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Cooperative properties of cytochromes P450

Ilia G. Denisov, Daniel J. Frank, and Stephen G. Sligar

Department of Biochemistry, University of Illinois, Urbana, IL, 61801

Abstract

Cytochromes P450 form a large and important class of heme monooxygenases with a broad spectrum of substrates and corresponding functions, from steroid hormone biosynthesis to the metabolism of xenobiotics. Despite decades of study, the molecular mechanisms responsible for the complex non-Michaelis behavior observed with many members of this super-family during metabolism, often termed ‘cooperativity,’ remain to be fully elucidated. Although there is evidence that oligomerization may play an important role in defining the observed cooperativity, some monomeric cytochromes P450, particularly those involved in xenobiotic metabolism, also display this behavior due to their ability to simultaneously bind several substrate molecules. As a result, formation of distinct enzyme-substrate complexes with different stoichiometry and functional properties can give rise to homotropic and heterotropic cooperative behavior. This review aims to summarize the current understanding of cooperativity in cytochromes P450, with a focus on the nature of cooperative effects in monomeric enzymes.

Keywords

P450; allosteric effects; cooperativity; drug-drug interactions

1. Introduction

Cytochromes P450 constitute a superfamily of heme monooxygenases with more than 8000 isozymes identified in organisms representing all biological kingdoms (<http://drnelson.utmem.edu/CytochromeP450.html>). All cytochromes P450 share a common fold, have a molecular weight of 45 – 60 kDa and contain a single b-type heme (iron protoporphyrin IX). This prosthetic group is deeply buried inside the protein globule (Johnson & Stout, 2005; von Koenig & Schlichting, 2007). Cytochromes P450 are able to catalyze various oxidative chemical transformations, such as hydroxylation of hydrocarbons, oxidation and dealkylation of heteroatoms, olefin epoxidation, dehydrogenation and desaturation (Guengerich, 2001). A large group of P450 enzymes is involved in the biosynthesis of steroid hormones, as well as of some antibiotics, vitamins and cofactors such as retinoids, eicosanoids and fatty acid derivatives. Other cytochromes P450, which metabolize xenobiotics, are capable of catalyzing multiple reactions with unusually broad substrate specificity. Many of these enzymes, such as human CYP3A4, have large and flexible substrate binding pockets capable of accommodating relatively large substrates with molecular weights >1000 Da, or

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Corresponding author: Stephen G. Sligar, Department of Biochemistry, 116 Morrill Hall, University of Illinois, 505 South Goodwin Avenue, Urbana, IL 61801, Tel. 217 244-7395, Fax: 217-265-4073, Email: s-sligar@uiuc.edu.

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alternatively two or three smaller organic molecules. Binding of several substrates and their mutual interference often gives rise to deviations from simple hyperbolic Michaelis-Menten kinetics and is often referred to as homotropic (interaction of two or more of the same substrate molecules) or heterotropic (different substrates binding to the same cytochrome P450 molecule) cooperativity.

Sigmoidal kinetics of 6 β -hydroxylation of androstenedione by purified individual cytochromes P450 was observed in 1980 for the rabbit "LM2", "LM3", and "LM4" P450 isozymes reconstituted in phospholipid vesicles (Ingelman-Sundberg & Johansson, 1980). Later, heterotropic effects of ANF and progesterone on the spin shift titration and activity of rabbit P450 "3c" were described (Johnson, Schwab, & Dieter, 1983; Johnson, Schwab, & Vickery, 1988; Schwab, Raucy, & Johnson, 1988). In the following years cooperative behavior of many other cytochromes P450 has been detected and related to the clinically important phenomenon of drug-drug interactions (Guengerich, 1999; Bachmann, 2003; Guengerich, 2005; Rock, 2008). Considerable efforts are devoted to the studies of potentially adverse interactions of substrates and/or inhibitors mediated by cytochromes P450 to improve predictions of such effects in clinical practice (Brown et al., 2006; Obach et al., 2006; Youdim et al., 2008). Cooperativity of cytochromes P450 has been documented *in vivo* (Tang & Stearns, 2001), in liver microsomes (Oda & Kharasch, 2001; Zhang et al., 2004; Di Marco et al., 2005; Niwa, Murayama, & Yamazaki, 2008a) and in reconstituted systems with purified and isolated individual enzymes, as reviewed in (Guengerich, 1999; Houston & Kenworthy, 2000; Guengerich, 2005; Houston & Galetin, 2005; Atkins, 2006; Tracy, 2006) (Hlavica & Lewis, 2001) (Davydov & Halpert, 2008). In addition to the textbook examples of cooperative cytochromes P450 such as CYP107, CYP3A4, and CYP2C9, cooperativity has also been reported for mammalian xenobiotic metabolizing enzymes CYP1A2 (Sohl et al., 2008), CYP2A6 (Harrelson, Atkins, & Nelson, 2008), CYP2B1 (Scott, He, & Halpert, 2002), CYP2B6, and CYP2E1 (Spatzenegger et al., 2003), for bacterial enzymes CYP102 (Gustafsson et al., 2004; van Vugt-Lussenburg et al., 2006), CYP130 (Ouellet, Podust, & Ortiz de Montellano, 2008), CYP158A2 (Zhao et al., 2005), P450 crpE (Ding et al., 2008), and even for chloroperoxidase (Torres & Aburto, 2005; Aburto, Correa-Basurto, & Torres, 2008).

