Brief Definitive Report

ON THE OCCURRENCE OF CYTOCHROME P-450 AND ARYL HYDROCARBON HYDROXYLASE ACTIVITY IN RAT BRAIN*

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Liver microsomes contain a mixed function oxidase system capable of metabolizing a broad range of biologically active substances, including drugs, steroids, and environmental chemicals such as polycyclic hydrocarbons.

Certain hemoproteins, collectively referred to as cytochrome P-450, function as the terminal oxidase in this system. The fact that this hepatic mixed function oxidase system is sensitive to induction and inhibition by many of its substrates and other agents has provided an approach to understanding a major mechanism by which endogenously- and environmentally-derived substances may affect each other's disposition and biological impact (1).

Mixed function oxidase activities which depend on cytochrome P-450 are present in many tissues other than liver, including such endocrine organs as adrenal, testes, and placenta, such sites of direct xenobiotic exposure as lung, intestine, and skin, and such sites of drug action as heart and kidney. Cytochrome P-450 has also been detected in each of these tissues. Aryl hydrocarbon hydroxylase (AHH) is an example of a mixed function oxidase involving cytochrome P-450 whose distribution and properties have been widely studied. Benzo[*a*]pyrene (BP), which is a carcinogenic constituent of cigarette smoke and charcoal-broiled meat, is both a substrate and an inducer of this enzyme activity.

Because many of the substrates of the hepatic cytochrome P-450 system have major actions on the central nervous system, this study was undertaken to determine whether this cytochrome, and the related AHH activity, could be detected in brain.

Materials and Methods

Microsomes were prepared from rat brains as follows: male, Sprague-Dawley, 125–150-g rats were decapitated and the brains were immediately removed and transferred to cold saline. Large blood vessels and clots were excised and the pooled brains were homogenized in 4 vol of KCl (1.15%), by using a glass Teflon homogenizer. This homogenate was centrifuged for 20 min at 11,000 *g*; the supernate was then centrifuged for 60 min at 105,000 *g*; the resulting microsome pellet was washed in KCl and then suspended in buffer (0.1 M potassium phosphate, pH 7.4).

AHH activity was determined by using BP as the substrate by the method of Nebert and Gelboin (2), as modified by Alvares et al. (3). Brain microsomal suspensions were incubated for 30

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min at 37°C in 1.05 ml buffer containing 100 nmol BP, 1 μmol NADPH, 3 μmol $MgCl_2$, and 1 mg bovine serum albumin. In other experiments, homogenate equivalent to 100 mg brain wet weight was also incubated as above, except that albumin was omitted. The reaction was stopped by adding 1 ml cold acetone, either before (for blank determinations) or after incubation. BP metabolites were then extracted from the mixture, first into hexane, and then into 1 N NaOH, in which the concentration of hydroxylated (OH-) BP was determined by measuring the fluorescence at 522 nm during excitation at 396 nm. Hydroxy-BP standards were prepared by adding genuine 8-OH-BP instead of BP to incubation mixtures and extracting as for blank determinations. The activation and fluorescence spectra of the extracted metabolites after incubation of brain microsomes or homogenates with BP, and the spectra of 8-OH-BP, added to and immediately extracted from unincubated mixtures, were identical.

Protein was determined by using bovine serum albumin as the standard (4).

The carbon monoxide (CO) complex of reduced cytochrome P-450 strongly adsorbs light at 450 nm. Because both the reduced cytochrome, alone, and the CO-complex of the oxidized cytochrome do not strongly absorb light of this wavelength, difference spectra permit detection of the cytochrome in turbid suspensions (5–7).

