HEPATIC AMINOPYRINE N-DEMETHYLASE SYSTEM: INTERACTION OF AMINOPYRINE WITH MICROSONAL CYTOCHROME P-4501)

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Abstract—Interaction of aminopyrine with microsomal membrane-bound cytochrome P-450 was studied spectrophotometrically at various pH. Aminopyrine-induced type I spectral change in untreated rat microsomes was observed in neutral and alkaline media, and the absorption magnitude between peak and trough in the spectra increased markedly by increasing pH. On the other hand, an anomalous spectral change ($\lambda_{max}$, 425 nm; $\lambda_{min}$, 410 nm) was obtained in acid medium, and the absorption magnitude of the anomalous spectral change was enhanced by decreasing pH. The spectral dissociation constant for the anomalous aminopyrine-binding reaction at pH 6.32 was about one order of magnitude greater than that for the type I binding reaction at pH 8.22. The type of aminopyrine-induced spectral change differed depending upon the age and pretreatment of animals. Neonatal microsomes elicited only the anomalous spectral change in all pH media. Liver microsomes from 3-methylcholanthrene-pretreated rats showed a reverse type I spectral change. Antipyrine produced only a reverse type I spectral change in all microsomes tested, and the absorption magnitude was enhanced by decreasing the pH. In the presence of a saturated concentration of a reverse type I compound, i.e., ethanol or antipyrine, aminopyrine induced the type I spectral change, even in acid medium. The binding mechanism of cytochrome P-450 with aminopyrine is discussed on the basis of these results.

The hepatic microsomal monooxygenase system, containing cytochrome P-450 as a terminal oxidase, is functional in the oxidative biotransformation of endogenous and exogenous substrates (1, 2). The first step of the cytochrome P-450 reaction cycle during oxidative metabolism is the binding of substrate with ferric cytochrome P-450 to form the substrate adduct (3, 4), and this interaction has been studied spectrophotometrically using membrane-bound and purified cytochrome P-450 (5–7).

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Probably one of the most widely employed enzyme assays to measure the microsomal cytochrome P-450 containing monooxygenase system, is the oxidative demethylation reaction of aminopyrine (4-dimethylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) (8–10). Although aminopyrine is generally classified as a type I compound (5, 11). Imai and Sato reported
induction of a reverse type I spectral change (12). Furthermore, fetal and neonatal microsomes have also been reported to produce a reverse type I spectrum (13–15). Therefore, the spectral change due to the interaction of aminopyrine with microsomal cytochrome P-450 remains to be elucidated.

In previous work (16), we found that the binding of substrate(s) with microsomal cytochrome P-450 is most sensitive to the pH, and intense substrate-induced difference spectra are observed in alkaline or acid medium, depending on the chemical structure of the substrates. The present work was an attempt to assess the aminopyrine-induced spectral change of microsomal cytochrome P-450 as affected by pH, age of the animal and by the pretreatment of animals with 3-methylcholanthrene.

MATERIALS AND METHODS

Animals and preparation of hepatic microsomes: Adult and neonatal Wistar strain rats were used. Neonates (17–38 days old) of both sexes were used without determination of individual sex, while male adult rats (9–11 weeks old) were used. Some adult rats were given 3-methylcholanthrene in sesame oil (40 mg/kg, i.p.) for the 3 days prior to sacrifice.

The rats were exsanguinated following cervical dislocation, and liver microsomes were prepared by differential centrifugation techniques as described previously (16). Microsomal fractions were prepared immediately before the substrate-binding study.

Microsomal concentrations were determined by the biuret reaction (17) using bovine serum albumin as standard. Cytochrome P-450 content was determined according to the method of Omura and Sato (18).

Spectrophotometric study for the interaction of substrate with microsomal cytochrome P-450: Substrate-induced difference spectra were recorded using a Union High-Sens Spectrophotometer SM 401 with a cuvette of 1.0 cm light path. Spectral titration to obtain kinetic parameters was carried out as described by Schenkman (19).

