Differential Interaction of Erythromycin with Cytochromes P450 3A1/2 in the Endoplasmic Reticulum: A CO Flash Photolysis Study

Aditya P. Koley,‡ Renke Dai,‡ Richard C. Robinson,‡ Allen Markowitz,§ and Fred K. Friedman*,‡

Laboratory of Molecular Carcinogenesis, National Cancer Institute, and Biomedical Instrumentation and Engineering Program, National Institutes of Health, Bethesda, Maryland 20892

Received August 21, 1996; Revised Manuscript Received January 14, 1997⊗

ABSTRACT: The kinetics of CO binding to cytochromes P450, measured by the flash photolysis technique, were used to probe the interaction of erythromycin with cytochromes P450 in rat liver microsomes. Addition of erythromycin generates substrate difference spectra using microsomes from rats treated with phenobarbital or dexamethasone but not from untreated rats, showing that it binds to P450s induced by these agents. In contrast, erythromycin and/or a monoclonal antibody to P450 3A1/2 accelerated CO binding to microsomes from rats treated with phenobarbital but had no effect on microsomes from untreated or dexamethasone-treated rats. Based on the differential amounts and inducibilities of the P450 3A1 and 3A2 forms in these microsomal samples, these results indicate that erythromycin increased the rate for P450 3A2 but not P450 3A1. The divergent effects of erythromycin on these P450s, which exhibit 89% sequence similarity, were consistent with a model of the P450 substrate binding site in which erythromycin forms a more rigid complex with P450 3A1 than P450 3A2. These results demonstrate the sensitivity of P450 conformation/dynamics to substrate binding, and show that CO binding kinetics can distinguish among closely related P450s in a microsomal environment.

Cytochrome P450 is a superfamily of heme protein enzymes that catalyze the oxidation of a wide variety of lipophilic compounds. P450[1] substrates include exogenous chemicals such as drugs and carcinogens, and endogenous compounds such as steroids and prostaglandins (Ryan & Levin, 1990; Ortiz de Montellano *et al*., 1995). The individual forms of P450 exhibit unique catalytic activity profiles toward various substrates. In particular, the 3A subfamily metabolizes an array of structurally diverse substrates, including many clinically important drugs [see Koley *et al*. (1995a) and references cited therein]. Several laboratories have identified multiple members of the P450 3A subfamily (Nelson *et al*., 1996), and their regulation has been extensively studied in the rat (Gonzalez *et al*., 1986; Hostetler *et al*., 1987; Halpert, 1988; Imaoka *et al*., 1988; Nagata *et al*., 1990; Telhada *et al*., 1992; Cooper *et al*., 1993). The differential regulation of these P450s in response to different inducers is exemplified by two members of this subfamily, P450s 3A1 and 3A2, which exhibit 89% amino acid sequence homology (Gonzalez *et al*., 1986). P450 3A2 but not 3A1 is constitutively expressed. In addition, while PB and DEX both induce P450 3A1, only PB significantly induces P450 3A2 (Gonzalez *et al*., 1986).

In order to gain insight into the nature of the P450−substrate interactions of these two closely related P450s, we examined the effect of erythromycin, a substrate for the P450 3A subfamily (Wrighton *et al*., 1985a,b), on the kinetics of CO binding to these P450s in rat liver microsomes. The kinetics reflect the rate of CO diffusion through the protein matrix to the heme and are a unique probe of P450 conformation and dynamics. This technique has been applied to examine mammalian P450−substrate interactions using both purified and microsomal-bound P450s [see Koley *et al*. (1994) and references cited therein]. Owing to the recent development of a difference method of kinetic analysis, this approach has also provided valuable details into the interactions of substrates with rat P450s 1A1 and 2B1 in their microsomal-bound state in the presence of other microsomal P450s (Koley *et al*., 1994, 1995b), as well as the interaction of drugs with human P450 3A4 expressed in a baculovirus expression system (Koley *et al*., 1995a). In this report, we apply this approach to define the interactions between erythromycin and P450s 3A1 and 3A2. The results revealed different modes of erythromycin binding to these two closely related P450s, and were interpreted via molecular modeling of their substrate binding sites.

