

NON-MICHAELIS-MENTEN KINETICS IN CYTOCHROME P450-CATALYZED REACTIONS

William M. Atkins

*Department of Medicinal Chemistry, University of Washington, Seattle,
Washington 98195-7610; email: winky@u.washington.edu*

Key Words allosterism, drug metabolism, enzyme kinetics, CYP

■ **Abstract** The cytochrome P450 monooxygenases (CYPs) are the dominant enzyme system responsible for xenobiotic detoxification and drug metabolism. Several CYP isoforms exhibit non-Michaelis-Menten, or “atypical,” steady state kinetic patterns. The allosteric kinetics confound prediction of drug metabolism and drug-drug interactions, and they challenge the theoretical paradigms of allosterism. Both homotropic and heterotropic ligand effects are now widely documented. It is becoming apparent that multiple ligands can simultaneously bind within the active sites of individual CYPs, and the kinetic parameters change with ligand occupancy. In fact, the functional effect of any specific ligand as an activator or inhibitor can be substrate dependent. Divergent approaches, including kinetic modeling and X-ray crystallography, are providing new information about how multiple ligand binding yields complex CYP kinetics.

OVERVIEW

The cytochrome P450 monooxygenases (CYPs) are ubiquitous heme-containing enzymes that catalyze an immense range of chemical reactions in prokaryotes, plants, and animals (1, 2). CYPs participate in the biosynthesis of hormones, second messengers, and other natural products. CYPs also dominate xenobiotic detoxification and human drug metabolism. As a result, CYPs are of primary importance in the pharmaceutical industry (3–6). In fact, characterization of the interactions between new drugs and human CYPs is now a routine component of early drug development. An enigmatic behavioral characteristic of CYPs, which has only recently been appreciated fully, is their tendency to exhibit “atypical” steady-state kinetic patterns in vitro, and possibly in vivo. In fact, several excellent recent reviews have focused on this atypical behavior, also referred to as allosterism, thus highlighting its perceived importance (7–11). This review explores some recent observations, while minimizing duplication with the previous reviews, and it considers mechanistic aspects of the atypical kinetics in the context of recently determined X-ray structures.

From a historical perspective, it is interesting that nonhyperbolic CYP kinetics were documented as early as the 1980s (12–14), but this received little attention.

Subsequently, Korzekwa et al. (15, 16) provided thoughtful accounts of the relationship between atypical kinetics observed with CYP3A4 and the possibility that multiple ligands could occupy the active site simultaneously. Today, it is widely accepted that several CYP isoforms, including 3A4, 1A2, 2E1, 2D6, and 2C9, can exhibit nonartefactual atypical kinetics *in vitro*. Furthermore, it is highly likely that the kinetic behavior is related in some cases to simultaneous binding of multiple ligands to a single active site, as elaborated here. Also, it is notable that experimental evidence for multiple ligand binding to CYP101 (P450cam) was provided by Sligar and coworkers as early as 1994, based on NMR approaches (17).

In contrast to the widespread acceptance of this behavior *in vitro*, examples of *in vivo* kinetics that deviate from Michaelis-Menten kinetics are sparse. Examples include interactions between diclofenac and quinidine in monkeys (18), carbamazepine and felbamate in humans (19), and a marginal effect between flurbiprofen and dapsone in humans (20). Although examples of *in vivo* allosteric CYP interactions are limited, they are likely to become more widespread as awareness of their possibility increases and with improved analytical methods. Regardless, the apparent universality of allosteric effects across several CYP isoforms and many drugs *in vitro* (21–26) demands a mechanistic understanding that could dramatically enhance *in vitro* predictability of drug-drug interactions. Presumably, this understanding would translate directly into increased predictive power *in vivo*.

The behavior of CYPs also is extremely important from an academic perspective because it demands significant revision of the paradigms of traditional allosteric enzymes. Nearly all allosteric proteins are multisubunit oligomers (27–29). Moreover, allosteric behavior of normal enzymes can be rationalized within their biological niche as a mechanism for achieving metabolic control through highly specific molecular recognition. In contrast, although CYPs may sample several aggregation states (30, 31), they can exhibit non-Michaelis-Menten kinetics under conditions in which they are predominantly monomeric. Although CYP-CYP, CYP-reductase, and CYP-Cyt b_5 interactions may provide an additional mechanism by which allosteric effects occur, they are considered only briefly here.

Also, according to traditional paradigms, allostery requires specificity. However, as detoxification enzymes, CYPs do not utilize specific molecular recognition. Rather, they are extraordinarily substrate diverse. The resulting nonspecific allostery is also of academic interest because it deviates from well-understood allostery of substrate-specific enzymes. It is not clear what biological advantage, if any, is gained from the allostery of CYPs, wherein some toxic substrates are metabolized more efficiently and others less efficiently in the presence of allosteric effectors. Both the mechanism and the biological purpose of CYP allostery are challenging (32).

What Are Atypical Kinetics and Why Do They Matter?

At the simplest level, atypical has become synonymous with a wide range of situations wherein nonhyperbolic plots of velocity versus $[S]$ are obtained. Common

types of CYP allostery are summarized below. Throughout this review, the term allostery is used interchangeably with atypical kinetics because both require formation of a ternary complex, $[CYP \bullet S \bullet S]$ or $[CYP \bullet S \bullet E]$, where S and E are substrate and effector, respectively, and these complexes have kinetic properties that differ from $[CYP \bullet S]$.

The implication of nonhyperbolic kinetics is that the Michaelis-Menten steady state model is insufficient to describe drug clearance, CL_{int} . The Michaelis-Menten model describes the velocity of product formation, v , as

$$v = \frac{V_{max} K_M}{[S] + K_M},$$

where V_{max} and K_M have their usual meanings. When the Michaelis-Menten relationship does apply, the clearance of a drug may accurately be estimated, in principle, from the V_{max}/K_M . This parameter, approaches the intrinsic drug clearance ($Cl_{int} = v/[S]$) or the slope of a hyperbolic Michaelis-Menten plot at low $[S]$. Furthermore, the in vitro clearance is frequently used to estimate in vivo clearance, after appropriate scaling for the CYP capacity of the liver or other tissue. Obviously, the accuracy of the in vivo prediction is limited by the accuracy of the model used to extract metabolic velocities from the in vitro data (6, 9).

TYPES OF ALLOSTERIC KINETICS

Homotropic Effects

Allosteric effects may result from homotropic substrate interactions in which the [substrate] versus velocity curve is nonhyperbolic, as summarized previously by others (7, 9, 11, 16) and as schematized in Figure 1. Homotropic effects may yield velocity versus [substrate] curves that are either sigmoidal (also called autoactivation), biphasic with continuously increasing velocity at high [substrate] (implying a low-affinity second substrate site and referred to as biphasic), or concave downward with a decrease in velocity at high [substrate] after an initial hyperbolic increase (substrate inhibition). Apparent biphasic kinetics with decreasing rate at high [substrate] may be observed also with product inhibition, but this does not represent allosteric kinetics by any definition, because it does not require simultaneous binding of multiple ligands. Without quantitative models of homotropic effects, in vitro kinetics will be inaccurately parameterized and in vivo drug clearance may be estimated incorrectly.

Heterotropic Effects

Alternatively, heterotropic effects occur when one drug alters the CYP interactions with a second drug, either activating or inhibiting the rate of product formation (33, 34). Here, the drug acting as substrate may yield classic hyperbolic velocity versus [substrate] curves, but the second drug changes the parameters V_{max} or

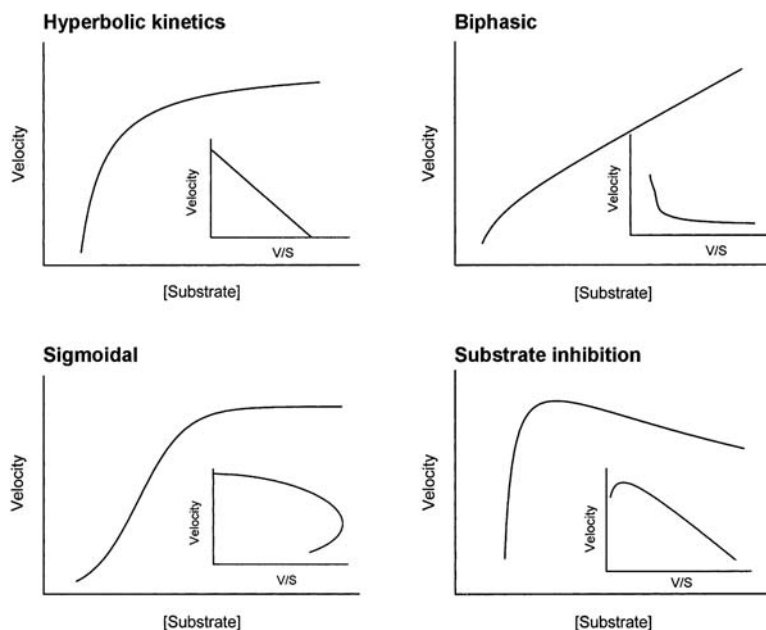


Figure 1 Velocity versus [substrate] plots depicting possible kinetic profiles with homotropic effects. Top left: hyperbolic kinetics with no allosterism. Bottom left: sigmoidal kinetics resulting from homotropic activation. Top right: biphasic kinetics resulting from a low-affinity second ligand site. Bottom right: substrate inhibition, wherein binding of the second substrate decreases V_{\max} . In each case, the inset depicts an Eadie-Hoffstee plot (V versus $V/[S]$) corresponding to the velocity curves.

