HOMOTROPIC VERSUS HETEROTROPIC COOPERATIVITY OF CYTOCHROME P450eryF: A SUBSTRATE OXIDATION AND SPECTRAL TITRATION STUDY

(Received November 18, 2002; accepted January 8, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:
P450eryF is the only bacterial P450 to show cooperativity of substrate binding and oxidation. However, the studies reported so far have provided evidence only for homotropic cooperativity of P450eryF but not for heterotropic cooperativity. Therefore, oxidation of 7-benzyloxyquinoline (7-BQ) and 1-pyrenebutanol (1-PB) by P450eryF A245T and spectral binding of 9-aminophenanthrene (9-AP) to wild-type P450eryF were investigated in the presence of various effectors. The addition of steroids and flavones caused no stimulation but rather moderate inhibition of 7-BQ or 1-PB oxidation by P450eryF A245T. However, the binding affinity of 9-AP was significantly increased in the presence of androstenedione or α-naphthoflavone (ANF). A comparative study with CYP3A4 revealed a similar increase in the binding affinity of 9-AP for the enzyme at low ANF concentrations but some competition at higher ANF concentrations. These studies, to our knowledge, provide the first report of heterotropic cooperativity in P450eryF as well as spectroscopic evidence for simultaneous presence of two ligand molecules in the CYP3A4 active site.

Short Communication

Cooperativity of cytochrome P450 (P4501) remains one of the most complex phenomena of this superfamily of hemoproteins. In the vast majority of cases of enzyme cooperativity, binding of a ligand at a distal regulatory site induces a conformational change in the active site that leads to activation or inhibition. However, steady-state kinetic analysis and site-directed mutagenesis studies of mammalian cytochromes P450 have suggested that the effector site is contiguous with the substrate binding site (Shou et al., 1994; Harlow and Halpert, 1997, 1998; Ueng et al., 1997; Korzekwa et al., 1998; Domanski et al., 2000, 2001; Hosea et al., 2000). A recent X-ray crystallographic study of P450eryF (CYP107A1), the only bacterial P450 to show cooperativity, has demonstrated that two molecules of androstenedione or 9-aminophenanthrene (9-AP) could bind simultaneously in the active site (Cupp-Vickery et al., 2000). However, the absence of a conserved threonine found in the I-helix of other P450s renders P450eryF ineffective in oxidizing even some closely related derivatives of the physiological substrate, 6-deoxyerythronolide B. The availability of only a single physiological substrate for P450eryF has previously posed a major hurdle in using this enzyme as a model system to better understand cooperativity and structure-function relationships of mammalian P450s. More recently, it has been shown that the substitution of Ala-245 with threonine leads to a significant gain-of-function that confers on P450eryF the ability to oxidize testosterone (Xiang et al., 2000), 7-benzyloxyquinoline (7-BQ) (Khan and Halpert, 2002), and 1-pyrenebutanol (1-PB) (Davydov et al., 2002). The steady-state oxidation kinetics of all three substrates by P450eryF A245T showed sigmoidal behavior, indicative of homotropic cooperativity. However, so far no evidence for heterotropic cooperativity of P450eryF has been reported. Therefore, oxidation of 7-BQ and 1-PB by P450eryF A245T and spectral titration of wild-type P450eryF with 9-AP were investigated in the presence of various effectors.

Experimental Procedures

The details of expression and purification of wild-type P450eryF and A245T, 7-BQ oxidation assays, and 1-PB oxidation assays have been described previously (Davydov et al., 2002; Khan et al., 2002; Khan and Halpert, 2002). Binding spectra were recorded on a Shimadzu-2600 spectrophotometer fitted with a temperature controller (TCC-240Al; Shimadzu, Kyoto, Japan). For the titration, the sample chamber contained 0.5 μM protein in 50 mM phosphate, pH 7.4, and the reference chamber contained the buffer. A fixed amount of effector (in methanol) was then added to both cuvettes, and a baseline was recorded between 350 and 500 nm. Subsequently, various amounts of 9-AP (in methanol, 2.5–75 μM) were added to both the cuvettes. The maximal methanol concentration used was 2%. The difference spectra were obtained after the system reached equilibrium (3 min). All spectra were recorded at 25°C.

