SUBSTRATE INTERACTION WITH HYDROXYLASE SYSTEM IN LIVER MICROSOMES

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Recent studies have established that a hemoprotein called P-450 (Omura and Sato, 1964a) is involved as the oxygen activating enzyme in certain NADPH-requiring mono-oxygenase reactions such as drug hydroxylations by liver microsomes and steroid hydroxylations by adrenal cortex microsomes and mitochondria (Estabrook et al., 1963; Cooper et al., 1965; Sato et al., 1965; Omura et al., 1965). However, little is as yet known of the mechanism by which substrates interact with the hydroxylase systems. Recently Narasimhulu et al. (1965) have observed characteristic spectral changes occurring on addition of the substrate, 17-hydroxyprogesterone, to the steroid C-21 hydroxylase system in adrenal cortex microsomes. We have also demonstrated other types of spectral changes on addition of aniline and other drugs to liver microsomes (Imai and Sato, 1965). The present paper presents evidence that P-450 is involved in the drug-induced spectral changes which appear to be closely connected to the mechanism of drug hydroxylation by liver microsomes.

Methods. Liver microsomes free from hemoglobin contamination were prepared from rabbits, guinea pigs, and rats in isotonic KCl as described by Omura and Sato (1964a). P-420 was partially purified from rabbit liver microsomes up to Step 4 of the procedure of Omura and Sato (1964b); the product was estimated to be about 70 % pure as compared with the purest preparation reported by Omura and Sato (1964b). Difference spectra were recorded at room temperature with a Cary 14 spectrophotometer. P-450 and P-420 were determined from the CO-differ-

ence spectra of dithionite-reduced samples (Omura and Sato, 1964a). Aniline hydroxylation and aminopyrine demethylation were measured essentially as described by Imai and Sato (1959) and Orrenius and Ernster (1964) respectively.

Results and Discussions. The addition of aniline to liver microsomes caused the appearance of a difference spectrum having a Soret peak at about 427 mμ and two smaller peaks at about 550 and 584 mμ (Fig. 1, Curve A), indicating the combination of aniline with a component in aerobic microsomes. The extent of combination, as measured from the magnitude of spectral change, was dependent on the concentration of aniline added, and the apparent dissociation constant (K_s) determined by a Lineweaver-Burk plot was 2 - 3 mM in case of rabbit liver microsomes. This value is in excellent agreement with the apparent Michaelis constant (K_m) of 1.5 - 2 mM determined for aniline hydroxylation by the same microsomes. Similar coincidence between K_s and K_m was also obtained with guinea pig and rat liver microsomes, though the constants determined for guinea pig (5 - 6 mM) and for rat (0.5 - 1 mM) differed considerably from those obtained for rabbit liver microsomes.

The shape of aniline difference spectrum (Fig. 1, Curve A) suggests the involvement of a hemoprotein in the observed interaction. In view of the established role of P-450 in drug hydroxylations, it is likely that this hemoprotein is the site of aniline binding. This consideration was in fact supported by several lines of evidence obtained in the present study. First, the magnitude of spectral change induced by an excess of aniline (20 mM) was proportional to the content of P-450 (but not cytochrome b_5) in a number of different preparations of liver microsomes containing various amounts of P-450. The preparations examined included liver microsomes prepared from rats in which the P-450 content was increased by the phenobarbital treatment (Orrenius et al. 1964). It was confirmed further that partially purified cytochrome b_5 shows no spectral interactions with aniline. Secondly, ethyl isocyanide competed spectro-

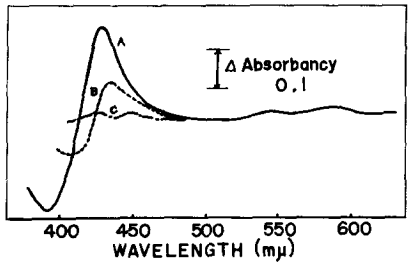

Fig. 1. Aniline difference spectra of rabbit liver microsomes. Curve A: The sample and control cuvettes contained 3.2 mg of microsomal protein (8.3 mμmoles of P-450) per ml of 0.1 M Tris acetate buffer, pH 8.0. Aniline (final concentration, 20 mM) was added in the sample cuvette. Curve B: Same as A, except that the both cuvettes contained a NADPH-generating system (NADP, 0.5 mM; glucose-6-phosphate, 5 mM; glucose-6-phosphate dehydrogenase; $MgCl_2$, 2.5 mM; nicotinamide, 10 mM). Curve C: A few mg of solid sodium dithionite was added to both cuvettes in A.

photometrically with aniline for liver microsomes. This finding is also indicative of P-450 involvement in aniline binding, since this reagent has been shown to combine not only with reduced P-450 (Omura and Sato, 1964a) but also with the oxidized pigment in aerobic microsomes to give a spectral change with a peak at 434 mμ (Omura, Nishibayashi and Sato, unpublished observations). Thirdly, partially purified P-420, which is a spectrally modified conversion product of P-450 (Omura and Sato, 1964a,b), gave rise to a difference spectrum illustrated in Fig. 2 on addition of aniline. Although the main peak in this spectrum is located at somewhat shorter wavelengths (417 - 420 mμ) as compared with that for microsomal bound P-450 (427 mμ) and the affinity of aniline is greatly decreased (K_s about 100 mM), it is clear that partially purified P-420 still retains the aniline binding capacity. These changes in spectral shape and affinity are not surprising, since the conversion of P-450 to P-420 has been shown to accompany profound changes also in the CO and ethyl isocyanide spectra of the reduced pigment (Omura and Sato, 1964a). Finally, all the treatments causing the conversion of microsomal P-450 to P-420 resulted in the disappearance of the 427 mμ peak and corresponding appearance of the new peak at 417 - 420 mμ in the

aniline difference spectrum of microsomes; moreover, such spectral conversion roughly paralleled the extent of P-420 formation. The treatments applied included those with trypsin, PCMB, urea (Mason et al., 1965), guanidine hydrochloride, high concentration of neutral salts, and lysolecithin. The effects of these conversion reagents on the microsomal hemoprotein will be reported in detail elsewhere.