Results and Discussion

In Fig. 1 A, the difference spectrum between the CO-complex of dithionite-reduced brain microsomes and the CO-complex of oxidized microsomes is depicted. The absorbance maximum (425 nm) and minimum (408 nm) in this spectrum correspond to the oxidized-minus-reduced spectrum of cytochrome b_5, which has been previously detected in brain microsomes (8). Cytochrome b_5 does not bind CO. However, the deflection in absorbance near 450 nm is only pronounced in the presence of CO, as in Fig. 1 A, and is not characteristic of cytochrome b_5. The effect of NADH on the dithionite-difference spectrum of CO-bound brain microsomes supports the idea that this deflection is due to cytochrome P-450. NADH transfers its reducing equivalents directly to cytochrome b_5 via the flavoprotein, cytochrome b_5 reductase (E.C. 1.6.2.2.), which has been detected in brain microsomes (8). Cytochrome P-450 differs from cytochrome b_5 in that it cannot be directly reduced by NADH aerobically (5). For this reason, the addition of NADH to both the sample and reference cuvettes which were compared in Fig. 1 A should result in a diminished contribution to the absorbance difference by cytochrome b_5 (which would become reduced in the reference as well as the sample cuvette), and an unaffected contribution by cytochrome P-450 (which would remain oxidized in the reference cuvette). Fig. 1 B depicts the difference spectrum after adding NADH to the cuvettes compared in Fig. 1 A. As expected, the addition of NADH resulted in a difference spectrum with a diminished minimum at 408 nm and a diminished maximum at 425 nm, yielding resolution of the absorbance at 450 nm as a distinct peak. A spectrum indistinguishable from that in Fig. 1 B was obtained either when more NADH was added (to a final concentration of 1 mM) or when NADH was added to the microsomes before exposure to CO, adjustment of the base line, and addition of dithionite to the sample cuvette only.

The CO-difference spectrum of dithionite-reduced brain microsomes provided direct evidence that cytochrome P-450 was present in these suspensions. Such a spectrum is presented in Fig. 2 A. The absorbance maximum at 454 nm reflects the absorbance difference between the CO-complex of reduced cytochrome P-450 and the reduced cytochrome, alone. The other absorbance maximum (420 nm) and the minimum (438 nm) in this spectrum may be due to cytochrome P-420 or

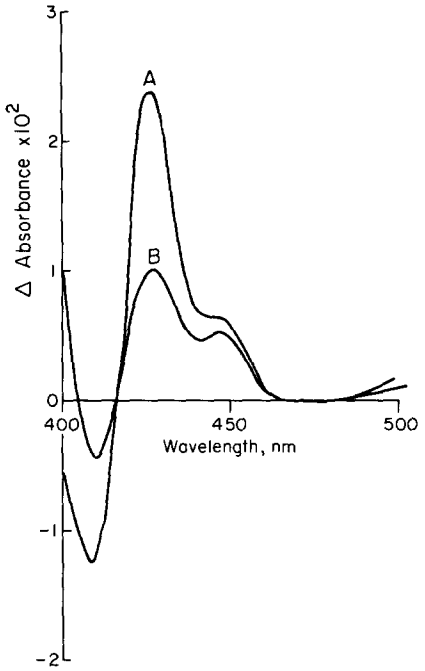

FIG. 1. The effect of NADH on the dithionite-difference spectrum of CO-bound brain microsomes. Microsomes (0.7 mg protein in 0.4 ml) were added to both cuvettes, CO was bubbled in each, and the base line was adjusted. A. Dithionite and more CO were then added to the sample only and the difference spectrum recorded after 3 min. B. 25 μl NADH (5 mg/ml buffer) and more CO were then added to both cuvettes and the difference spectrum was recorded after 5 min.

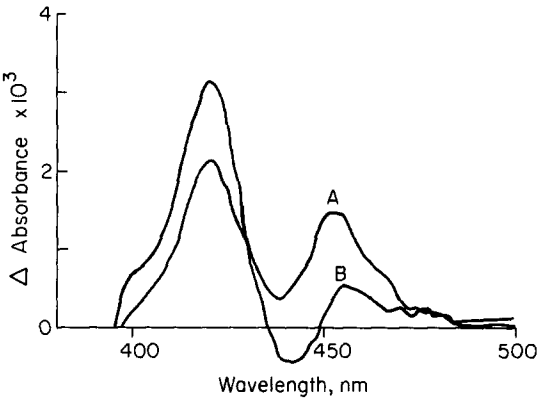

FIG. 2. The effect of mercury on the CO-difference spectrum of reduced brain microsomes. Microsomes were mixed with dithionite and added to each cuvette. A. The base line was adjusted, CO was added to the sample only, and the difference spectrum was recorded. B. 25 μl mercury (4 mg/ml methylmercury chloride in 1.15% KCl) was then added to each cuvette and the difference spectrum was again recorded after 2 min.

hemoglobin. When examined by this method, the absorbance peak of placental cytochrome P-450 is also at 454 nm, instead of precisely 450 nm (6).