2. Cooperative ligand binding

In biochemistry and biophysics, cooperativity is typically defined as the interaction between the binding sites on a macromolecule (see (Di Cera, 1998) and several excellent books (Hill, 1985; Connors, 1987; Winzor, 1995; Ben-Naim, 2001; Woodbury, 2008) for comprehensive reviews). Positive cooperativity is present if the binding of a substrate to one site increases the affinity of other binding sites, and it is negative in the opposite case. Cooperative enzymes typically display a sigmoid plot of the reaction rate against substrate concentration. Note that in the normal vernacular, cooperativity implies changes in ligand affinity induced in a macromolecule by binding at one or more sites. If the site is not involved in catalytic action, this effect is often termed "allosteric". On binding an allosteric effector molecule, the catalytic activity of the enzyme towards the substrate may be enhanced, in which case the effector is an activator, or reduced, as in the case of an inhibitor.

Definitions of cooperativity are, in general, based either on the purely phenomenological approach to the analysis of experimental data, or on an *a priori* model of two or more identical binding sites with interactions between them. The first approach relies on the non-Michaelis (or non-Langmuir) shape of the plot of the enzyme property (binding isotherm or steady-state kinetics of product formation) as a function of the substrate concentration (Woodbury, 2008). The simple hyperbolic binding isotherm is characteristic for the ensemble of macromolecules each having one or several identical binding sites with dissociation constant K. For one binding

site on each macromolecule, the binding scheme consists of only one step: and yields the equation (1) for the binding isotherm is a hyperbolic Langmuir isotherm:

$$Y = \frac{[ES]}{[E_0] + [ES]} = \frac{[S]}{K + [S]} = \frac{\frac{[S]}{K}}{1 + \frac{[S]}{K}} \quad (1)$$

For two binding sites, which are not identical (Scheme 2), there are four site-specific (microscopic) equilibrium dissociation constants, k_{11} , k_{21} , k_{12} , k_{22} , three of which are independent, because the relation $k_{11} \cdot k_{12} = k_{21} \cdot k_{22}$ always holds in equilibrium due to thermodynamic energy conservation.

The corresponding stoichiometric (macroscopic) dissociation constants K_1 and K_2 can be expressed as $K_1 = (k_{11} + k_{21})$, $K_2 = k_{11} \cdot k_{12} / (k_{11} + k_{21})$. Concentrations of the binding intermediates $[E_0]$, $[ES_1]$, and $[ES_2]$, and the relative populations of the enzyme with zero, one, and two substrate molecule bound, y_0 , y_1 , and y_2 , can be calculated from the stoichiometric equilibrium provided in Scheme 2:

$$[ES_1] = [E_0] \cdot [S] / K_1, [ES_2] = [ES_1] \cdot [S] / K_2 = [E_0] \cdot [S]^2 / K_1 \cdot K_2,$$

$$[E_{\text{total}}] = [E_0] + [ES_1] + [ES_2] = [E_0] (1 + [S] / K_1 + [S]^2 / K_1 \cdot K_2)$$

The overall binding isotherm (BI) is expressed by equation (2):

$$Y = \frac{\frac{[S]}{K_1} + \frac{2[S]^2}{K_1 K_2}}{2 \left(1 + \frac{[S]}{K_1} + \frac{[S]^2}{K_1 K_2} \right)} = \frac{1}{2} y_1 + y_2 \quad (2)$$

Here y_1 and y_2 are fractions of the binding intermediates $[ES_1]$ and $[ES_2]$, i.e. the relative populations of the enzyme with one or two ligand molecules bound, and Y is the fraction of binding sites occupied by ligands.