K_M , or it induces nonhyperbolic behavior. Alternatively, if the substrate alone exhibits nonhyperbolic kinetics, the heterotropic effector may restore hyperbolic kinetics or maintain them but change the shape of the velocity versus $[S]$ curve or shift it along the $[S]$ axis. A further case, which can occur through heterotropic interactions when there is either hyperbolic or atypical kinetics, is partial inhibition, wherein an effector bound at the same time as substrate may partially inhibit the enzymatic reactions. Partial inhibition may also be observed for the homotropic substrate inhibition mentioned above. Both the heterotropic and homotropic partial inhibition cases are incompatible with simple competitive inhibition and require allosteric interactions of some type.

Substrate and Effector Dependence

A particularly interesting aspect of CYP allostery is the context dependence of heterotropic effects. Any individual compound may activate CYP-dependent metabolism of one drug, yet inhibit or have no effect on the metabolism of a

second drug cleared by the same CYP isoform. Equally important, a single effector molecule may change from an activator at low concentration to an inhibitor at higher concentration. Thus, the behavior of any effector compound depends on the substrate that is being metabolized, as well as on the concentrations of effector and substrate (35, 36). For example, testosterone inhibits with different apparent potencies the metabolism of terfenadine and midazolam by CYP3A4. In contrast, testosterone does not inhibit metabolism of nifedipine, but terfenadine does. Moreover, testosterone itself is a substrate for CYP3A4, and its metabolism is partially inhibited by nifedipine (35–37). Similar nonreciprocal effects have been observed with CYP3A4-dependent interactions between α -naphthoflavone (α -NF) and aflatoxin B1. The α -NF activates metabolism of the aflatoxin, but the latter has no effect on the metabolism of aflatoxin B1 (38). Houston and coworkers have initiated the categorization of various CYP3A4 substrates into subgroups based on kinetic traits and heterotropic effects in which they participate (37). Clearly, the behavior of any substrate or inhibitor depends on what other compounds are present, and this is a major challenge for describing CYP allosterism. Moreover, the heterotropic effects of any ligand pair are CYP isoform dependent. For example, the highly homologous CYPs 3A4 and 3A5 exhibit different heterotropic interactions for several ligand pairs (39).

At least two molecular mechanisms may contribute to context-dependent ligand effects. The first is ligand-dependent conformational change, wherein the enzyme is sufficiently flexible that each combination of ligands induces a different enzyme conformation with different kinetic properties. This contrasts the case with substrate-specific enzymes in which only a few specific conformations are coupled to a few specific ligands (27, 28). If a wide range of ligand-dependent conformational space is available to the enzyme, this will promote context-dependent ligand effects (40).

Based on flash photolysis and CO recombination experiments, it was proposed that slowly equilibrating conformations of a single CYP isoform could differentially interact with ligands (41–43). This possibility has been reconsidered based on studies using hydrostatic pressure (44). Such persistent conformations could cause allosteric kinetics, even in the absence of multiple ligand binding, just as mixtures of isoforms can yield non-Michaelis-Menten kinetics. In contrast, ligand-induced conformational changes, in the absence of nonequilibrating conformational states, cannot cause allosteric kinetics. In the absence of persistent conformations with different properties, ligand-dependent conformational change is neither a necessary nor sufficient condition for allosteric kinetics. Multiple ligand binding, however, is a necessary but not a sufficient condition for allosterism. Conformational change provides one mechanism by which multiple ligand binding can yield complex kinetics (40).

Conformational changes induced by nonactive site ligands may also contribute. For example, Schrag & Wienkers (45) found that addition of Mg^{2+} to CYP3A4 incubations with the substrate pyrene resulted in the conversion from positive homotropic kinetics to hyperbolic patterns, and this correlated with a change in

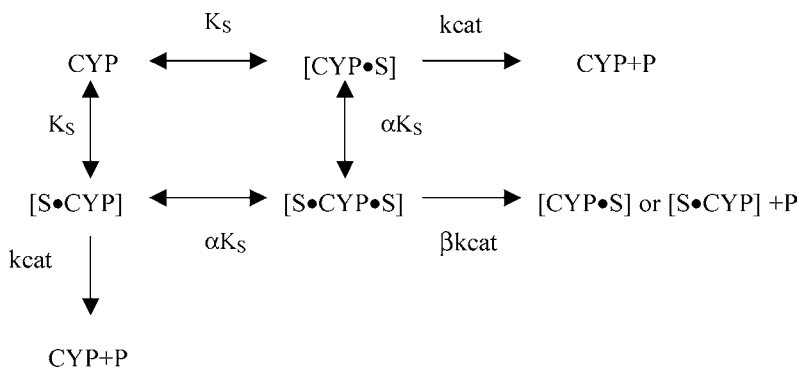
reiosselectivity of heme adduction by phenyldiazene. Similarly, carbonate anion, but not other common buffer salts, altered the O-dealkylation activity of CYP2D6, but not its N-dealkylation activity (46). Possibly, different oxidative intermediates, either iron-peroxy or iron-oxo $[\text{FeO}]3+$, are differentially populated owing to subtle conformational changes induced by carbonate. CYP3A4 is particularly sensitive to nonactive site ligands and buffer conditions (47, 48). As noted above, cytochrome b_5 (Cyt b_5 ; 49), possibly apo-Cyt b_5 (50–53), or possibly the CYP reductase (54, 55) may also contribute to the conformational landscape of CYPs, and thus provide additional mechanisms of allostereism. In fact, each of these effects may be ligand- and CYP isoform-dependent, as well (56). For example, Cyt b_5 -CYP4B7 interactions are modulated differently by various CYP ligands.

A second mechanism for context-dependent effector behavior is direct ligand-ligand interactions. Few proteins allow multiple ligands to bind in a single active site that promotes direct hydrophobic bonds, electrostatic effects, or hydrogen bonds between the ligands. As elaborated further below, X-ray crystal structures of CYP_{eryF} clearly support this possibility for CYPs (57). Also, a recent computational docking study based on a homology model for CYP3A4 suggests the possibility of hydrogen bonds between the amide groups of two carbamazepine molecules simultaneously bound (58). When one molecule is bound, it may directly contribute to the binding site for a second ligand, even if no significant protein conformational change takes place. Each ligand can change the active site constraints directly, wherein the second ligand can exploit handles presented by the first ligand. If ligand-ligand interactions are stronger than ligand-protein interactions, they may control orientation of the complex within the active site. Evidence for strong ligand-ligand interactions is limited, but one example is the aromatic stacking of pyrenes simultaneously bound to CYP3A4 (59). The important point is that direct ligand-ligand contacts might provide a mechanism for context-dependent allosteric effects.

THE PROGRESSION OF KINETIC MODELS

To improve in vitro-in vivo correlations, several steady-state kinetic models have been developed that account for homotropic interactions and the possibilities that (a) identical substrate molecules may have different affinities for free CYP versus $[\text{CYP}\bullet\text{S}]$, thus yielding two K_S values, and (b) the $[\text{CYP}\bullet\text{S}]$ and $[\text{CYP}\bullet\text{S}\bullet\text{S}]$ complexes may yield product at different rates, thus yielding different V_{max} values for each complex. Similarly, for heterotropic interactions the effector may have different affinities for CYP versus $[\text{CYP}\bullet\text{S}]$, thus yielding two K_I or K_A values. It is beyond the scope of this review to summarize all of the possible models that may describe CYP atypical kinetics, but it is instructive to consider a few as a means to highlight the strengths and weaknesses of kinetic modeling in general. For a more comprehensive survey of multisite kinetic models, the reader is referred to other recent reviews (7–10) or to Segel's classic book (60), which has become a standard reference for those doing CYP research.