Previous studies in this laboratory have demonstrated that hepatic cyto-

chrome P-450 is quantitatively degraded in vitro to its inactive form, cytochrome P-420, by methylmercury. To confirm that brain microsomes contain a chromophore which is susceptible to such degradation, 0.4 μmol mercury as methylmercury chloride was added to each of the cuvettes compared in Fig. 2 A, and the difference spectrum was again recorded. During the next few minutes, the absorbance maximum near 450 nm gradually diminished, and the extrema at 420 and 438 nm correspondingly increased in magnitude. 2 min after adding mercury, these changes were pronounced, as shown in Fig. 2 B. These changes in the CO difference spectrum of reduced brain microsomes are precisely those which were observed when hepatic microsomes were similarly treated (9), and document the simultaneous loss of one cytochrome (P-450) and formation of another (P-420). This formation of cytochrome P-420 from brain microsomes exposed to mercury is clear evidence that these microsomes originally contained cytochrome P-450.

An independent approach to the question of whether brain contains cytochrome P-450 is to examine the ability of this organ to metabolize substances which are substrates of the cytochrome in other tissues. BP was selected as a model substrate because its hydroxylated metabolites are intensely fluorescent. We have found that brain microsomes do metabolize BP at a rate sufficient to produce appreciable fluorescence: incubation of 2 mg microsomal protein for 30 min resulted in about five times the fluorescence of blank determinations, which is equivalent to the formation of about 30 pmol OH-BP/mg protein per h. As controls, mixtures from which NADPH or microsomes were omitted, or which were incubated at 0°C produced no net fluorescence, relative to blank determinations.

In other experiments, this activity was detected at a lower specific activity in brain homogenates. By using this enzyme source, brain AHH was estimated in individual rats and the effect of pretreatment with an inducer of hepatic AHH, 3-methylcholanthrene (3-MC), was studied. Five rats were injected intraperitoneally with 3-MC (25 mg/kg/d) for 6 days before sacrifice. The mean (± standard deviation) brain homogenate AHH activity in these 3-MC-pretreated rats was 25 ± 2 pmol OH-BP formed/mg protein per h. This is a four-fold increase over the activity in five untreated rats (5 ± 1 pmol OH-BP/mg protein per h); this increase is statistically significant ($P < 0.001$) with Student's t test.

In this study, cytochrome P-450 and AHH activity were identified for the first time in brain microsomal suspensions from normal animals. The physiologic significance of the small amounts of this cytochrome present in brain microsomes would depend on several properties of the cytochrome in this tissue, such as its substrate specificity, inducibility, and distribution within the central nervous system. Because brain microsomes contain large amounts of NADPH-cytochrome reductase (E.C. 1.6.2.4., [8]), the transfer of reducing equivalents to cytochrome P-450 would probably not be limiting for oxidative biotransformations. Localization of cytochrome P-450 within the central nervous system is likely, although not examined in this study. Such localization is strongly suggested by the recent observation of Fishman and associates that the mixed function oxidation of morphine occurs in vivo in specific areas of the brain only (10).

Summary

The difference spectra of the carbon monoxide-complex of dithionite-reduced rat brain microsomes, compared with both reduced microsomes, alone, and the carbon monoxide-complex of oxidized microsomes, indicate the presence of small amounts of cytochrome P-450 in brain. As in liver, cytochrome P-450 in brain is degraded in vitro to its inactive form, cytochrome P-420, by methylmercury chloride.

Aryl hydrocarbon hydroxylase activity is also present in rat brain microsomes and, at lower specific activity, in brain homogenates. This carcinogen metabolizing activity is increased four-fold in rats pretreated with 3-methylcholanthrene.

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