MATERIALS AND METHODS

Rat Liver Microsomes. Male Sprague-Dawley rats (6 weeks old) were injected intraperitoneally daily with DEX (100 mg/kg of body weight for 3 days) or PB (80 mg/kg of body weight for 3 days). Liver microsomes were prepared as described (Koley *et al*., 1994).

Substrate Binding Difference Spectra. Substrate binding difference spectra were obtained using an Aminco DW2000 spectrophotometer; 3.5 mL of DEX- or PB-microsomes [0.33 mg/mL, suspended in 0.1 M sodium phosphate buffer, pH 7.5, 20% glycerol (w/v)] was added to both sample and reference cuvettes. After recording a base line, 53 μL of erythromycin (from a 20 mM stock solution in methanol to yield a final concentration of 0.3 mM) and methanol were added to the sample and reference cuvettes, respectively, and

* Address correspondence to this author at the NIH, Building 37, Room 3E-24, Bethesda, Maryland 20892. Telephone: 301-496-6365. FAX: 301-496-8419. E-mail: fkfried@helix.nih.gov.

‡ Laboratory of Molecular Carcinogenesis.

§ Biomedical Instrumentation and Engineering Program.

⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: P450, cytochrome P450; PB, phenobarbital; PB-microsomes, microsomes from PB-treated rats; DEX, dexamethasone; DEX-microsomes, microsomes from DEX-treated rats; MAb, monoclonal antibody.

S0006-2960(96)02110-1

the spectrum was recorded. Erythromycin-induced spectral changes are presented as the magnitude of the 420−390 nm absorbance couple. When MAb 2-13-1 to P450s 3A1/2 (Park *et al*., 1986; Waxman *et al*., 1987) was present, the microsomes were incubated with the antibody (0.082 mg/mL) prior to addition of erythromycin.

CO Flash Photolysis. Reactions were carried out using 0.33 mg/mL microsomes and 20 μM CO, at 23 °C in deoxygenated 0.1 M sodium phosphate (pH 7.5), 20% glycerol (w/v), essentially as previously described (Koley *et al*., 1994). When present, erythromycin (from a 20 mM stock solution in methanol) or MAb 2-13-1 was added to yield a final concentration of 0.3 mM for erythromycin or 0.082 mg/mL for the antibody, respectively, as initial experiments showed that these concentrations produced maximal effects on the CO binding kinetics. The mixture was incubated for 20 min before adding CO and reducing with dithionite. The instrumentation for photodissociation of the P450−CO complex and monitoring of reassociation kinetics at 450 nm was also previously described (Markowitz *et al*., 1992). Static spectral measurements showed that neither erythromycin nor MAb 2-13-1 altered the absorbance of the P450−CO complex at 450 nm. Flash photolysis did not destabilize P450, as static spectral measurements before and after photolysis resulted in the same P450−CO spectra, and successive (up to five) flash photolyses of the same sample yielded the same CO binding kinetics.

Kinetic Data Analysis. Since microsomes contain a multiplicity of P450s, classical multiexponential analysis of CO binding kinetics yields parameters for mixtures of kinetically unresolvable P450s rather than individual P450s. To overcome this problem, we developed a kinetic difference method (Koley *et al*., 1994). Briefly, kinetic progress curves are obtained in the absence and presence of a substrate or other P450 effector. The difference between these curves is computed to generate a kinetic difference curve which is solely dependent on, and can be used to define, the kinetic behavior of the effector-specific P450. Using this approach in the case where the effector perturbs a single P450, kinetic parameters for individual P450s can be obtained by least-squares fitting of the data according to eq 1:

$$\Delta A_t' - \Delta A_t = a_i' e^{-k_i' t} - a_i e^{-k_i t} \quad (1)$$

where $\Delta A_t'$ and ΔA_t are the absorbance changes observed at time t for the reactions in the presence and absence of effector. a_i' and a_i are the absorbance changes, and k_i' and k_i are the pseudo-first-order rate constants for the effector-specific P450 in the presence and absence of effector, respectively. Data were processed and analyzed with RS/1 software (BBN Software Products, Cambridge, MA).