For identical non-interacting binding sites (no cooperativity), $k_{11} = k_{21} = k_{12} = k_{22} = k$, the shape of binding isotherm is the same as in Equation (1), with k as a binding constant, but the stepwise (stoichiometric) binding constants become:

$$K_1 = 2k, \\ K_2 = k/2 = K_1/4.$$

If the two sites are distinct and independent:

$$k_{11} = k_{22} = k_1$$

$$k_{21} = k_{12} = k_2$$

and

$$K_1 = (k_1 + k_2)$$

$$K_2 = k_1 \bullet k_2 / (k_1 + k_2)$$

Using these relations, it is easy to show that $K_2 < K_1/4$, if $k_1 \neq k_2$. Therefore, for two-site binding with intrinsically different sites, the BI is indistinguishable from the presence of negative cooperativity in the system with identical sites.

In the presence of a positive cooperative interaction between sites, the second binding step occurs with higher affinity than it would have had the first site been unoccupied, meaning that $k_{12} > k_{21}$, and $k_{22} > k_{11}$ (Scheme 2). For the two-site system one can define a cooperativity coefficient $\alpha = k_{12}/k_{21} = k_{22}/k_{11}$, where $\alpha > 1$ corresponds to positive cooperativity, and $\alpha < 1$ to negative cooperativity. Then the site-specific binding constants are:

$$k_{11} = k_1, k_{22} = \alpha \bullet k_1,$$

$$k_{21} = k_2, k_{12} = \alpha \bullet k_2,$$

and the stoichiometric binding constants:

$$K_1 = (k_1 + k_2)$$

$$K_2 = \alpha \bullet k_1 \bullet k_2 / (k_1 + k_2)$$

Note that the same cooperativity coefficient α appears as the measure of cooperativity for the stoichiometric stepwise binding constants K_1 and K_2 , and for the microscopic site-specific binding constants k_{ij} . See Figure 1A for illustration. If the site-specific microscopic binding constants differ by factor p , so that $k_1 = pk_2$, then $K_1 = (p+1)k_2$ and $K_2 = k_2 \alpha p / (p+1)$ and there is a positive cooperative interaction between these sites with $\alpha = (p+1)^2 / 4p$. Then $K_2 = K_1/4$, and the binding isotherm will be hyperbolic, with no apparent cooperativity. Thus, for the system with intrinsically different sites, the presence of site-specific cooperativity has to be evaluated through additional independent measurements of site-specific binding constants, and only the overall apparent cooperative or non-cooperative behavior of the system is indicated by the shape of binding isotherm.

These equations help one to understand and evaluate cooperativity in terms of experimentally derived quantities, i.e. the equilibrium binding constants, which can be measured directly using various methods. (Connors, 1987; Winzor, 1995). Typically, the stepwise stoichiometric binding constants K_i can be derived from experimental data in more straightforward manner than microscopic site-specific constants k_{ij} . The stoichiometric binding scheme, which is sometimes termed a sequential binding model, does not rely on any specific assumptions about the nature and mutual influence of binding sites, but assumes the certain overall binding stoichiometry, or maximum number of ligands which can bind. Moreover, for the systems with more than two binding sites complete resolution of all site-specific constants from analysis of the binding isotherm is in general case impossible, because of the number of independent parameters to fit: seven for three sites, and fifteen for four sites (for further analysis see (Klotz & Hunston, 1975; Klotz, 1997)).

For three binding sites, the binding isotherm can be expressed through stoichiometric binding constants, similarly to the equation (2) for two sites:

$$Y = \frac{\frac{[S]}{K_1} + \frac{2[S]^2}{K_1 K_2} + \frac{3[S]^3}{K_1 K_2 K_3}}{3 \left(1 + \frac{[S]}{K_1} + \frac{[S]^2}{K_1 K_2} + \frac{[S]^3}{K_1 K_2 K_3} \right)} = \frac{1}{3} y_1 + \frac{2}{3} y_2 + y_3 \quad (3)$$

The multipliers 1/3, 2/3, and 3/3 at the right side of equation (3) yield the fractional contributions to the BI from each binding intermediates, corresponding to the fraction of binding sites occupied by ligand. Note, that in the most general case, if the measured signal is not directly proportional to the fraction of occupied binding sites, this equation (3) will look differently:

$$Y_a = \frac{a_0 + a_1 \frac{[S]}{K_1} + a_2 \frac{[S]^2}{K_1 K_2} + a_3 \frac{[S]^3}{K_1 K_2 K_3}}{1 + \frac{[S]}{K_1} + \frac{[S]^2}{K_1 K_2} + \frac{[S]^3}{K_1 K_2 K_3}} = a_0 y_0 + a_1 y_1 + a_2 y_2 + a_3 y_3 \quad (3a)$$

Equation (3a) provides a useful representation of BI as a sum of contributions of binding intermediates, and will be used further in analysis of functional cooperativity operating in the cytochromes P450.