RESULTS

Aminopyrine-induced spectral change: Addition of aminopyrine to microsomes obtained from untreated adult rats resulted in a type I spectral change exhibiting a peak at 385 nm and a trough at 420 nm (Fig. 1a). The absorption magnitude between peak and trough in the spectrum was enhanced by increasing the pH of the reaction medium. In acid pH, an anomalous difference spectrum, exhibiting a peak and trough at 425 and 410 nm, respectively, was observed (Fig. 1a). Similar pH-dependent alteration of aminopyrine-induced spectral change was also obtained using liver microsomes prepared from phenobarbital-pretreated rats, although the absorption magnitude was increased depending upon increase in the content of cytochrome P-450. The aminopyrine-induced difference spectrum observed in acid medium resembled a type II difference spectrum, the magnitude between peak and trough in the difference spectrum being enhanced when the pH in the medium was lowered.

When liver microsomes prepared from 3-methylcholanthrene-pretreated rats were used, the addition of aminopyrine resulted in a reverse type I spectral change exhibiting a peak at 424 and 390 nm, respectively, regardless of the pH, while the absorption magnitude between peak and trough in the difference spectrum increased when the pH in the medium was decreased.

In addition, the pH-dependent spectral change was reversible (16), suggesting that the spectral change shown in Fig. 1a indicates the interaction of aminopyrine with cytochrome P-450, but not with cytochrome...
P-420, as based on the result that the conversion of cytochrome P-450 to P-420 was not detected by judging the CO difference spectrum of dithionite-pretreated microsomes.

Antipyrine-induced spectral change: Antipyrine (2,3-dimethyl-1-phenyl-3-pyrazolin-5-one), which is one of the analogues of aminopyrine (20), is also a substrate for the microsomal cytochrome P-450 containing monooxygenase system. Antipyrine-induced difference spectra of microsomes was also studied in order to compare the spectral changes with those of aminopyrine. As shown in Fig. 1b, addition of antipyrine to normal microsomes produced the reverse type I spectral change ($\lambda_{\text{max}}$, 424 nm; $\lambda_{\text{min}}$, 390 nm). The spectral pattern was not altered by changing the pH, but the absorption magnitude between peak and trough in the difference spectrum increased on decreasing the pH of the medium (Fig. 1b). Similar reverse type I spectral changes were also observed in microsomes obtained from 3-methylcholanthrene-pretreated rats, the absorption magnitude being dependent upon the cytochrome P-450 (P-448) content of the microsomes.

Effect of pH on the absorption magnitude of aminopyrine- or antipyrine-induced spectral change: In the presence of a constant concentration of aminopyrine, the substrate-induced difference spectrum was observed at various pH using liver microsomes from untreated rats, and the absorption magnitude between peak and trough in the resulting spectrum was plotted as a function of pH (Fig. 2). The magnitude of the
aminopyrine-induced type I spectral change increased gradually when the pH was increased from 6.60 to 8.22, a maximum value being obtained at a pH over 7.77 (Fig. 2).

Table 1. Effect of pH on kinetic parameters for aminopyrine and antipyrine binding reactions with liver microsomes

| pH  | Kinetic parameters | Untreated rat microsomes | 3-methylcholanthrene-treated rat microsomes |
|-----|-------------------|--------------------------|------------------------------------------|
|     |                   | Aminopyrine | Antipyrine | Aminopyrine | Antipyrine |
| 8.22| $K_s$ (mM)        | 0.328       | 88.5       | 31.3        | 9.99       |
|     | $\Delta A_{max}$  | 0.028       | 0.058      | 0.018       | 0.026      |
| 7.20| $K_s$ (mM)        | 0.472       | 52.6       | 11.1        | 9.09       |
|     | $\Delta A_{max}$  | 0.015       | 0.058      | 0.018       | 0.032      |
| 6.32| $K_s$ (mM)        | 3.01        | 25.6       | 3.66        | 8.49       |
|     | $\Delta A_{max}$  | 0.003       | 0.058      | 0.018       | 0.035      |