Molecular Modeling of P450s 3A1/2. The models were based on the crystallographic structure of P450cam (Poulos *et al*., 1987), whose coordinates (2cpp) were obtained from the Brookhaven Protein Database. Active site molecular models of P450s 3A1 and 3A2 were generated similarly to that previously reported for P450 3A4 (Ferenczy *et al*., 1989) by replacing residues in the P450cam substrate binding region by those of P450 3A1/2, using previous sequence alignments (Ouzounis & Melvin, 1991; Lewis *et al*., 1992). These residues were first mutated to those of P450 3A1 (Table 3) using the Protein Design module of the Quanta 4.0 program (MSI, Cambridge, MA). The structure of erythromycin was built with this program's Builder application, and its energy was minimized with 1000 steps of steepest descents using CHARMm 22.2. Replacement of camphor by erythromycin was performed via overlay of the N atom in erythromycin with the C5 atom in camphor. After generating a P450 3A1 model, an additional four residues (Table 3) were mutated to yield a P450 3A2 model. Polar hydrogens were used in building both models. The energies of both P450 structures were minimized by 3000 steps of steepest descents followed by 3000 steps of the conjugate gradient method. The adopted-basis Newton Raphson method was further applied until the energy gradient converged to a value of 0.01 kcal mol^{-1} Å^{-1}.

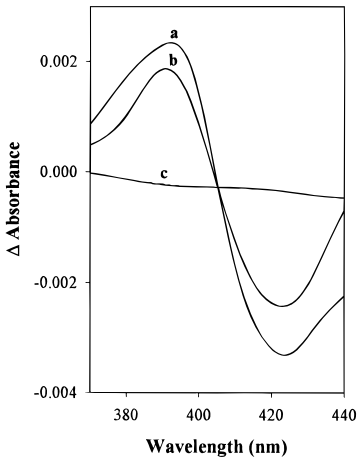

FIGURE 1: Erythromycin-induced difference spectra of (a) DEX-, (b) PB-, and (c) control microsomes. Spectra of microsomes (0.33 mg/mL) were measured in the absence and presence of erythromycin (0.3 mM) as described under Materials and Methods.

RESULTS AND DISCUSSION

The binding of erythromycin to microsomal P450s is most simply illustrated via the difference spectrum generated by the substrate-induced shift in the P450 spin state. Spectral measurements were performed using control microsomes from untreated rats as well as microsomes from rats treated with PB and DEX. The resulting difference spectra are shown in Figure 1. Erythromycin elicited a larger spectral change from DEX- than PB-microsomes, and had little effect on control microsomes. These results show that DEX- and PB-microsomes both contain erythromycin binding P450s, and are consistent with the known inducibility of erythromycin-metabolizing P450s of the 3A subfamily by DEX and PB (Wrighton *et al*., 1985a,b). However, such classical difference spectroscopy neither distinguishes among the different members of this subfamily nor provides details concerning their modes of binding to erythromycin.

In order to distinguish among erythromycin binding microsomal P450s, we employed the kinetics of CO binding as a probe of the P450−erythromycin interaction, as this technique had previously provided insight into substrate interactions with rat P450s 1A1 and 2B1 in their microsomal environment (Koley *et al*., 1994, 1995b). We examined the effect of erythromycin on the kinetics of CO binding to PB-, DEX-, and control microsomes. A typical CO binding curve for PB-microsomes is shown in panel A of Figure 2 along with the curve obtained in the presence of erythromycin. These data show that erythromycin accelerated CO binding to PB-microsomes. In contrast, erythromycin had no effect