Results and Discussion

7-BQ and 1-PB Oxidation in the Absence and Presence of Flavones and Steroids. The rates of 7-BQ and 1-PB oxidation by P450eryF A245T were determined in the absence and presence of various flavones [flavone, α-naphthoflavone (ANF), and β-naphthoflavone] and steroids (testosterone, androstenedione, progesterone, dehydroepiandrosterone, androstenediol, and pregnenolone) to investigate whether these compounds cause activation. The rates of 7-BQ oxidation were determined at 50, 100, and 200 μM substrate and at least two different effector concentrations. In contrast to other P450s, which show stimulation by a number of these compounds (Johnson et al., 1988; Schwab et al., 1988; Shou et al., 1994; Harlow and Halpert,
1997; Ueng et al., 1997; Korzekwa et al., 1998), no stimulation of P450eryF A245T was observed. Instead as shown in Fig. 1A, B, and C the presence of effectors caused small decreases in the rate of 7-BQ oxidation by P450eryF A245T. The inhibition was more pronounced at higher 7-BQ concentrations than at lower. Among all the compounds tested, progesterone caused the maximal inhibition. As illustrated in Fig. 1D the inhibition was due to a small decrease in the $k_{cat}$ as well as an increase in $S_{50}$ value. The $k_{cat}$, $n$, and $S_{50}$ values of 7-BQ oxidation by A245T in the absence of progesterone were $0.78 \pm 0.04$ min$^{-1}$, $2.9 \pm 0.5$, and $100 \pm 7$ $\mu$M, respectively, whereas these values were $0.57 \pm 0.04$ min$^{-1}$, $1.9 \pm 0.2$, and $139 \pm 13$ $\mu$M, respectively, in the presence of 25 $\mu$M progesterone.

The effects of testosterone, progesterone, and androstenedione on 1-PB oxidation by P450eryF A245T were similar to those observed with 7-BQ. Once again, none of these compounds showed any activation but rather small decreases in 1-PB oxidation (Fig. 1E). The effect of addition of flavones on 1-PB oxidation could not be determined because of a significant overlap between the fluorescence spectra of 1-PB and the flavones.

**Spectral Binding Studies of P450eryF with 9-AP and a Comparison with CYP3A4.** The titration of P450eryF with androstenedione is known to produce a type-I spectrum, whereas 9-AP demonstrates type-II binding (Cupp-Vickery et al., 2000; Khan and Halpert, 2002; Khan et al., 2002). Spectral titrations have also revealed sigmoidal binding curves for the interaction of androstenedione or 9-AP with P450eryF, indicating positive homotropic cooperativity. The fact that the titration of the enzyme with androstenedione and 9-AP produces two completely different spectra (type I versus type II) made it possible to evaluate the effect of addition of one compound on the binding affinity of the other. Quite interestingly, the presence of increasing concentrations of androstenedione enhanced the $A$ at lower 9-AP concentrations and caused a continuous shift in trough position toward lower wavelength (Fig. 2). A complete titration of
P450eryF with 9-AP also revealed a shift from a sigmoidal \( \Delta A \) versus \( S \) plot to a hyperbolic one and a significant increase in the binding affinity of 9-AP with increasing androstenedione concentrations (Table 1). The shift in the trough position of the difference spectra of P450eryF in the presence of androstenedione indicates that the enzyme is first converted to high-spin, and then binds to 9-AP to generate the modified type II spectra. Thus, the observed increase in the binding affinity of 9-AP and the shift in trough position on addition of androstenedione provide a clear indication of the simultaneous existence of androstenedione and 9-AP in the active site.

ANF is known to be one of the best effectors of CYP3A4 and also binds to P450eryF (Khan and Halpert, 2002). The presence of increasing concentrations of ANF caused a decrease in the apparent dissociation constant (\( K_a \)) of 9-AP at lower 9-AP concentrations compared with the free enzyme but no shift in the trough position. The plot of \( \Delta A \) versus \( S \) also remained sigmoidal, however the \( S_{50} \) value for 9-AP binding decreased very significantly with increasing concentrations of ANF (Table 2). The increase in the binding affinity of 9-AP on addition of ANF as well as the observation of sigmoidal 9-AP binding even in the presence of ANF indicates the presence of more than two bound ligands, as has been suggested in the case of CYP3A4 (Domanski et al., 2000, 2001; Hosea et al., 2000).