Liver microsomes prepared from untreated or 3-methylcholanthrene-pretreated adult rats were suspended in 50 mM potassium phosphate buffer of various pH to a final protein concentration of 1.5 or 1.0 mg/ml (1.63 or 1.42 $\mu$M cytochrome P-450), respectively. Substrate-induced spectral change was determined by the addition of aliquots of aminopyrine or antipyrine. The parameters were calculated from the aminopyrine-induced type I and anomalous spectral changes of normal rat microsomes at pH 8.22–7.20 and 6.32, respectively. In the case of 3-methylcholanthrene-pretreated rat microsomes, the reverse type I spectral change was used for the calculation. Antipyrine exhibited only the reverse type I spectra in both untreated and 3-methylcholanthrene-pretreated rat microsomes.
2, open circles). Apparent spectral dissociation constants (Ks), obtained as described in MATERIALS AND METHODS, were 0.328 and 0.472 mM at pH 8.22 and 7.20, respectively, and the maximum absorption change, calculated at pH 8.22, was much greater than that at pH 7.20 (Table 1). When the pH was changed to 6.32 or 6.01, the absorption magnitude of the anomalous spectral change between peak and trough increased with decrease in the pH (Fig. 2, closed circles). Apparent spectral dissociation constant obtained at pH 6.32 showed a higher value when compared to those for a type I change obtained at pH 8.22 or 7.20 (Table 1). When liver microsomes prepared from 3-methylcholanthrene-pretreated rats were used, the values of apparent spectral dissociation constants were decreased by decreasing the pH (Table 1).

On the other hand, addition of antipyrine elicited the reverse type I spectral change in both untreated and 3-methylcholanthrene-pretreated microsomes. The absorption magnitude between peak and trough was depressed gradually by increasing the pH (Fig. 2, open triangles), and the apparent spectral dissociation constant showed higher values in alkaline medium (Table 1). Interestingly, Ks values of untreated microsomes showed much higher values, as compared with those from 3-methylcholanthrene-pretreated rats (Table 1).

**Aminopyrine- and antipyrine-induced spectral changes in microsomes of developing rats:** Several researchers have found that aminopyrine-induced difference spectra of fetal and neonatal microsomes do not elicit typical type I spectral changes, under the experimental conditions of physiological pH (13–15). Since an anomalous aminopyrine-induced spectral change (λ_max, 425 nm; λ_min, 410 nm) was observed in acid pH, even in the case of adult microsomes (Fig. 1a), aminopyrine-induced spectral changes at various pH were checked using microsomes prepared from rats of various ages (Table 2). An anomalous spectral change exhibiting peak and trough at 425 and 410 nm, which was detected only at acid pH with adult microsomes (63 days old), was observed at all the pH values tested (8.22–6.32), by adding aminopyrine to microsomes obtained from 17 day old rats. Using microsomes from 33 or 38 day old rats, an aminopyrine-induced type I spectral change was observed only at pH 8.22 with microsomes from the 33 day old rats, and again was only observed at pH 7.20 with microsomes from the 38 day old rats. Transition of the pattern was observed at pH 8.22 in the case of 33 day rat microsomes and at pH 7.20 with 38 day rat microsomes. Type I and the anomalous spectral change were observed with the addition of lower and higher concentrations of aminopyrine, respectively (Table 2). Apparent spectral dissociation constants calculated from the aminopyrine-induced anomalous spectral change in neonatal

| pH  | 17 | 33 | 38 | 63 |
|-----|----|----|----|----|
| 8.22| 'II' | 'II' | 'II' | 'II' |
| 7.20| 'II' | 'II' | 'II' | 'II' |
| 6.32| 'II' | 'II' | 'II' | 'II' |

Liver microsomes obtained from untreated rats of various ages were suspended in 50 mM potassium phosphate buffer and aminopyrine-induced spectral changes were observed, as shown in Fig. 1. Aminopyrine-induced type I and anomalous spectral changes are represented in the Table as I and 'II', respectively. The mark of 'II' in the Table represents the induction of type I and anomalous spectra by adding lower and higher concentrations of aminopyrine, respectively. Microsomal concentrations obtained from 17, 33, 38 and 63 days old rats were 3.0, 3.0, 1.0 and 1.0 mg protein/ml, respectively.
microsomes were 27.8-8.77 mM at pH 7.20, which values from 8.33-6.90 mM were obtained at pH 6.32. The Ks values from aminopyrine-induced type I spectral changes in neonatal microsomes (33-38 days old) were 0.839-0.946 mM at pH 8.22, that is about three times greater than changes seen in the case of microsomes from adult rats.

The addition of antipyrine to microsomes from neonatal (17-38 day old) rats elicited only a reverse type I spectral change regardless of the pH. An intense absorption in the difference spectrum was always obtained in acid medium, as observed in Fig. 1b.