Table 1: Effect of Erythromycin and MAb 2-13-1 on the Kinetics of CO Binding to P450s in PB-Microsomes[a]

addition	a_1'	k_1' (s^{-1})	a_1	k_1 (s^{-1})
erythromycin	0.0032 ± 0.0011	425.3 ± 83.5	0.0029 ± 0.0009	9.6 ± 1.6
MAb 2-13-1	0.0023 ± 0.0010	458.1 ± 67.4	0.0022 ± 0.0007	9.9 ± 1.2
MAb 2-13-1 + erythromycin	0.0037 ± 0.0011	497.2 ± 54.0	0.0037 ± 0.0012	9.9 ± 1.0

[a] Parameters were determined according to eq 1. k_1 and k_1' are the pseudo-first-order rate constants, and a_1 and a_1' are the corresponding absorbances in the absence and presence of effector, respectively. Means and standard deviations are derived from at least three experiments.

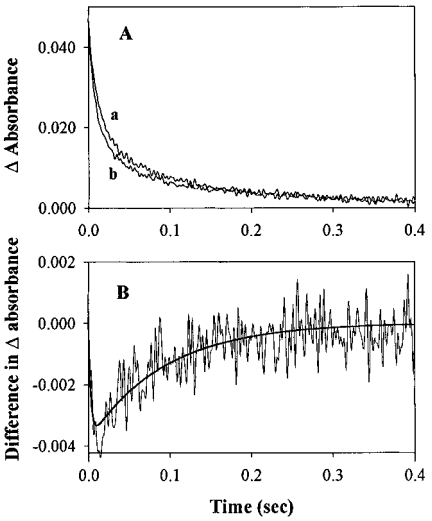

FIGURE 2: (A) Effect of erythromycin on the kinetics of CO binding to P450s in PB-microsomes. Progress curves (a) in the absence and (b) in the presence of erythromycin, respectively. The CO concentration was 20 μM; the erythromycin concentration was 0.3 mM. Microsomal concentration, 0.33 mg/mL in 0.1 M sodium phosphate buffer (pH 7.5) containing 20% (w/v) glycerol; temperature, 23 °C. (B) Kinetic difference curve for the effect of erythromycin on CO binding to PB-microsomes. Difference between traces b and a in panel 2A. The solid line represents the best fit according to eq 1.

on the CO binding kinetics of control or DEX-microsomes (data not shown). Although these experiments were routinely performed using 300 μM erythromycin, the differential effect of this drug on the CO binding kinetics of PB-, DEX-, and control microsomes was also observed using 100 μM erythromycin.

As an additional P450 effector, we assessed the effect of MAb 2-13-1 to P450s 3A1/2. This MAb, as well as a combination of the MAb and erythromycin, accelerated CO binding to PB-microsomes indistinguishably from erythromycin (data not shown). In addition, neither of these effectors changed the CO binding kinetics of DEX- or control microsomes (data not shown). This MAb thus behaved similarly to erythromycin in its effect on PB-, DEX-, and control microsomes.

Interpretation of the above kinetic data in terms of specific P450s is not straightforward since multiple microsomal P450s contribute to the overall reaction, complicating extraction of kinetic parameters for a specific P450. We therefore applied a recently developed kinetic difference method (Koley *et al*., 1994) to these data. This approach evaluates the difference between the kinetic profiles obtained in the presence and absence of an effector and thus effectively cancels out the contributions from P450s that do not bind the effector. Figure 2B shows the resultant difference curve for the data in panel A along with the least-squares curve fit to eq 1. This procedure yields k_1 and k_1', which represent the CO binding rates for erythromycin-specific P450 in the absence and presence of erythromycin, respectively. The kinetic parameters are presented in Table 1 and reveal that erythromycin accelerated the rate of its target P450 by 44-fold (from 9.6 to 425.3 s^{-1}) while MAb 2-13-1 similarly accelerated the rate by 46-fold (from 9.9 to 458.1 s^{-1}). When both erythromycin and MAb 2-13-1 were present simultaneously, the rate was similarly increased by 50-fold (from 9.9 to 497.2 s^{-1}). The similar k_1 values (9.6−9.9 s^{-1}) suggest a common target P450 for erythromycin and MAb 2-13-1.