If the number of binding sites and binding stoichiometry is unknown, the Hill equation is commonly used to characterize the binding cooperativity with one averaged parameter, the Hill coefficient. Originally, this equation was derived to describe the cooperative binding of oxygen and carbon monoxide to hemoglobin, assuming that the protein is composed of several subunits, which bind ligands simultaneously (Hill, 1913; Pauling, 1935; Coryell, 1939), i.e., with very high cooperativity:

The BI for such binding scheme is described by the Hill equation:

$$Y = \frac{[ES_n]}{[E_0] + [ES_n]} = \frac{[S]^n}{K^n + [S]^n} \quad (4)$$

Here K is the dissociation constant for binding of one ligand, so that the n-th order reaction of simultaneous binding of n ligands is defined by the n-th power of K.

For a two-site binding model the Hill coefficient can be expressed through the stoichiometric binding constants K_1 and K_2 (Forsen & Linse, 1995):

$$n_H = 4 \cdot (K_2/K_1)^{1/2} / (1 + 2 \cdot (K_2/K_1)^{1/2}) = 2 / (1 + \alpha^{-1/2})$$

Depending on α , or the ratio of K_1 and K_2 , the values of the Hill coefficient for a two-site model can be anywhere between 0 and 2, Figure 2A. In the absence of cooperativity, $\alpha = 1$, and $n_H = 1$. In case of strong positive cooperativity, α is large, and n_H approaches 2, the maximum possible value for the system with two sites.

By definition, Hill coefficient n_H represents the number of subunits acting simultaneously (Hill, 1913; Hill, 1985), i.e. with an infinite cooperativity between sites, which is unrealistic. However, fitting of experimental data with the Hill equation (4) is widely used as a simplest two-parameter approximation without additional knowledge of the binding stoichiometry. The position of the midpoint of the binding isotherm $S_{50} = K$ characterizes the averaged affinity, and the Hill coefficient n_H , being a quantitative measure of apparent cooperativity, defines the sigmoid shape of binding isotherm. Physically, such interpretation of the Hill coefficient is equivalent to the average size of cooperative domain in the studies of thermotropic phase transitions in lipid bilayers, calculated as a ratio of van't Hoff and calorimetric enthalpies (Hinz & Sturtevant, 1972), or the similar mean size of cooperative unit calculated as an average length of the helical segment at the helix - coil transition point in the Zimm-Bragg model (Zimm & Bragg, 1959; Jones, 1979). All these representations provide a useful quantitative measure of cooperativity by comparison of the real experimental system to the simple model of the small but infinitely cooperative system, which has the same shape of the transition curve. However, the Hill coefficient is not equal to the number of binding sites or subunits of the real macromolecule. It indicates only the minimal number of binding sites, which cannot be lower than n_H . The value of S_{50} is also not a binding constant by itself, but a composite of the real binding constants.

A useful general analysis of the shape of BI as an indicator of cooperative interactions in the system is given in (Bardsley & Wyman, 1978). Importantly, it shows that in general the sign of Hessian of the binding polynomial *at the given substrate concentration* defines the curvature of the binding isotherm and thus the presence and sign of cooperativity *at the given point*. Clearly, in cases of more than two binding sites, the sign of cooperativity may be different as the interactions between binding sites can be favorable or unfavorable (Di Cera, 1990, 1995, 1998; Ben-Naim, 2001). Note here that the unambiguous conclusion of the presence of cooperativity in the system can be reached based solely on the shape of binding isotherm only in case of positive cooperativity, while the presence of apparent negative cooperativity may be due to the heterogeneity of the binding sites. The application and validity of the Hill plot and equation to the analysis of cooperative binding to dimers and tetramers have been analyzed by Cornish-Bowden and Koshland (Cornish-Bowden & Koshland, 1975), and by Bardsley (Bardsley, 1977) for heterogeneous three-site binding.