Effect of ethanol and antipyrine on aminopyrine-induced anomalous spectral change: Aminopyrine is generally considered to be a type I substrate (5, 11) but this compound induced an anomalous and reverse type I spectral change, under the conditions described above (Fig. 1, Tables 1 and 2). Since the reverse type I spectral change was observed by adding ethanol (7) and also antipyrine (Fig. 1b), the effect of ethanol and antipyrine on aminopyrine-induced anomalous spectral change was studied in acid medium in order to obtain information on the interaction of aminopyrine with cytochrome P-450 under these conditions. As shown in Fig. 3 (solid line), addition of aminopyrine to microsomes produced an anomalous spectral change at pH 6.32. When the microsomal suspension contained ethanol (1.21 M), the addition of aminopyrine resulted in the appearance of a typical type I spectral change even in acid medium (Fig. 3, dashed line). Addition of antipyrine (23.8 mM), produced a similar change in the aminopyrine-induced difference spectrum. The results suggest that there are several binding site(s) for aminopyrine on the cytochrome P-450 molecule, one of which probably competes with ethanol or antipyrine.

DISCUSSION

The formation of a complex between a substrate and the oxidized form of cytochrome P-450 seems to be an initial and obligatory step in the hydroxylation process by this monoxygenase (3, 4). The interaction of chemical compounds with cytochrome P-450 produces characteristic absorption changes in the difference spectrum (5-7, 21, 22), and the spectral changes have been classified into three groups: type I, type II and reverse type I (5, 7, 22). Although aminopyrine is generally considered to be a type I substrate (5), different types of spectral change ($\lambda_{\text{max}}$, 420 nm; $\lambda_{\text{min}}$, 385 nm) have also been reported (12, 21). Several workers also reported the anomalous aminopyrine-induced spectral change in fetal and neonatal animals (13-15). In previous paper (16), we
reported the interaction of exogenous substrates with microsomal cytochrome P-450 at various pH and the interaction was influenced markedly by changing the pH of the microsomal suspension. In the present work, aminopyrine induced an anomalous spectral change (λ_{max} = 425 nm, λ_{min} = 410 nm) at acid pH (Fig. 1a and Table 2), although the typical type I spectral change was obtained at physiological and alkaline pH. The presence of the above two different patterns of spectral change suggests the existence of at least two different binding mechanisms for the interaction of aminopyrine with microsomal cytochrome P-450.

The existence of multiple forms of cytochrome P-450 in liver microsomes is well established (23–25). However, it is not clear whether all the forms of cytochrome P-450 interact with aminopyrine or only some forms of the hemoprotein interact with the substrate, with a specific mechanism for each hemoprotein. Recently, Gibson and Schenkman reported the purification of two forms of cytochrome P-450 (P-450_{I} and P-450_{II}) from untreated rat liver microsomes (25). Aminopyrine-induced spectral change of P-450_{I} and P-450_{II} determined at physiological pH were a type I and an anomalous type, respectively, and the values for spectral dissociation constants of these cytochromes were found to be very different. The aminopyrine-induced anomalous spectral change observed in acid medium (Fig. 1a) was similar to that obtained in P-450_{II}. The spectral dissociation constant for an anomalous aminopyrine-binding reaction at pH 6.32 showed about one order of magnitude greater than that for the type I spectral change obtained at pH 8.22 (Table 1), which was consistent with the previous report. When liver microsomes prepared from phenobarbital-, 3-methylcholanthrene- or β-naphthoflavone-pretreated rats and/or alloxan-diabetic rats were used, the absorption magnitude between peak and trough in the anomalous and reverse type I spectral change observed was closely correlated to microsomal cytochrome P-450 content, while the type I spectral change was observed to depend upon the pretreatment of animals (or molecular forms of cytochrome P-450) but not cytochrome P-450 content. Apparent correlation between aminopyrine N-demethylase activity and the absorption magnitude of type I spectral change was obtained using those microsomes (T. Matsubara and Y. Masuda, unpublished data). These results indicate that all forms of cytochrome P-450 can interact with aminopyrine, but the mechanism of interaction differs depending on the molecular forms of the cytochrome used. Some forms of cytochrome P-450 cause preferentially type I spectral change and others elicit reverse type I or anomalous spectral change. pH Change may introduce preferential interaction of aminopyrine with microsomal cytochrome P-450, eliciting specifically the type I or the reverse type I (or the anomalous) spectral change (Figs. 1 and 2). Thus, under the present experimental conditions, we obtained type I or reverse type I (or anomalous) spectral change with aminopyrine. The alteration of pH-dependent aminopyrine-induced spectral change with the age of the rat (Table 2) suggests the alteration of cytochrome P-450 species during development, and such warrants further investigation.