Table 2: Effect of MAb 2-13-1 on Erythromycin Binding Spectra[a] of DEX- and PB-Microsomes

addition	DEX-microsomes	PB-microsomes
erythromycin	0.0046 ± 0.0011	0.0036 ± 0.0005
erythromycin +MAb 2-13-1	0.0033 ± 0.0002	0.0016 ± 0.0002

[a] The 420−390 nm absorbance differences are presented as the means and standard deviations derived from at least three experiments.

The data thus far reveal that PB-microsomes, but not control or DEX-microsomes, contain a P450 whose kinetics are accelerated by erythromycin. Since difference spectroscopy (Figure 1) showed that erythromycin binds P450s in both PB- and DEX-microsomes, the kinetic data point to differential modes of erythromycin binding to its target P450(s) in these microsomes. The known metabolism of erythromycin by members of the P450 3A subfamily (Wrighton *et al*., 1985a,b) as well as recognition of a P450 with similar kinetic properties by MAb 2-13-1 strongly suggests that P450 3A1 and/or 3A2 are responsible for the erythromycin-induced rate acceleration in PB-microsomes. Distinguishing between these P450s requires consideration of the relative inducibilities of these P450s by PB and DEX: while PB induces both P450s 3A1 and 3A2, DEX induces only P450 3A1 (Gonzalez *et al*., 1986). These considerations indicate that P450 3A2 is the P450 whose rate is accelerated by erythromycin in PB-microsomes.

As an independent probe for erythromycin binding to P450s 3A1 and 3A2, we assessed the effect of MAb 2-13-1 on the substrate-induced difference spectra of PB- and DEX-microsomes, which differ in the relative amounts of these P450s. The MAb inhibited the spectral changes, albeit to different extents (Table 2). While only 28% inhibition was evident with DEX-microsomes, 56% inhibition was observed with PB-microsomes. Addition of the MAb 2-13-1 alone had no effect on either microsomal sample. This differential inhibition of erythromycin binding by the MAb again points to differences in the interaction between erythromycin and its target P450s: the higher inhibition observed with PB-microsomes suggests that the MAb more effectively perturbs the substrate-induced shift in spin state of P450 3A2, which is present in these microsomes but not in DEX-microsomes, than P450 3A1. These results are thus consistent with the CO binding kinetics in demonstrating P450 3A1/2 specific differences in the interaction with erythromycin.

Table 3: Residue Replacements in P450cam Used To Generate Molecular Models of the P450 3A1 and P450 3A2 Active Sites

position in P450cam	residue in P450cam	position in P450 3A1/2	residue in P450 3A1/2[a]	location within secondary structure
86	P	107	D	B-B′ loop
96−101	YDFIPT	113−118	IMGKAV	B′-C loop
119	V	137	F	helix C
181	T	226	F	helix F
184	M	229	L	helix F
193	F	238	I	helix G
196	A	241	F	helix G
200	L	245	S	helix G
241−253	MCGLLLVGGLDTV	299−311	QS(I/V)IFIFAGYE(P/T)T	helix I
295−296	VA	371−372	GN	β-sheet 3
322	Q	399	(S/T)	β-sheet 3
349	T	435	P	before heme peptide
355−360	HLCLGQ	441−446	RNCIGM	heme peptide
395−396	IV	483−484	(L/I)L	β-sheet 5

[a] When the residues in P450s 3A1 and 3A2 differ, their respective amino acids are presented in parentheses, separated by a slash.