One of the primary aims of studying P450eryF cooperativity is to use this as a model to better understand cooperativity of mammalian P450s. Therefore for comparison, we titrated CYP3A4 with 9-AP in the presence of increasing concentrations of ANF. In contrast to P450eryF, the titration of CYP3A4 with 9-AP showed a hyperbolic \( \Delta A \) versus \( S \) plot, even in the absence of ANF (Table 3). However, similar to the effect of androstenedione on P450eryF, the presence of ANF enhanced \( \Delta A \) of CYP3A4 at lower 9-AP concentrations (2.5–15 \( \mu M \)) and caused a shift in trough position toward lower wavelength. Furthermore, in the case of CYP3A4 low concentrations of ANF caused a decrease in the apparent dissociation constant (\( K_a \)) of 9-AP with the maximal effect at 5 \( \mu M \); after that \( K_a \) value increased but remained lower than the \( K_a \) value in the absence of ANF. These observations can be explained by earlier studies indicating that ANF has two different binding sites within the large 3A4 active site. At lower concentrations, ANF generally binds to an effector site, and at higher concentration it starts to compete for the substrate binding site (Domanski et al., 2000, 2001). The observed increase in the binding affinity of 9-AP and the shift in trough position on addition of ANF provide, to our knowledge, the first spectral evidence of the simultaneous existence of two different ligands in the CYP3A4 active site.

### Conclusions

The present study clearly demonstrated that the addition of androstenedione and ANF to P450eryF causes an increase in the binding affinity of 9-AP, although these compounds show no stimulation of 7-BQ or 1-PB oxidation by P450eryF A245T. One of the likely explanations for this anomaly could be that although the binding affinity of the substrate is increased in the presence of an effector, the competition between the substrate and effector for the reactive oxygen species leads to decrease in the rate of substrate oxidation (Shou et al., 1994; Korzekwa et al., 1998). The observed decrease in \( K_{cat} \) of 7-BQ oxidation by P450eryF A245T in the presence of progesterone substrates such a possibility (Fig. 1D). The complementarity between substrate and effector is known to be important for the atypical kinetics of P450s (Shou et al., 1994; Korzekwa et al., 1998). There have been a number of reports on CYP3A4, in which the addition of an effector has been shown to activate, inhibit, or have no effect on oxidation of substrates as well as to differentially stimulate/inhibit formation of two different metabolites of the same substrate (e.g., aflatoxin B1, Ueng et al., 1997; midazolam, Maenpää et al., 1998). Therefore, the use of different pairs of substrate and effector as well as use of wild type versus P450eryF A245T in substrate oxidation and binding studies could be another reason for our experimental observations. The study of cooperativity and structure-function relationships of P450eryF is still in its infancy. However the known structures of a number of its enzyme-ligand complexes, the high water solubility, high-level expression in Escherichia coli, and simple reaction

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<th>Androstenedione (µM)</th>
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<th>( S_{50} ) (µM)</th>
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<tr>
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<th>ANF (µM)</th>
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<th>( S_{50} ) (µM)</th>
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*These data fit best to the hyperbolic equation.

2 Recently, Dabrowski et al. (2002) provided spectroscopic evidence for the presence of pyrene dimers in the CYP3A4 active site.
systems could be of tremendous advantage in our quest for better understanding of atypical kinetics of P450s using P450eryF as a model system. Use of P450eryF along with a combination of structural, functional, and theoretical approaches and many more substrate/effector pairs may be required to solve the intriguing problem of P450 cooperativity, with its implications for drug-drug and drug-food interactions as well as in vitro and in vivo correlations involving this superfamily of enzymes.

Acknowledgments. We thank Dr. Santosh Kumar for help in generating 1-PB oxidation data.

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References