Since microsomal hydroxylase reactions require the presence of NADPH which reduces P-450 by way of a special electron transfer pathway (Sato et al., 1965), it was of interest to examine the effect of NADPH on the aniline binding by liver microsomes. As shown in Fig. 1 (Curve B), the intensity of aniline spectrum was greatly reduced (to 1/2 - 1/3 of control depending on the microsomal preparations used) when an NADPH-generating system was added to aerobic microsomes, though the band position and the affinity of aniline were not appreciably altered. In the presence of dithionite, however, the addition of aniline produced only a very feeble spectral change (Fig. 1, Curve C). The almost complete loss of spectral effect of aniline in dithionite-treated microsomes may be taken as an indication of the inability of reduced P-450 to bind aniline. It appears therefore likely that the partial appearance of aniline spectrum on addition of NADPH to aerobic microsomes is a reflection of the partial reduction of P-450 under these conditions. The variable intensity of aniline spectrum in the presence of NADPH may also be accounted for by the varying degree of P-450 reduction by NADPH in different microsomes. However, the possibility can not be ruled out that reduced P-450 is also capable of binding aniline.

Besides aniline, aminopyrine and phenobarbital which are also metabolizable by liver microsomes were found to cause another type of spectral change in aerobic microsomes (Fig. 3, Curves A and B). The spectra thus obtained were similar to each other, having peaks at about 420, 535 and 570 mμ; the spectral intensities were smaller than that caused by aniline. As in the case of aniline

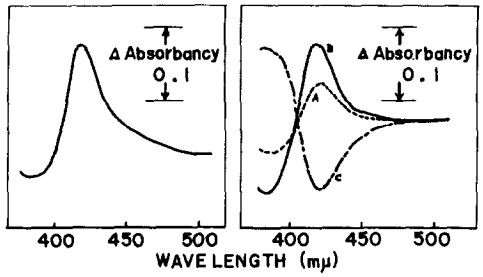

Fig. 2. Aniline difference spectra of partially purified P-420. The sample and control cuvettes contained 8.4 mμmoles of P-420 per ml of 0.1 M potassium phosphate buffer, pH 7.0. Aniline (final concentration, 67 mM) was added to the sample cuvette.

Fig. 3. Aminopyrine, phenobarbital, and phenacetin difference spectra of rabbit liver microsomes. The sample and control cuvettes contained 3.2 mg of microsomal protein (8.3 mμmoles of P-450) per ml of 0.1 M Tris acetate buffer, pH 8.0. Aminopyrine and phenobarbital (each 20 mM) were added to the sample cuvettes in Curve A and Curve B respectively. In Curve C 10 mM of phenacetin dissolved in ethanol (final concentration, 5%) was added to the sample cuvette; the control cuvette received only the same concentration of ethanol.

binding, these spectra were profoundly diminished in dithionite-treated microsomes. Furthermore, the K_s of aminopyrine binding was similar to the K_m for aminopyrine demethylation. A third type of interaction was obtained on addition of another hydroxylatable substrate, phenacetin, to liver microsomes; the difference spectrum thus observed was characterized by a trough at about 420 mμ (Fig. 3, Curve C). Although benzene and barbital were oxidatively metabolized by liver microsomes, these compounds (10 mM) induced no spectral changes in aerobic microsomes. Diethylamine and amino acids, though possessing amino groups, did not cause any spectral changes in microsomes. This last observation lends further support to the correlation between hydroxylation reactions and the spectral change reported here, since diethylamine and amino acids are not the substrates of the microsomal hydroxylase systems.

From the results described above it may be concluded that the oxidized form of hemoprotein P-450 in microsomes combines with aniline and other drugs to

induce several types of spectral changes and these interactions are essential for the oxidative metabolism of these drugs. Although the difference spectra caused by aniline, aminopyrine, and phenobarbital are indicative of the direct interaction of these drugs with the heme moiety of P-450, the phenacetin spectrum is unusual in this respect and benzene and barbital produce no spectral changes. An explanation for these multiple types of interaction would be to assume that the metabolizable drugs combine with a specific site or sites located on the protein moiety of P-450. It is not difficult to infer that such combinations exert various secondary effects on the heme moiety to produce multiple types of spectral alterations. A possible secondary effect induced by the drug binding is the change in protein conformation, which results in the alteration in the state of heme, since it has been shown in preliminary experiments that the addition of moderate concentrations of organic solvents to microsomes can cause spectral changes similar to those produced by certain drugs. By excluding P-450 heme from the possible substrate binding site, it is easier to visualize the role of the heme as the oxygen activating site. Further studies are in progress to define more clearly the molecular mechanisms of substrate and oxygen interactions with P-450.

When the work presented in this paper was completed, Dr. R. W. Estabrook kindly sent us a copy of manuscript of the work of his group (Remmer et al., 1966). In this manuscript they describe two types of spectral interactions of drugs with P-450 and report the coincidence between K_s for drug binding and K_m for drug hydroxylation. Although their results are similar to ours, there are differences in certain important respects, for instance in the effect of NADPH and the shape of aminopyrine spectrum. The reasons for such discrepancy are now in investigation.

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