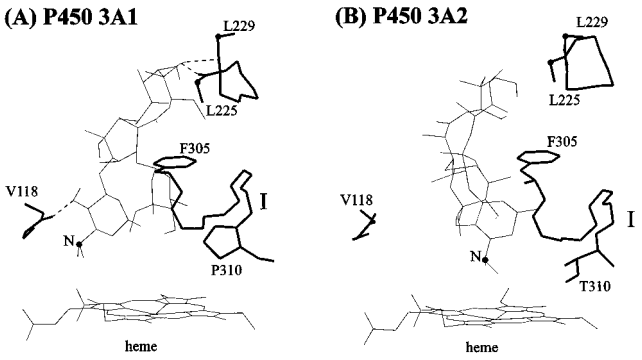

FIGURE 3: Molecular models of the active sites of the erythromycin−P450 complex for P450s 3A1 (A) and 3A2 (B). Identical views are presented for both P450s, based upon superimposition of their heme groups. The heme is at the bottom, while erythromycin is at the center and represented by thin lines. The tertiary amine in erythromycin, immediately above the heme, is shown as a filled circle and denoted by an **N**. Boldface lines represent the protein backbone, including a segment of helix I (residues 305−310). The side chains of F305 and P310 in P450 3A1, as well as F305 and T310 in P450 3A2, are also shown in boldface. In P450 3A1, hydrogen bonds between erythromycin and residues V118, L225, and L229 are represented by dashed lines; for ease of identification, the Cα atoms in L225 and L229 are shown as filled circles.

In order to gain more insight into the differential modes of erythromycin binding to P450s 3A1 and 3A2, we constructed molecular models of these P450s as detailed under Materials and Methods. This approach has proven successful in explaining the substrate specificity of the related P450 3A4 (Ferenczy *et al.*, 1989). The residues in the substrate binding region and their relation to P450cam are presented in Table 3. Although P450s 3A1 and 3A2 exhibit amino acid differences at four positions, three of these involve conservative substitutions: I/V at position 301, S/T at position 399, and L/I at position 483, for P450s 3A1/2, respectively. The remaining difference, occurring at position 310, is a proline in P450 3A1 and threonine in P450 3A2. This nonconservative substitution would be expected to have different conformational implications in this region of helix I. Comparison of the P450 3A1 and 3A2 models indeed reveals fundamental differences in the erythromycin interaction with P450 active site residues, of which the most prominent are differential positioning of erythromycin, and hydrogen bonding of the peptide backbone of residues V-118, L-225, and L-229 with erythromycin in P450 3A1 (Figure 3A) but not P450 3A2 (Figure 3B). These differences are most simply explained by the aforementioned P/T difference, as proline in P450 3A1 bends helix I such that both P-310 and F-305 are positioned closer to the heme iron in P450 3A1 than the corresponding T-310 and F-305 in P450 3A2. This displaces erythromycin, especially the atoms near the tertiary amine which most closely contacts the heme iron, from the region immediately above the heme in P450 3A1. The net result is closer contact and additional hydrogen bonding between erythromycin and the peptide backbone in P450 3A1 but not P450 3A2. The relatively greater flexibility of the erythromycin−P450 3A2 complex is thus consistent with the observed erythromycin enhancement of CO binding to the heme in P450 3A2 but not P450 3A1.

We point out that the P450 3A2 referred to in this work corresponds to PCN2 (Gonzalez *et al*., 1986), PCNc (Halpert, 1988), and PB-1 (Imaoka *et al*., 1988), which are all induced by PB but not by glucocorticoids. On the other hand P450 3A1 corresponds to PCN1 (Gonzalez *et al*., 1986), PCNa (Halpert, 1988), and 6β-4 (Nagata *et al*., 1990).

The substrate-induced increase in CO binding to P450 3A2 extends our previous findings that benzphetamine accelerated CO binding to P450 2B1/2 in PB-microsomes (Koley *et al*., 1994) and polycyclic aromatic hydrocarbons increased the CO binding rate of P450 1A1 in MC microsomes (Koley *et al*., 1995b). This common rate acceleration for P450s in three different families indicates that these substrates primarily exert their effect via a conformational change that enlarges the ligand access channel, rather than by steric factors which would decrease the rate (Koley *et al*., 1995b). In addition, we have for the first time successfully applied CO binding kinetics as a probe for distinguishing P450−substrate interactions for two closely related P450s in a single microsomal sample. We have thus experimentally demonstrated, with the support of molecular modeling, that P450s 3A1 and 3A2 engage in fundamentally different interactions with erythromycin.