Scheme 1 depicts the simplest generic model for homotropic effects, which has been used by numerous investigators. Here S is substrate; P is product; K_S is the affinity of the CYP for substrate; k_{cat} is the rate of formation of product from the $[\text{CYP}\bullet\text{S}]$ complex; and V_{max} is defined as $2k_{\text{cat}}/[\text{E}]_t$, where $[\text{E}]_t$ is the total enzyme concentration. The equation describing the fraction of maximal velocity at any $[\text{S}]$ is



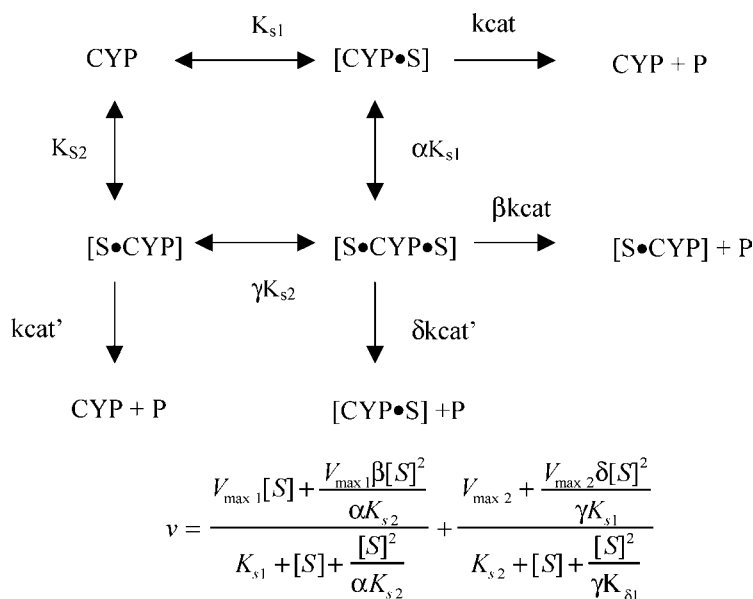
$$\frac{v}{V_{\text{max}}} = \frac{\frac{[\text{S}]}{K_S} + \frac{\beta[\text{S}]^2}{\alpha K_S^2}}{1 + \frac{2[\text{S}]}{K_S} + \frac{[\text{S}]^2}{\alpha K_S^2}}$$

Scheme 1

In this model, the substrate can bind in either of two sites, as indicated by $[\text{S}\bullet\text{CYP}]$ versus $[\text{CYP}\bullet\text{S}]$, and these complexes have identical dissociation constants for substrate, K_S , and identical k_{cat} values for product formation. The widespread use of the two-site model in Scheme 1 in the CYP literature, or variations of it, reflects the popular belief that at low occupancy, the bound ligand is localized in a discrete binding site, rather than sampling all parts of the active site, i.e., that $[\text{CYP}\bullet\text{S}]$ and $[\text{S}\bullet\text{CYP}]$ are two different molecular species that can only interconvert via substrate dissociation and rebinding, but neither is preferred thermodynamically. Regardless, binding of the second substrate leads to a complex $[\text{CYP}\bullet\text{S}\bullet\text{S}]$ with different kinetic properties, αK_S and βk_{cat} . Here, α is the effect that the first substrate has on the K_S for the second substrate, and β is the effect that the presence of the first substrate has on the k_{cat} for the second. Thus when either $\alpha < 1$ or $\beta > 1$, positive homotropic cooperativity may be apparent and velocity curves will be sigmoidal. Alternatively, if $\alpha > 1$ the curves may appear biphasic, and if $\beta < 1$ substrate inhibition will be evident. The detailed shape of the corresponding velocity versus $[\text{S}]$ plot will be determined by K_S , k_{cat} , α , and β . Although this model has been extremely useful for conceptualizing homotropic allosteric kinetics for CYPs, it is inherently oversimplified because of the kinetic equivalence of $[\text{S}\bullet\text{CYP}]$ and $[\text{CYP}\bullet\text{S}]$, which form with equal apparent affinities

and generate product at equivalent velocities. Such kinetic symmetry is useful for reducing the complexity of the system, but in the absence of any structural symmetry of the CYP enzymes, it is likely to be an inaccurate depiction of what really occurs at the molecular level. This model is more suitable for normal allosteric enzymes with multiple copies of identical active sites.

A more likely scenario, possibly, is that multiple $[\text{CYP}\bullet\text{S}]$ complexes are formed, with multiple orientations of S in rapid equilibrium, $[\text{S}\bullet\text{CYP}]$ and $[\text{CYP}\bullet\text{S}]$, which form with different affinities and different k_{cat} values associated with them, as in Scheme 2. In this case, the system behaves like a mixture of enzyme-substrate complexes, with the fractional contribution of $[\text{S}\bullet\text{CYP}]$ versus $[\text{CYP}\bullet\text{S}]$ determined by K_{s1}/K_{s2} , and with the reaction velocity equation shown. Here $V_{\text{max}1} = k_{\text{cat}}[\text{E}_T]$, $V_{\text{max}2} = \beta k_{\text{cat}}[\text{E}_T]$, and $[\text{E}_T]$ is the total enzyme concentration, and α , δ , and γ are scaling factors that modulate the K_{s1} , K_{s2} , and k_{cat}' , respectively. The parameters $V_{\text{max}1}$ and $V_{\text{max}2}$ are virtual parameters that represent the rate of product formation if all of the enzyme could be forced into the $[\text{CYP}\bullet\text{S}]$ or the $[\text{S}\bullet\text{CYP}]$ states; however, this cannot actually occur. Note that the number of fitting parameters has increased to eight (K_{s1} , K_{s2} , k_{cat} , k_{cat}' , α , β , γ , δ). This model was used recently to explore the metabolism of verapamil by CYP3A4 (61). It was found that formation of several metabolites could be described by Scheme 2, wherein negative cooperativity was associated with β and δ values less than 1 and α and γ values greater than 1.



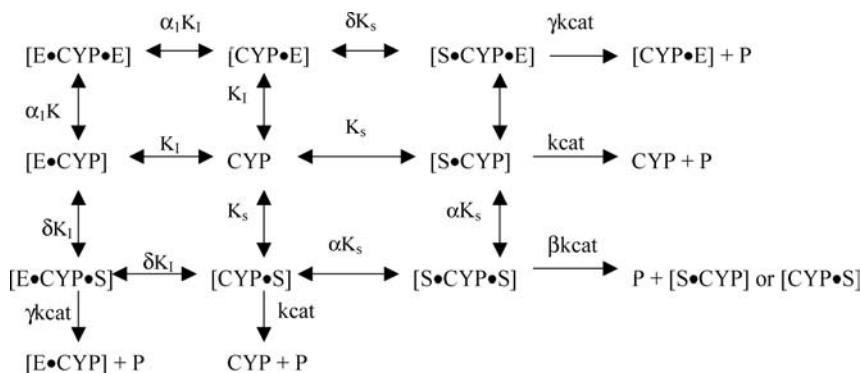
Scheme 2

Comparison of Schemes 1 and 2 reveals the compromise that must accompany a choice between models. The model in Scheme 1 suffers from potentially unrealistic features, such as the existence on a single unsymmetrical CYP enzyme

with physically distinguishable, kinetically indistinguishable, binding sites. The more realistic and general model in Scheme 2 suffers from the possibility of over-parameterization if a sufficient number of data points are not included. With two experimental observables (velocity and $[S]$) and ten fitting parameters, the recovered parameters for the best fit may not represent a unique solution. There are likely to be other combinations of parameters that yield a fit that is very nearly as good based on standard statistical criteria. Although the curve-fitting procedures yield standard errors or standard deviations for each individual parameter, they do not indicate how the overall goodness of fit for the model varies with each parameter. To date, no kinetic models have included a rigorous analysis of the uniqueness of the best fit or the sensitivity of the fit to parameter changes as is routinely performed with complex fluorescence decay data (62).

For the case of homotropic effects, Shou and coworkers have used a variation of these schemes to provide a detailed survey of several examples of substrate inhibition, including CYP1A2-catalyzed O-deethylation of ethoxyresorufin, CYP2C9-dependent hydroxylation of celecoxib, O-demethylation of dextromethorphan by CYP2D6, and other CYP-drug combinations (63). This analysis provides an extended description of substrate inhibition, which is observed with many combinations of CYPs and substrates.

Heterotropic effects are significantly more problematic to model owing to the additional parameters required to describe the effector interactions with the enzyme in multiple states, in addition to the substrate-enzyme interactions. For example, the simplest general heterotropic model that allows for multiple binding of both substrate and effector is shown in Scheme 3, along with the velocity equation.



$$\frac{v}{V_{\max}} = \frac{\frac{[S]}{K_S} + \frac{\beta[S]^2}{\alpha K_S^2} + \frac{\gamma[S][I]}{\delta K_S K_I}}{1 + \frac{2[S]}{K_S} + \frac{[S]^2}{K_S^2} + \frac{2[S][I]}{\delta K_S K_I} + \frac{2[I]}{K_I} + \frac{[I]^2}{\alpha_1 K_I^2}}$$

Scheme 3

The model in Scheme 3 accounts for multiple substrate binding with homotropic effects, heterotropic binding, and multiple effector (E) binding with homotropic

effects. Differential affinity of both substrate and effector is caused by the presence of another effector or inhibitor, and differential effects on k_{cat} are allowed. This model, and variations, have been applied to examine context-dependent heterotropic effects (7, 36, 37, 64, 65).

