube and frozen in liquid nitrogen. Spectra were determined, with the sample cooled to liquid nitrogen temperature, using a Varian Model 4500-10A EPR spectrometer with 100-kH_z modulation. Curve A represents the spectrum obtained with untreated microsomes, while curve B represents the spectrum of a sample of microsomes to which aniline (final concentration, 5 mM) was added.

spectrum have been labeled g_1, g_2, and g_3 (Fig 2). The g_2 and g_3 components of the low spin hemoprotein at 2.26 and 2.42 were nearly identical for the three different types of preparations. The g_1 component at 1.92, while similar for adrenal and liver microsomes, could not be observed with adrenal mitochondria, since it was obscured by the g = 1.94 component of the non-heme iron protein present in adrenal mitochondria (3,11). The addition of aniline to rat liver microsomes caused a shift in the location of the g_1 and g_3 components, as illustrated by the first derivative spectra presented in Figure 2. The g_1 component shifted to higher field (1.92 to 1.90) while a major part of the g_3 component shifted to lower field (2.42 to 2.45). When the appropriate steroid substrates were added to adrenal cortex microsomes or mitochondria, the g_3 component was also modified, but in a direction opposite to that illustrated in Figure 2 for aniline addition to liver microsomes. Substrates such as aminopyrine or hexobarbital were therefore tested with liver microsomes, since they produced optical spectral changes different from those seen with aniline

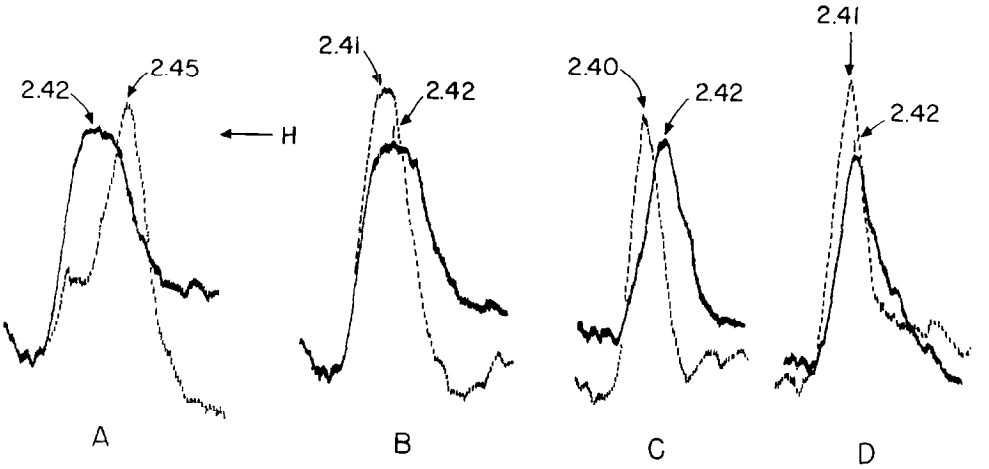

Figure 3. The EPR spectra (first derivative) of the g_3 component of rat liver microsomes, adrenal cortex microsomes, and adrenal cortex mitochondria. Conditions were similar to those described in Fig. 2.

A. Rat liver microsomes (38 mg/ml) as prepared (solid curve) and microsomes to which aniline (5 mM) was added (dashed curve).
B. Rat liver microsomes as prepared (solid curve) and microsomes to which hexobarbital (5 mM) was added (dashed curve).
C. Beef adrenal cortex microsomes (30 mg/ml) as prepared (solid curve) and adrenal cortex microsomes to which 17-hydroxyprogesterone (100 μM) was added (dashed curve).
D. Adrenal cortex mitochondria (34 mg/ml) as prepared (solid curve) and adrenal cortex mitochondria treated with deoxycorticosterone (3mM)(dashed curve).

but similar to those seen when steroids were added to adrenal cortex preparations. Again the EPR spectrum was modified, and in this case the change was similar to that observed when steroids were added to the adrenal cortex fractions. These EPR spectral changes of the g_3 component are presented in Figure 3.

These initial studies suggest a correlation between the two types of optical spectral changes observed on substrate addition to mixed function oxidases and the two types of changes in the EPR spectra. In addition the present EPR data strengthen the earlier suggestion (5) that substrates of mixed function oxidations interact at or near the heme of cytochrome P-450, resulting in a modification of ligand interaction with the heme iron. Such an interaction might permit the spatial proximity of the substrate for hydroxylation during oxygen activation by cytochrome P-450.

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