REFERENCES

Cooper, K. O., Reik, L. M., Jayyosi, Z., Bandiera, S., Kelley, M., Ryan, D. E., Daniel, R., McCluskey, S. A., Levin, W., & Thomas, P. E. (1993) *Arch. Biochem. Biophys. 301*, 345−354.

Ferenczy, G. G., & Morris, G. M. (1989) *J. Mol. Graphics 7*, 206−211.

Gonzalez, F. J., Song, B.-J., & Hardwick, J. P. (1986) *Mol. Cell. Biol. 6*, 2969−2976.

Halpert, J. R. (1988) *Arch. Biochem. Biophys. 263*, 59−68.

Hostetler, K. A., Wrighton, S. A., Kremers, P., & Guzelian, P. S. (1987) *Biochem. J. 245*, 27−33.

Imai, Y., Iizuka, T., & Ishimura, Y. (1982) *J. Biochem. 92*, 67−75.

Imaoka, S., Terano, Y., & Funae, Y. (1988) *J. Biochem. 104*, 481−487.

Koley, A. P., Robinson, R. C., Markowitz, A., & Friedman, F. K. (1994) *Biochemistry 33*, 2484−2489.

Koley, A. P., Buters, J. T. M., Robinson, R. C., Markowitz, A., & Friedman, F. K. (1995a) *J. Biol. Chem. 270*, 5014−5018.

Koley, A. P., Robinson, R. C., Markowitz, A., & Friedman, F. K. (1995b) *Biochemistry 34*, 1942−1947.

Lewis, D. F. V., & Moereels, H. (1992) *J. Comput.-Aided Mol. Des. 6*, 235−252.

Markowitz, A., Robinson, R. C., Omata Y., & Friedman, F. K. (1992) *Anal. Instrum. 20*, 213−221.

Nagata, K., Gonzalez, F. J., Yamazoe, Y., & Kato, R. (1990) *J. Biochem. 107*, 718−725.

Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., & Nebert, D. W. (1996) *Pharmacogenetics 6*, 1−42.

Ortiz de Montellano, P. R., Ed. (1995) *Cytochrome P-450: Structure, Mechanism and Biochemistry*, 2nd ed., Plenum Press, New York.

Ouzounis, C. A., & Melvin, W. T. (1991) *Eur. J. Biochem. 198*, 307−315.

Park, S. S., Waxman, D. J., Miller, H., Robinson, R., Attisano, C., Guengerich, F. P., & Gelboin, H. V. (1986) *Biochem. Pharmacol. 35*, 2859−2867.

Poulos, T. L., Finzel, B. C., & Howard, A. J. (1987) *J. Mol. Biol. 195*, 687−700.

Ryan, D. E., & Levin, W. (1990) *Pharmacol. Ther. 45*, 153−239.

Shimizu, T., Ito, O., Hatano, M., & Fujii-Kuriyama, Y. (1991) *Biochemistry 30*, 4659−4662.

Telhada, M. B., Pereira, T. M., & Lechner, M. C. (1992) *Arch. Biochem. Biophys. 298*, 715−725.

Waxman, D. J., Lapenson, D. P., Park, S. S., Attisano, C., & Gelboin, H. V. (1987) *Mol. Pharmacol. 32*, 615−624.

Wrighton, S. A., Maurel, P., Schuetz, E. G., Watkins, P. B., Young, B., & Guzelian, P. S. (1985a) *Biochemistry 24*, 2171−2178.

Wrighton, S. A., Schuetz, E. G., Watkins, P. B., Maurel, P., Barwick, J., Bailey, B. S., Hartle, H. T., Young, B., & Guzelian, P. (1985b) *Mol. Pharmacol. 28*, 312−321.

BI962110H