The steady-state kinetics provide a powerful tool for conceptualizing the possible mechanisms responsible for the variety of atypical kinetics observed, and they accurately predict metabolism rates. The examples described above demonstrate the necessary compromise between a level of complexity adequate to describe the experimental data and the need to use many data points to avoid overparameterization.

How Many Ligands Bind?

Several investigators have suggested that more than two ligands can bind simultaneously with the active site of CYP3A4 (23, 25, 37, 66–68). This is based on inhibition studies and site-directed mutagenesis approaches. For example, it was found that a peptide inhibitor of CYP3A4 yields differential K_I values with respect to different products from midazolam, which is presumed to bind at two different subsites. The authors propose two separate binding sites for midazolam, a site for testosterone that overlaps one of the midazolam sites and a site for α -NF in the active site of CYP3A4 (68). Also with CYP3A4, kinetic modeling suggests the presence of three sites wherein diazepam and testosterone each bind to specific sites and both can bind to the third site (66, 69, 70). Perhaps the strongest support for a third binding site on a single CYP molecule comes from inhibitor studies in which plots of fractional inhibition versus $[S]$ change slope with changing $[I]$. That is, with increasing inhibitor concentration the slopes of relative velocity versus substrate, for example, become greater (37). The change in slope indicates a cooperative interaction between inhibitor molecules owing to simultaneous binding of inhibitors. However, the inhibition is not purely competitive, implying that two inhibitors and one substrate can simultaneously bind, $[CYP \bullet S \bullet I \bullet I]$. Similarly, a scenario diagnostic for two S molecules bound simultaneously with an inhibitor is the persistence of sigmoidal kinetics (positive homotropic) even at saturating concentrations of inhibitor. If the inhibitor shifts the curve to higher $[S]$ without converting it to a hyperbolic curve, then a $[CYP \bullet S \bullet S \bullet I]$ complex is implied. It will be particularly interesting to search for direct evidence of three ligands simultaneously binding within a CYP active site.

STRUCTURAL AND MECHANISTIC ASPECTS

Although the kinetic models can accurately predict rates of product formation, they do not address directly specific mechanistic aspects of multiple ligand binding. This is because K_M , k_{cat} , α , β , etc., are nearly impossible to interpret in molecular terms given the complexity of the CYP reaction cycle. For example, there may be no

cooperativity or negative cooperativity at the level of ligand binding, per se, and positive cooperativity on the ligand-dependent spin state shift (69, 70). It is unclear how these results relate mechanistically to changes in the K_s parameter used in kinetic models, as described above.

Several conceptual models that are based on multiple ligand binding to a single active site remain viable. It is possible that there are discrete and static subsites within the large active site, and each subsite has its own personality. Each subsite may have a characteristic affinity for each ligand and hold it in a preferred orientation, which is static on the timescale of oxidative turnover (66, 68, 71, 72). In this extreme case, multiple ligands bind sequentially to the highest affinity available subsite and then to the lowest affinity site. From their respective binding sites, ligands may alter the metabolism of other ligands by inducing conformational changes, causing minor shifts in the distances between oxidizable sites on the drug and the heme iron-oxo species, or by altering relative uncoupling rates to nonproductive formation of superoxide. Support for discrete static subsites has come, partly, from mutagenesis studies as championed by Halpert and coworkers (69–72). For example, midazolam appears to be an example of this type of ligand wherein distinct subsites within the active site are responsible for the formation of the 1'-hydroxy- versus 4-hydroxy-midazolam products. The results with midazolam support the unlikely suitability of Scheme 1 as a model; the different binding subsites for midazolam, if they exist, are proposed to have different V_{\max} 's and substrate affinities, and to even generate different products.

Alternatively, the large active site may be fluid and multiple bound ligands may sample several subsites within the large active site, either dynamically or through a static heterogeneity. Evidence for a fluid active site has come mainly from kinetic deuterium isotope effects. Trager and coworkers have provided numerous examples, and appropriate theory, to understand CYP substrates as moving within the active site and presenting several points of oxidation on a single molecule to the $[\text{FeO}]^{3+}$ intermediate (73–75). In effect, they have varied distances between deuterium- and hydrogen-bearing benzylic methyl groups on ring systems of increasing size. The substrate size could be correlated to the extent of masking of the isotope effect (k_H/k_D), as expected if smaller substrates rapidly reorient within the active site. In principle, this approach could be used to determine the effect of multiple ligand binding on substrate dynamics. Both homotropic and heterotropic effects should modulate the magnitude of the observed isotope effects if they change the effective size of the active site. In fact, deuterium isotope effects have already been used to observe multiple ligand binding to CYP BM3 (CYP102), wherein deuteration of laurate caused a change in the regioselectivity of hydroxylation of palmitate (76). This could only occur if both ligands bound simultaneously. As with the kinetic modeling, the mechanism of multiple ligand binding within CYP active sites may be context-dependent. Some ligands may dynamically or statically sample several parts of the active site, whereas others may occupy well-defined subsites.

An additional mechanistic complexity arises from the possibility that uncoupling may occur. Many [CYP•substrate] complexes generate superoxide anion, hydrogen peroxide, or water as the reduction products of O₂ at the expense of substrate oxidation (77–79). As competing processes, these pathways decrease the apparent k_{cat} for product (oxidized substrate) formation. Hutzler and coworkers recently demonstrated that the branching ratios between substrate oxidation and uncoupling could be altered by the addition of an effector (80). Specifically, dapsone caused a decrease in the uncoupling of [CYP2C9•flurbiprofen], thus explaining the activation by dapsone of flurbiprofen metabolism. Allosteric effects on coupling are likely to be common.

Recent elegant strategies, and nearly heroic efforts, have led to the successful solubilization of several mammalian CYP isoforms by engineering the membrane binding regions (81–85). Specifically, the N-terminal membrane anchor has been partially truncated, and the F-G region, thought to be a peripheral membrane-binding patch, has been mutated or chimerized in several ways. The resulting soluble proteins have been crystallized and they have afforded X-ray structural models. The structures provide an obvious tool to look for mechanistic clues concerning the atypical kinetic behavior described above, so they are briefly discussed here.

First, it is useful to highlight an important relevant aspect of the crystal structure of the bacterial CYP_{eryF} as it relates to atypical kinetics. Cupp-Vickery and coworkers provided a crystal structure of CYP_{eryF} complexed with either androstenedione or 9-amino-phenanthrene (57). For both, clear electron density revealed the simultaneous presence of two molecules in the active site cavity proximal to the heme. Interestingly, for both complexes, direct ligand-ligand interactions were observed, suggesting a possible contribution to positive homotropic cooperativity as noted above. Also, for both cases, only one of the bound ligands appears to be in a location that would allow metabolism (of course, the 9-aminophenanthrene forms a 6-coordinate nitrogen-ligated complex that is not expected to be metabolized, but if the exocyclic amine were not present, only one of the phenanthrene rings would be sterically accessible to the heme iron-oxo complex). In short, the structures demonstrated for the first time the possibility that two ligands could simultaneously occupy a CYP active site, although only one could be a target for oxidation with reorientation on the timescale of turnover.

The first X-ray structure of a mammalian CYP was that of rabbit CYP2C5 (86). Perhaps the single most important conclusion resulting from this structure was that mammalian CYPs are structural homologs of the bacterial CYPs, for which a wealth of structural data already exists (87–91). This was not a surprising conclusion, but its experimental validation was comforting and important. Subsequent structures of the engineered rabbit CYP2C5 have allowed for a comparison of ligand-free enzyme with complexes of diclofenac (92) or a benzenesulfonamide (DPZ) derivative (93). This comparison reveals the likely ligand-dependent conformations of the protein in the B'-C loop and the F-G loop, and it has prompted the use of induced fit models for discussing CYP dynamics. These results extend to the mammalian CYPs the notion that ligand binding alters the conformational dynamics, particularly in these regions, as expected from the bacterial structures.