Another approach to define cooperativity is based on the explicit theoretical model of several binding sites on macromolecule. Detailed analysis can be found in several excellent books (Hill, 1985; Di Cera, 1995; Ben-Naim, 2001). If these multiple sites are independent, and binding of the ligand at any one does not depend on the occupation of other, the system is non-cooperative. However, if the affinity of the site changes as a result of the presence of ligands at another site, then there is a cooperative interaction between them. This cooperativity is often called site-specific (Di Cera, 1998). This definition is less ambiguous, although it requires knowledge of the binding stoichiometry and the separate measurement of the site-specific binding constants unperturbed by their cooperative interactions. The measurement of these constants can be achieved using site-specific mutations (Di Cera et al., 2007; Di Cera, 2008), but such experiments rely on the detailed structural information which is unavailable in most cases.

In the case of different functional response from two binding sites, for example a different spin shift caused by the first and the second ligand binding by cytochromes P450, the substrate concentration dependence of this property ('dose - response' function) may appear cooperative even without binding cooperativity. If the first binding is 'spectrally or functionally silent', the second provides most of the signal and generates an apparently cooperative response. An example of such a case is shown in Figure 2B. As discussed below, this is the most relevant case with respect to the several mammalian cytochromes P450, including human CYP3A4

(Fernando, Halpert, & Davydov, 2006; Denisov et al., 2007a; Denisov et al., 2007b; Nath et al., 2008a), human CYP2C9 (Wei, Locuson, & Tracy, 2007), rabbit CYP1A2 (Sohl et al., 2008), and several others, like CYP107 (Davydov, Kumar, & Halpert, 2002; Davydov et al., 2005), in which the binding of the first substrate molecule with higher affinity was shown to be spectrally silent with low or no product formation, while the second, lower affinity, binding event resulted in the formation of a catalytically competent enzyme-substrate complex.

In the P450 literature there are also well known phenomena of heterotropic cooperativity; when ligands of two different types can interact simultaneously with one enzyme. The ability of one ligand (effector) to *enhance the metabolism* of another (substrate) is often called positive heterotropic cooperativity, even if there is no information on the enhancement of *binding* of the substrate in the presence of the effector. Heterotropic cooperativity in cytochromes P450 is reviewed in Section 7.

3. Cooperativity in the kinetics of metabolism

Very often the catalytic steps in the reaction cycle of cytochromes P450 are rate determining, i.e. substrate binding and product release are fast, and all binding steps are in equilibrium. In such cases, the overall kinetics of the reaction catalyzed by enzyme is a sum of fractional contributions of each of the binding intermediates, as shown in equation (3a). In this case, the catalytic rate of each binding intermediate determines the “weight” of this intermediate in the overall activity, together with the concentration, which depends on the stoichiometric binding constants. However, in the more general case, the rates of binding and dissociation of substrate and product molecules may be slow enough to become comparable to the rate of catalytic step. In this case, the dependence of the product turnover rate on the substrate concentration is more complex, and deviations from the Michaelis-Menten kinetics can be observed as apparently positive or negative cooperativity even for a single substrate binding site if it can exist in two conformational states with different functional properties (Ainslie, Shill, & Neet, 1972). This may be relevant to the idea of cooperative catalysis in several P450s as a result of multistep substrate binding, as discussed by Isin and Guengerich (Isin & Guengerich, 2006; Isin & Guengerich, 2007, 2008; Isin et al., 2008).

A steady-state approximation (or Bodenstein approximation) (Segel, 1975; Hill, Eisenberg, & Chalovich, 1981; Reinhart, 1983; Heinrich, Schuster, & Holzhuetter, 1991) is usually used to interpret the substrate turnover data (Houston, 2003; Houston & Galetin, 2005; Tracy, 2006). This means that concentrations of all of the functionally important intermediates must remain the same during the incubation time of the reaction mixture, when the rate of product formation is measured. To hold to this approximation, the substrate binding kinetics should not be rate-limiting at any substrate concentrations used in the experimental measurements of the rate of P450 catalysis. If, at low substrate concentrations, or in case of diffusion limited substrate access to the active center of the enzyme, the apparent first-order substrate binding rates are comparable to the rate of the subsequent step in P450 cycle, fractional populations of binding intermediates will no longer be the function of only the substrate concentration, but will also depend on kinetic parameters. In such cases, the apparent stepwise binding constants will be lower than the true equilibrium binding constants, implying the tighter substrate binding at higher concentrations. The plot of the steady-state rate of product formation as a function of substrate concentration will deviate from Michaelis-Menten shape indicating the presence of positive cooperativity.