As shown in Table 1, liver microsomes obtained from 3-methylcholanthrene-pretreated rats showed only the reverse type I spectral change, although these microsomes was functioning to N-demethylate aminopyrine (10). Antipyrine and ethanol, well known substrates for microsomal drug-metabolizing enzymes (20, 26), elicit only the reverse type I spectral change, when microsomes obtained from untreated and
3-methylcholanthrene-pretreated rats are added (Fig. 1b). It may nevertheless be significant that an aminopyrine-induced type I spectral change was obtained even in acid medium without any conversion of cytochrome P-450 to P-420, when saturated concentrations of a reverse type I substrate, i.e., ethanol or antipyrine, were added to the microsomal suspension (Fig. 3). It is thus supposed that an aminopyrine-induced type I spectral change is hidden in the case of 3-methylcholanthrene-pretreated rat microsomes, and also microsomes from untreated rats at acid pH. A similar mechanism has been reported for aniline binding in which a type I component has been shown to be hidden within the type II spectral change (19). These results also support the idea that aminopyrine binding to cytochrome P-450 probably involves two different mechanisms, but only one of these interactions would preferentially proceed, under the conditions used and the results could be detected spectrophotometrically. Evidence of the duality of interactions between microsomal cytochrome P-450 and substrates has also been reported by Orrenius et al. who used various concentrations of didesmethylimipramine and d-amphetamine (22).

Another factor to consider is the influence of endogenous substrate(s) and microsomal membrane structure (or lipids). Recently, Gibson et al. demonstrated the interaction of cytochrome P-450 with membrane lipids, particularly unesterified fatty acids (27). Furthermore, Jefcoate and Boyde indicated the pH-dependent interaction of adrenal mitochondrial cytochrome P-450 with endogenous substrate(s) accompanied by spin-state changes (28). Since pH-dependent spectral change was also observed in liver microsomal system without any addition of exogenous substrate (Y. Hachino, T. Matsubara and B. Hagihara, unpublished data), endogenous substrate(s) and membrane lipids are assumed to interfere to some extent in the interaction of cytochrome P-450 with aminopyrine. Although the interference of endogenous substrate(s) and lipids to the exogenous substrate-binding reaction at various pH is not clear, membrane lipids do not alter the extinction coefficient of cytochrome P-450 (27). Therefore, we consider that the interaction of cytochrome P-450 with aminopyrine, even in microsomal membrane systems, can be detected spectrophotometrically.

As shown in this paper, the spectral change of the aminopyrine-induced difference spectrum was altered by pH, the age of animals, and the pretreatment of rats with 3-methylcholanthrene, thereby suggesting that the interaction of aminopyrine with microsomal cytochrome P-450 is influenced by several factors. Aminopyrine may interact on at least two different sites of the hemoprotein, and various factors such as pH, molecular species of cytochrome P-450 and membrane lipids, may introduce preferential interactions of aminopyrine with one site but not the other(s). The pH-dependence of the spectral change may thus reflect the degree of the binding of aminopyrine to microsomal cytochrome P-450. When liver microsomes obtained from untreated adult rats were used, aminopyrine bound preferentially to a binding site on the protein moiety of cytochrome P-450 and was accompanied by a type I spectral change. In the case of microsomes obtained from neonatal and 3-methylcholanthrene-pretreated rats, aminopyrine interacted preferentially with heme-iron, and thus the reverse type I or the anomalous spectral change was observed. In acid medium, the binding site on the protein moiety may be modified slightly followed by a decreased ability to elicit the type I spectral change. Whether or not similar binding mechanisms exist in neonatal and 3-methylcholanthrene-pretreated microsomes.
even at physiological pH remains to be investigated. Further studies on this line are in progress.

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