In addition, the complex with DPZ suggests the possibility that the substrate binds in two different orientations, with each providing electron density in partially occupied complexes. Interestingly, neither orientation, including the one with substrate several angstroms from the heme iron and expected to yield less product, revealed water bound to the iron. Thus, the crystallographic models are at odds with solution spectroscopy, wherein addition of diclofenac or DPZ to 2C5 does not induce a high spin transition (92, 93). Crystallographic evidence for multiple binding orientations of a single substrate has also been provided with a [CYP101• nicotine complex] (94). Here the major substrate orientation is nonproductive, with a substrate-heme coordinate bond. Upon reduction and stabilization with CO, the nicotine orientation changes to a productive one, consistent with the metabolism of nicotine. This clearly demonstrates the complexity of simple ligand binding with CYPs.

A structure of human CYP2C9 was recently solved, and reveals a striking behavior that is particularly relevant to CYP allostery (95). The [CYP2C9•warfarin] complex positions warfarin in a corner of the active site, far from the heme iron, and in an orientation inconsistent with the experimentally established regiospecificity of warfarin hydroxylation (96). Based on this complex, the authors performed docking experiments to demonstrate that there is ample room within the active site for two ligands, suggesting that productive and nonproductive binding modes may be available for any substrate, and that the relative population of these modes will change with single occupancy versus multiple occupancy, i.e., [CYP•S] versus [CYP•S•S]. For example, it is tempting to speculate that the first ligand can merely take up space, without being a good target for oxidation, as suggested by the crystal structure. Williams et al. note (see 95 for supporting information) that in their attempt to obtain a ligand-free structure they observed undefined electron density in the active site directly adjacent to the heme iron. Although they were unable to identify the species yielding this density, it demonstrates that this part of the active site is accessible to ligands, as required for metabolism. This amplifies the possible preference of warfarin to not bind near the heme iron. In this case, the bound warfarin may only occasionally sample portions of the active site closer to the heme iron. However, at higher occupancy [CYP•S•S], the second ligand is forced into more productive binding modes, thus providing a structural model for positive homotropic or positive heterotropic effects. However, it should be emphasized that warfarin does not demonstrate atypical kinetics when metabolized by CYP2C9, so this intuitive model is either incorrect or oversimplified.

A structure of CYP2B4 provides evidence for the possible contribution of conformational dynamics in atypical kinetics (97). Owing to structural rearrangements in the B' region and the F-G loops, an open conformation is captured in the crystal state and stabilized by dimerization with a second CYP2B4. In fact, the cleft is sufficiently pronounced to allow heme ligation by His-226 of the other CYP2B4 molecule of the dimer. Importantly, evidence for this dimer existing in solution as well is presented. Comparison with the CYP2C5 structures suggests a range of conformations in the B'-C and F-G regions, including a significantly altered conformation with a very large solvent-exposed crevice above the heme. Speculatively, the two structures, CYP2B4 and CYP2C5, may provide benchmarks for

the range of conformations accessible to any single isoform and underscore the extensive flexibility of the protein in these regions.

No structure of mammalian CYPs has revealed two ligands simultaneously bound at the active site, so these structures have not provided any direct clues about the mechanisms of atypical kinetics. In fact, a recent structure of human 2C8 reveals a nonsubstrate palmitic acid binding site that is peripheral to the active site, which could modulate catalytic properties (98). The apparent fatty acid binding site includes determinants within the F', G', and G helices, which are contiguous with ligand-sensitive regions of other isoforms, and this site communicates with substrate binding regions. At one level, this may be taken as evidence for a true allosteric binding site remote from the heme iron and the active site *per se*. However, without significant rearrangement, it is not obvious that this site could accommodate hydrophobic drugs, and it is unlikely to be responsible for the heterotropic effects discussed above. Rather, it supports the importance of nonsubstrate ligand-dependent conformational effects (47–54). Most recently, crystal structures of CYP3A4 have been solved, and they further complicate the existing paradigms (99, 100). Specifically, a structure of CYP3A4 complexed with progesterone indicates that this ligand also binds at a site remote from the active site, which suggests a separate “allosteric” site (99). Interestingly, with either progesterone at this remote site or with metyrapone coordinated to the heme iron, no dramatic ligand-induced conformational changes are evident, compared to the ligand-free CYP3A4 (99). However, the dimensions of the active site “cavity” are significantly greater for CYP3A4 than the 2C isoforms in the immediate vicinity of the heme (100). Thus, the possibility of multiple ligand binding within the active site remains. The available structures have not proven the central assumption of current models for mammalian CYP allostereism: multiple ligand binding within a single active site. However, collectively the available structures provide fundamentally important insights. For example, the presence of warfarin in a nonproductive binding mode that limits the space available for a second ligand on CYP2C9, if it binds, clearly demonstrates that each ligand can present new surfaces and handles to a second ligand, and direct ligand-ligand interactions can contribute, as suggested for pyrene binding to CYP3A4 (59).

CONCLUSIONS

Atypical steady-state kinetics are now commonly observed among CYPs directly involved in xenobiotic and drug metabolism for a wide range of drug structures. In the past few years, the notion that multiple ligands bind within a single active site of mammalian CYPs has evolved from an interesting speculation to a likely possibility for many CYP-drug combinations. Of the experimental approaches used to understand complex CYP kinetics, including kinetic modeling, crystallography, and spectroscopic approaches, none alone are likely to reveal the mechanism of CYP allostereism. Rather, there are likely to be multiple mechanisms spanning different combinations of CYP isoform, substrate, and effector. An understanding

of both ligand and protein dynamics will be necessary to fully understand CYP kinetics. The combination of these approaches may be required to learn any general rules of CYP allosterism, if they exist.

**The Annual Review of Pharmacology and Toxicology is online at
<http://pharmtox.annualreviews.org>**

LITERATURE CITED

1. Guengerich FP. 2002. Cytochrome P450 enzymes in the generation of commercial products. *Nat. Rev. Drug Discov.* 1:359–66
2. Coon MJ, Ding XX, Pernecky SJ, Vaz AD. 1992. Cytochrome P450: progress and predictions. *FASEB J.* 6:669–73
3. Wienkers LC. 2001. Problems associated with in vitro assessment of drug inhibition of CYP3A4 and other P-450 enzymes and its impact on drug discovery. *J. Pharmacol. Toxicol. Methods* 45(1):79–84
4. Szklarz GD, Halpert JR. 1998. Molecular basis of P450 inhibition and activation: implications for drug development and drug therapy. *Drug Metab. Dispos.* 26(12):1179–84
5. MacCoss M, Baillie TA. 2004. Organic chemistry in drug discovery. *Science* 303(5665):1810–13
6. Venkatakrishnan K, Von Moltke LL, Greenblatt DJ. 2001. Human drug metabolism and the cytochromes P450: application and relevance of in vitro models. *J. Clin. Pharmacol.* 41(11):1149–79
7. Shou M. 2002. Kinetic analysis for multiple substrate interaction at the active site of cytochrome P450. *Methods Enzymol.* 357:261–76
8. Shou M, Lin Y, Lu P, Tang C, Mei Q, et al. 2001. Enzyme kinetics of cytochrome P450-mediated reactions. *Curr. Drug Metab.* 2:17–36
9. Houston JB, Kenworthy KE. 2000. In vitro-in vivo scaling of CYP kinetic data not consistent with the classical Michaelis-Menten model. *Drug Metab. Dispos.* 28:246–54
10. Hlavica P, Lewis DF. 2001. Allosteric phenomena in cytochrome P450-catalyzed monooxygenations. *Eur. J. Biochem.* 268: 4817–32
11. Tracy TS. 2003. Atypical enzyme kinetics: their effect on in vitro-in vivo pharmacokinetic predictions and drug interactions. *Curr. Drug Metab.* 4:341–46
12. Lasker JM, Huang M-T, Conney AH. 1982. In vivo activation of zoxazolamine metabolism by flavone. *Science* 216:1419–21
13. Huang MT, Chang RL, Fortner JG, Conney AH. 1981. Studies on the mechanism of activation of microsomal benzo[a]pyrene hydroxylation by flavonoids. *J. Biol. Chem.* 256:6829–36
14. Schwab GE, Raucy JL, Johnson EF. 1988. Modulation of rabbit and human hepatic cytochrome P-450-catalyzed steroid hydroxylations by alpha-naphthoflavone. *Mol. Pharmacol.* 33:493–99
15. Shou M, Grogan J, Mencewicz JA, Krausz KW, Gonzalez FJ, et al. 1994. Activation of CYP3A4: evidence for the simultaneous binding of two substrates in a cytochrome P450 active site. *Biochemistry* 33:6450–55
16. Korzekwa KR, Krishnamachary N, Shou M, Ogai A, Parise RA, et al. 1998. Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry* 37:4137–47
17. Banci L, Bertini I, Marconi S, Pierattelli R, Sligar SG. 1994. Cytochrome P450 and

- aromatic bases: a ^1H NMR study. *J. Am. Chem. Soc.* 116(11):4866–73
18. Tang W, Stearns RA, Kwei GY, Iliff SA, Miller RR, et al. 1999. Interaction of diclofenac and quinidine in monkeys: stimulation of diclofenac metabolism. *J. Pharmacol. Exp. Ther.* 291:1068–74
 19. Egnell AC, Houston B, Boyer S. 2003. In vivo CYP3A4 heteroactivation is a possible mechanism for the drug interaction between felbamate and carbamazepine. *J. Pharmacol. Exp. Ther.* 305:1251–62
 20. Hutzler JM, Frye RF, Korzekwa KR, Branch RA, Huang SM, Tracy TS. 2001. Minimal in vivo activation of CYP2C9-mediated flurbiprofen metabolism by dapsone. *Eur. J. Pharm. Sci.* 14:47–57
 21. Ekins S, Ring BJ, Binkley SN, Hall SD, Wrighton SA. 1998. Autoactivation and activation of the cytochrome P450s. *Int. J. Clin. Pharmacol. Ther.* 36:642–51
 22. Tracy TS, Hutzler JN, Haining RL, Rettie AE, Hummel MA, Dickmann LJ. 2002. Polymorphic variants (CYP2C9*3 and CYP2C9*5) and the F114L active site mutation of CYP2C9: effect on atypical kinetic metabolism profiles. *Drug Metab. Dispos.* 30:385–90
 23. He YA, Roussel F, Halpert JR. 2003. Analysis of homotropic and heterotropic cooperativity of diazepam oxidation by CYP3A4 using site-directed mutagenesis and kinetic modeling. *Arch. Biochem. Biophys.* 409:92–101
 24. Hutzler JM, Hauer MJ, Tracy TA. 2001. Dapsone activation of CYP2C9-mediated metabolism: evidence for activation of multiple substrates and a two-site model. *Drug Metab. Dispos.* 29:1029–34
 25. Khan KK, He YA, Domanski TL, Halpert JR. 2002. Midazolam oxidation by cytochrome P450 3A4 and active-site mutants: an evaluation of multiple binding sites and of the metabolic pathway that leads to enzyme inactivation. *Mol. Pharmacol.* 61:495–506
 26. Ngui JS, Chen Q, Shou M, Wang RW, Stearns RA, et al. 2001. In vitro stimulation of warfarin metabolism by quinidine: increases in the formation of 4'- and 10-hydroxywarfarin. *Drug Metab. Dispos.* 29:877–86
 27. Edelstein SJ, Changeux JP. 1998. Allosteric transitions of the acetylcholine receptor. See Ref. 99, pp. 121–84
 28. Ackers GK. 1998. Deciphering the molecular code of hemoglobin allostery. See Ref. 99, pp. 185–253
 29. Wyman J, Gill SJ. 1990. Allosteric systems. In *Binding and Linkage: Functional Chemistry of Biological Macromolecules*, pp. 123–63. Mill Valley, CA: Univ. Sci. Books
 30. Szczesna-Skorupa E, Mallah B, Kemper B. 2003. Fluorescence resonance energy transfer analysis of cytochromes P450 2C2 and 2E1 molecular interactions in living cells. *J. Biol. Chem.* 278:31269–76
 31. Myasoedova KN, Tsuprun VL. 1993. Cytochrome P-450: hexameric structure of the purified LM4 form. *FEBS Lett.* 325:251–54
 32. Atkins WM, Lu WD, Cook DL. 2002. Is there a toxicological advantage for non-hyperbolic kinetics in cytochrome P450 catalysis? Functional allostery from “distributive catalysis.” *J. Biol. Chem.* 277(36):33258–66
 33. Galetin A, Clarke SE, Houston JB. 2002. Quinidine and haloperidol as modifiers of CYP3A4 activity: multisite kinetic model approach. *Drug Metab. Dispos.* 30:1512–22
 34. Tang W, Stearns RA. 2001. Heterotropic cooperativity of cytochrome P450 3A4 and potential drug-drug interactions. *Curr. Drug Metab.* 2(2):185–98
 35. Wang RW, Newton DJ, Scheri TD, Lu AY. 1997. Human cytochrome P450 3A4-catalyzed testosterone 6 beta-hydroxylation and erythromycin N-demethylation. Competition during catalysis. *Drug Metab. Dispos.* 25(4):502–7
 36. Wang RW, Newton DJ, Liu N, Atkins WM, Lu AY. 2000. Human cytochrome

- P-450 3A4: in vitro drug-drug interaction patterns are substrate-dependent. *Drug Metab. Dispos.* 28:360–66
37. Galetin A, Clarke SE, Houston JB. 2003. Multisite kinetic analysis of interactions between prototypical CYP3A4 substrates: midazolam, testosterone, and nifedipine. *Drug. Metab. Dispos.* 31(9):1108–16
38. Ueng YF, Kuwabara T, Chun YJ, Guengerich FP. 1997. Cooperativity in oxidations catalyzed by cytochrome P450 3A4. *Biochemistry* 36:370–81
39. Patki KC, Von Moltke LL, Greenblatt DJ. 2003. In vitro metabolism of midazolam, triazolam, nifedipine, and testosterone by human liver microsomes and recombinant cytochromes p450: role of cyp3a4 and cyp3a5. *Drug Metab. Dispos.* 31(7):938–44
40. Atkins WM, Wang RW, Lu AY. 2001. Allosteric behavior in cytochrome p450-dependent in vitro drug-drug interactions: a prospective based on conformational dynamics. *Chem. Res. Toxicol.* 14:338–47
41. Koley AP, Buters JTM, Robinson RC, Markowitz A, Friedman FK. 1997. Differential mechanisms of cytochrome P450 inhibition and activation by alpha-naphthoflavone. *J. Biol. Chem.* 272: 3149–52
42. Koley AP, Robinson RC, Markowitz A, Friedman FK. 1997. Drug-drug interactions: effect of quinidine on nifedipine binding to human cytochrome P450 3A4. *Biochem. Pharmacol.* 53:455–60
43. Koley AP, Buters JT, Robinson RC, Markowitz A, Friedman FK. 1995. CO binding kinetics of human cytochrome P450 3A4. Specific interaction of substrates with kinetically distinguishable conformers. *J. Biol. Chem.* 270(10):5014–18
44. Davydov DR, Halpert JR, Renaud JP, Hui Bon Hoa G. 2003. Conformational heterogeneity of cytochrome P450 3A4 revealed by high pressure spectroscopy. *Biochem. Biophys. Res. Commun.* 312:121–30
45. Schrag ML, Wienkers LC. 2000. Topological alteration of the CYP3A4 active site by the divalent cation Mg(2+). *Drug Metab. Dispos.* 28(10):1198–201
46. Hutzler JM, Powers FJ, Wynalda MA, Wienkers LC. 2003. Effect of carbonate anion on cytochrome P450 2D6-mediated metabolism in vitro: the potential role of multiple oxygenating species. *Arch. Biochem. Biophys.* 417(2):165–75
47. Maenpaa JVA, Hall SD, Ring BJ, Strom SC, Wrighton SA. 1998. Human cytochrome P450 3A (CYP3A) mediated midazolam metabolism: the effect of assay conditions and regioselective stimulation by α -naphthoflavone, terfenadine and testosterone. *Pharmacogenetics* 8:137–55
48. Guengerich FP. 1999. Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu. Rev. Pharmacol. Toxicol.* 39:1–17
49. Schenkman JB, Jansson I. 2003. The many roles of cytochrome b5. *Pharmacol. Ther.* 97(2):139–52
50. Yamazaki H, Johnson WW, Ueng YF, Shimada T, Guengerich FP. 1996. Lack of electron transfer from cytochrome b5 in stimulation of catalytic activities of cytochrome P450 3A4. Characterization of a reconstituted cytochrome P450 3A4/NADPH cytochrome P450 reductase system and studies with apo-cytochrome b5. *J. Biol. Chem.* 271(44):27438–44
51. Gilep AA, Guryev OL, Usanov SA, Estabrook RW. 2001. Apo-cytochrome b5 as an indicator of changes in heme accessibility: preliminary studies with cytochrome P450 3A4. *J. Inorg. Biochem.* 87(4):237–44
52. Yamazaki H, Nakamura M, Komatsu T, Ohyama K, Hatanaka N, et al. 2002. Roles of NADPH-P450 reductase and apo- and holo-cytochrome b5 on xenobiotic oxidations catalyzed by 12 recombinant human cytochrome P450s expressed in membranes of *Escherichia coli*. *Protein Expr. Purif.* 24(3):329–37

53. Guryev OL, Gilep AA, Usanov SA, Estabrook RW. 2001. Interaction of apocytochrome b5 with cytochromes P450-3A4 and P45017A: relevance of heme transfer reactions. *Biochemistry* 40(16): 5018–31
54. Modi S, Gilham DE, Sutcliffe MJ, Lian LY, Primrose WU, et al. 1997. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine as a substrate of cytochrome P450 2D6: allosteric effects of NADPH-cytochrome P450 reductase. *Biochemistry* 36(15): 4461–70
55. Hanna IH, Krauser JA, Cai H, Kim MS, Guengerich FP. 2001. Diversity in mechanisms of substrate oxidation by cytochrome P450 2D6. Lack of an allosteric role of NADPH-cytochrome P450 reductase in catalytic regioselectivity. *J. Biol. Chem.* 276(43):39553–61
56. Loughran PA, Roman LJ, Miller RT, Masters BS. 2001. The kinetic and spectral characterization of the *E. coli*-expressed mammalian CYP4A7: cytochrome b5 effects vary with substrate. *Arch. Biochem. Biophys.* 385(2):311–22
57. Cupp-Vickery J, Andersen R, Hatziris Z. 2000. Crystal structures of ligand complexes of P450eryF exhibiting homotropic cooperativity. *Proc. Natl. Acad. Sci. USA* 97(7):3050–55
58. Torimoto N, Ishii I, Hata M, Nakamura H, Imada H, et al. 2003. Direct interaction between substrates and endogenous steroids in the active site may change the activity of cytochrome P450 3A4. *Biochemistry* 42(51):15068–77
59. Dabrowski MJ, Schrag ML, Wienkers LC, Atkins WM. 2000. Pyrene-pyrene complexes at the active site of cytochrome P4503A4: evidence for a multiple substrate binding site. *J. Am. Chem. Soc.* 124(40):11866–67
60. Segel IH. 1993. Multisite and allosteric enzymes. In *Enzyme Kinetics*. pp. 346–404. New York: Wiley
61. Shen L, Fitzloff JF, Cook CS. 2004. Differential enantioselectivity and product-dependent activation and inhibition in metabolism of verapamil by human CYP3As. *Drug Metab. Dispos.* 32:186–96
62. Beechem JM, Gratton E, Ameloot M, Knutson JR, Brand L. 1991. The global analysis of fluorescence and intensity and anisotropy decay data: second generation theory and program. In *Topics in Fluorescence Spectroscopy*, ed. JR Lakowicz, vol. 2, pp. 241–98. New York: Plenum Press
63. Lin Y, Lu P, Tang C, Mei Q, Sandig G, et al. 2001. Substrate inhibition kinetics for cytochrome P450-catalyzed reactions. *Drug Metab. Dispos.* 29:368–74
64. Kenworthy KE, Clarke SE, Andrews J, Houston JB. 2001. Multisite kinetic models for CYP3A4: simultaneous activation and inhibition of diazepam and testosterone metabolism. *Drug Metab. Dispos.* 29:1644–51
65. Shou M, Dai R, Cui D, Korzekwa KR, Baillie TA, Rushmore TH. 2001. A kinetic model for the metabolic interaction of two substrates at the active site of cytochrome P450 3A4. *J. Biol. Chem.* 276(3):2256–62
66. Hosea NA, Miller GP, Guengerich FP. 2000. Elucidation of distinct ligand binding sites for cytochrome P450 3A4. *Biochemistry* 39:5929–39
67. Domanski TL, He YA, Khan KK, Rousset F, Wang Q, Halpert JR. 2001. Phenylalanine and tryptophan scanning mutagenesis of CYP3A4 substrate recognition site residues and effect on substrate oxidation and cooperativity. *Biochemistry* 40(34):10150–60
68. Khan KK, He YQ, Domanski TL, Halpert JR. 2002. Midazolam oxidation by cytochrome P450 3A4 and active-site mutants: an evaluation of multiple binding sites and of the metabolic pathway that leads to enzyme inactivation. *Mol. Pharmacol.* 61(3):495–506
69. Davydov DR, Kumar S, Halpert JR. 2002. Allosteric mechanisms in P450eryF

- probed with 1-pyrenebutanol, a novel fluorescent substrate. *Biochem. Biophys. Res. Commun.* 294(4):806–12
70. Yoon MY, Campbell AP, Atkins WM. 2004. "Allostherism" in the elementary steps of the cytochrome P450 reaction cycle. *Drug Metab. Rev.* 36:1–12
71. Harlow GR, Halpert JR. 1998. Analysis of human cytochrome P450 3A4 cooperativity: construction and characterization of a site-directed mutant that displays hyperbolic steroid hydroxylation kinetics. *Proc. Natl. Acad. Sci. USA* 95(12):6636–41
72. Domanski TL, He YA, Harlow GR, Halpert JR. 2000. Dual role of human cytochrome P450 3A4 residue Phe-304 in substrate specificity and cooperativity. *J. Pharmacol. Exp. Ther.* 293(2):585–91
73. Iyer KR, Jones JP, Darbyshire JF, Trager WF. 1997. Intramolecular isotope effects for benzylic hydroxylation of isomeric xylenes and 4,4'-dimethylbiphenyl by cytochrome P450: relationship between distance of methyl groups and masking of the intrinsic isotope effect. *Biochemistry* 36(23):7136–43
74. Korzekwa KR, Trager WF, Gillette JR. 1989. Theory for the observed isotope effects from enzymatic systems that form multiple products via branched reaction pathways: cytochrome P-450. *Biochemistry* 28(23):9012–18
75. Nelson SD, Trager WF. 2003. The use of deuterium isotope effects to probe the active site properties, mechanism of cytochrome P450-catalyzed reactions, and mechanisms of metabolically dependent toxicity. *Drug Metab. Dispos.* 31(12):1481–98
76. Rock DA, Perkins BN, Wahlstrom J, Jones JP. 2003. A method for determining two substrates binding in the same active site of cytochrome P450BM3: an explanation of high energy omega product formation. *Arch. Biochem. Biophys.* 416(1):9–16
77. Reed JR, Hollenberg PF. 2003. Comparison of substrate metabolism by cytochromes P450 2B1, 2B4, and 2B6: relationship of heme spin state, catalysis, and the effects of cytochrome b5. *J. Inorg. Biochem.* 93(3–4):152–60
78. Gorsky LD, Koop DR, Coon MJ. 1984. On the stoichiometry of the oxidase and monooxygenase reactions catalyzed by liver microsomal cytochrome P-450. Products of oxygen reduction. *J. Biol. Chem.* 259(11):6812–17
79. Atkins WM, Sligar SG. 1987. Metabolic switching in cytochrome p450cam: deuterium isotope effects on regiospecificity and the monooxygenase/oxidase ratio. *J. Am. Chem. Soc.* 109:3754–60
80. Hutzler JM, Wienkers LC, Wahlstrom JL, Carlson TJ, Tracy TS. 2003. Activation of cytochrome P450 2C9-mediated metabolism: mechanistic evidence in support of kinetic observations. *Arch. Biochem. Biophys.* 410(1):16–24
81. Wester MR, Stout CD, Johnson EF. 2002. Purification and crystallization of N-terminally truncated forms of microsomal cytochrome P450 2C5. *Methods Enzymol.* 357:73–79
82. Scott EE, Spatzenegger M, Halpert JR. 2001. A truncation of 2B subfamily cytochrome P450 yields increased expression levels, increased solubility, and decreased aggregation while retaining function. *Arch. Biochem. Biophys.* 395(1):57–68
83. Cosme J, Johnson EF. 2000. Engineering microsomal cytochrome P450 2C5 to be a soluble, monomeric enzyme. Mutations that alter aggregation, phospholipid dependence of catalysis, and membrane binding. *J. Biol. Chem.* 275(4):2545–53
84. Hsu PY, Wang LH. 2003. Protein engineering of thromboxane synthase: conversion of membrane-bound to soluble form. *Arch. Biochem. Biophys.* 416(1):38–46
85. Sueyoshi T, Park LJ, Moore R, Juvenon RO, Negishi M. 1995. Molecular engineering of microsomal P450 2a-4 to a stable, water-soluble enzyme. *Arch. Biochem. Biophys.* 322(1):265–71
86. Tennant M, McRee DE. 2001. The

- first structure of a microsomal P450—implications for drug discovery. *Curr. Opin. Drug Discov. Dev.* 4(5):671–77
87. Lepesheva GI, Waterman MR. 2004. CYP51—the omnipotent P450. *Mol. Cell Endocrinol.* 215(1–2):165–70
 88. Li H, Poulos TL. 1996. Conformational dynamics in cytochrome P450-substrate interactions. *Biochimie* 78(8–9):695–99
 89. Poulos TL. 1988. Cytochrome P450: molecular architecture, mechanism, and prospects for rational inhibitor design. *Pharm. Res.* 5(2):67–75
 90. Schlichting I, Berendzen J, Chu K, Stock AM, Maves SA, et al. 2000. The catalytic pathway of cytochrome p450cam at atomic resolution. *Science* 287(5458):1615–22
 91. Ravichandran KG, Boddupalli SS, Haser-mann CA, Peterson JA, Deisenhofer J. 1993. Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science* 261(5122):731–36
 92. Wester MR, Johnson EF, Marques-Soares C, Dijols S, Dansette PM, et al. 2003. Structure of mammalian cytochrome P450 2C5 complexed with diclofenac at 2.1 Å resolution: evidence for an induced fit model of substrate binding. *Biochemistry* 42(31):9335–45
 93. Wester MR, Johnson EF, Marques-Soares C, Dansette PM, Mansuy D, Stout CD. 2003. Structure of a substrate complex of mammalian cytochrome P450 2C5 at 2.3 Å resolution: evidence for multiple substrate binding modes. *Biochemistry* 42(21):6370–79
 94. Strickler M, Goldstein BM, Maxfield K, Shireman L, Kim G, et al. 2003. Crystallographic studies on the complex behavior of nicotine binding to P450cam (CYP101). *Biochemistry* 42(41):11943–50
 95. Williams PA, Cosme J, Ward A, Angove HC, Matak Vinkovic D, Jhoti H. 2003. Crystal structure of human cytochrome P450 2C9 with bound warfarin. *Nature* 424(6947):464–68
 96. Rettie AE, Eddy AC, Heimark LD, Gibaldi M, Trager WF. 1989. Characteristics of warfarin hydroxylation catalyzed by human liver microsomes. *Drug Metab. Dispos.* 17(3):265–70
 97. Scott EE, He YA, Wester MR, White MA, Chin CC, et al. 2003. An open conformation of mammalian cytochrome P450 2B4 at 1.6-Å resolution. *Proc. Natl. Acad. Sci. USA* 100(23):13196–201
 98. Schoch GA, Yano JK, Wester MR, Griffin KJ, Stout CD, Johnson EF. 2004. Structure of human microsomal cytochrome P450 2C8: Evidence for a peripheral fatty acid binding site. *J. Biol. Chem.* 279:9497–503
 99. Williams PA, Cosme J, Vinkovic DM, Ward A, Angove HC, et al. 2004. Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone. *Science* 305(5684):683–86
 100. Yano JK, Wester MR, Schoch GA, Griffin KJ, Stout CD, Johnson EF. 2004. The structure of human microsomal cytochrome P450 3A4 determined by X-ray crystallography to 2.05' resolution. *J. Biol. Chem.* In press

CONTENTS

FRONTISPIECE— <i>Minor J. Coon</i>	xii
CYTOCHROME P450: NATURE'S MOST VERSATILE BIOLOGICAL CATALYST, <i>Minor J. Coon</i>	1
CYTOCHROME P450 ACTIVATION OF ARYLAMINES AND HETEROCYCLIC AMINES, <i>Donghak Kim and F. Peter Guengerich</i>	27
GLUTATHIONE TRANSFERASES, <i>John D. Hayes, Jack U. Flanagan, and Ian R. Jowsey</i>	51
PLEIOTROPIC EFFECTS OF STATINS, <i>James K. Liao and Ulrich Laufs</i>	89
FAT CELLS: AFFERENT AND EFFERENT MESSAGES DEFINE NEW APPROACHES TO TREAT OBESITY, <i>Max Lafontan</i>	119
FORMATION AND TOXICITY OF ANESTHETIC DEGRADATION PRODUCTS, <i>M. W. Anders</i>	147
THE ROLE OF METABOLIC ACTIVATION IN DRUG-INDUCED HEPATOTOXICITY, <i>B. Kevin Park, Neil R. Kitteringham, James L. Maggs, Munir Pirmohamed, and Dominic P. Williams</i>	177
NATURAL HEALTH PRODUCTS AND DRUG DISPOSITION, <i>Brian C. Foster, J. Thor Arnason, and Colin J. Briggs</i>	203
BIOMARKERS IN PSYCHOTROPIC DRUG DEVELOPMENT: INTEGRATION OF DATA ACROSS MULTIPLE DOMAINS, <i>Peter R. Bieck and William Z. Potter</i>	227
NEONICOTINOID INSECTICIDE TOXICOLOGY: MECHANISMS OF SELECTIVE ACTION, <i>Motohiro Tomizawa and John E. Casida</i>	247
GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE, APOPTOSIS, AND NEURODEGENERATIVE DISEASES, <i>De-Maw Chuang, Christopher Hough, and Vladimir V. Senatorov</i>	269
NON-MICHAELIS-MENTEN KINETICS IN CYTOCHROME P450-CATALYZED REACTIONS, <i>William M. Atkins</i>	291
EPOXIDE HYDROLASES: MECHANISMS, INHIBITOR DESIGNS, AND BIOLOGICAL ROLES, <i>Christophe Morisseau and Bruce D. Hammock</i>	311

NITROXYL (HNO): CHEMISTRY, BIOCHEMISTRY, AND PHARMACOLOGY, <i>Jon M. Fukuto, Christopher H. Switzer, Katrina M. Miranda, and David A. Wink</i>	335
TYROSINE KINASE INHIBITORS AND THE DAWN OF MOLECULAR CANCER THERAPEUTICS, <i>Raoul Tibes, Jonathan Trent, and Razelle Kurzrock</i>	357
ACTIONS OF ADENOSINE AT ITS RECEPTORS IN THE CNS: INSIGHTS FROM KNOCKOUTS AND DRUGS, <i>Bertil B. Fredholm, Jiang-Fan Chen, Susan A. Masino, and Jean-Marie Vaugeois</i>	385
REGULATION AND INHIBITION OF ARACHIDONIC ACID (OMEGA)-HYDROXYLASES AND 20-HETE FORMATION, <i>Deanna L. Kroetz and Fengyun Xu</i>	413
CYTOCHROME P450 UBIQUITINATION: BRANDING FOR THE PROTEOLYTIC SLAUGHTER? <i>Maria Almira Correia, Sheila Sadeghi, and Eduardo Mundo-Paredes</i>	439
PROTEASOME INHIBITION IN MULTIPLE MYELOMA: THERAPEUTIC IMPLICATION, <i>Dharminder Chauhan, Teru Hideshima, and Kenneth C. Anderson</i>	465
CLINICAL AND TOXICOLOGICAL RELEVANCE OF CYP2C9: DRUG-DRUG INTERACTIONS AND PHARMACOGENETICS, <i>Allan E. Rettie and Jeffrey P. Jones</i>	477
CLINICAL DEVELOPMENT OF HISTONE DEACETYLASE INHIBITORS, <i>Daryl C. Drummond, Charles O. Noble, Dmitri B. Kirpotin, Zexiong Guo, Gary K. Scott, and Christopher C. Benz</i>	495
THE MAGIC BULLETS AND TUBERCULOSIS DRUG TARGETS, <i>Ying Zhang</i>	529
MOLECULAR MECHANISMS OF RESISTANCE IN ANTIMALARIAL CHEMOTHERAPY: THE UNMET CHALLENGE, <i>Ravit Arav-Boger and Theresa A. Shapiro</i>	565
SIGNALING NETWORKS IN LIVING CELLS, <i>Michael A. White and Richard G.W. Anderson</i>	587
HEPATIC FIBROSIS: MOLECULAR MECHANISMS AND DRUG TARGETS, <i>Sophie Lotersztajn, Boris Julien, Fatima Teixeira-Clerc, Pascale Grenard, and Ariane Mallat</i>	605
ABERRANT DNA METHYLATION AS A CANCER-INDUCING MECHANISM, <i>Manel Esteller</i>	629
THE CARDIAC FIBROBLAST: THERAPEUTIC TARGET IN MYOCARDIAL REMODELING AND FAILURE, <i>R. Dale Brown, S. Kelley Ambler, M. Darren Mitchell, and Carlin S. Long</i>	657

EVALUATION OF DRUG-DRUG INTERACTION IN THE HEPATOBILIARY AND RENAL TRANSPORT OF DRUGS, <i>Yoshihisa Shitara, Hitoshi Sato, and Yuichi Sugiyama</i>	689
DUAL SPECIFICITY PROTEIN PHOSPHATASES: THERAPEUTIC TARGETS FOR CANCER AND ALZHEIMER'S DISEASE, <i>Alexander P. Ducruet, Andreas Vogt, Peter Wipf, and John S. Lazo</i>	725
INDEXES	
Subject Index	751
Cumulative Index of Contributing Authors, Volumes 41–45	773
Cumulative Index of Chapter Titles, Volumes 41–45	776
ERRATA	
An online log of corrections to <i>Annual Review of Pharmacology and Toxicology</i> chapters may be found at http://pharmtox.annualreviews.org/errata.shtml	