Detailed analysis of binding cooperativity (with no kinetic difference between the first and the second site) and kinetic cooperativity (with no binding cooperativity) was given by Lane (Lane, 1982). In addition to the exact Hill equations derived for these cases, Lane introduced “sensitivity parameters”, i.e. the rate of change of the overall catalytic rate of the enzyme as a

function of substrate concentration. Sensitivity is maximal for the cooperative systems, and much higher in case of the kinetic cooperativity than for binding cooperativity. This aspect of kinetic cooperativity may provide an important functional advantage for such enzymes as xenobiotic metabolizing cytochromes P450 with a broad substrate specificity and low, if any binding cooperativity.

A two-site kinetic scheme was used by Korzekwa et al. (Korzekwa et al., 1998) for the analysis of cooperativity in CYP3A4:

The overall rate of product formation in this scheme is given by the following equation:

$$v = \frac{\frac{V_{\max 1}[S]}{K_{m1}} + \frac{V_{\max 2}[S]^2}{K_{m1}K_{m2}}}{1 + \frac{[S]}{K_{m1}} + \frac{[S]^2}{K_{m1}K_{m2}}} \quad (5)$$

This equation uses stoichiometric Michaelis constants, similar to Adair equation (2) for binding. As in the Adair equation, stoichiometric Michaelis constants represent statistical averages over steady-state (not necessarily identical to the equilibrium distribution corresponding to the stoichiometric binding constants) microscopically different distributions of substrate between binding sites. Corresponding values of $V_{\max 1}$ and $V_{\max 2}$ are also averaged over fractional contributions to the rate of product formation from all microscopically different binding configurations of the enzyme - substrate complex with the given stoichiometric enzyme - substrate ratio, i.e. from the given binding intermediate.

The equation used by Houston and Kenworthy (Kenworthy et al., 2001) is equivalent to the eq. 5 with parameters α and β indicating difference between Michaelis constants K_{m1} and K_{m2} , and catalytic rate constants k_1 and k_2 :

$$\frac{v}{V_{\max}} = \frac{\frac{[S]}{K_S} + \frac{\beta[S]^2}{\alpha K_S^2}}{1 + \frac{2[S]}{K_S} + \frac{[S]^2}{\alpha K_S^2}} \quad (5a)$$

Both equations contain four independent parameters, two stepwise binding constants and two catalytic rates, which can be derived by fitting experimental data set of product formation rates measured at different substrate concentrations.

A more detailed site-specific scheme (Scheme 5) with two active sites was suggested by Shou and coworkers (Shou et al., 1999) to describe metabolism of diazepam and its derivatives by CYP3A4.

The rate of overall product formation in such system is given by the following equation:

$$\frac{v}{[E]_{\text{total}}} = \frac{\frac{k_a}{K_{S1}} + \frac{k_a}{K_{S2}} + \frac{(k_\gamma + k_\delta)[S]}{K_{S1}K_{S3}}}{\frac{1}{[S]} + \frac{1}{K_{S1}} + \frac{1}{K_{S2}} + \frac{[S]}{K_{S1}K_{S3}}} \quad (6)$$

In this scheme four independent equilibrium constants and four rate constants have been fitted simultaneously for several experimental data sets. The additional constrain $K_{S1}K_{S3} = K_{S2}K_{S4}$, which is true at equilibrium but may not hold for the steady-state kinetics, was not mentioned in the original study of diazepam hydroxylation by CYP3A4 (Shou et al., 1999).

The results suggest substantial positive binding cooperativity with the K_S being 4 – 36 fold higher for the second binding. In addition, the rates of product formation appeared to be much faster for the enzyme with two substrate molecules bound. The results of this work confirm the predominant contribution of the CYP3A4 saturated with the substrate to the overall catalysis, with both binding and catalytic rate significantly enhanced at the second binding event.

Similar two-site and three-site models and corresponding equations have been used by Houston, Kenworthy, Galetin and collaborators to analyze homotropic and heterotropic cooperativity in kinetics of diazepam and TS metabolism by CYP3A4 (Houston & Kenworthy, 2000; Kenworthy et al., 2001; Galetin, Clarke, & Houston, 2002, 2003; Houston & Galetin, 2005). Their results reveal the same tendency as in other studies (Korzekwa et al., 1998; Shou et al., 1999), where the increase of binding affinity at the second step is mostly responsible for the cooperative kinetics of CYP3A4 catalyzed reactions. In some cases the catalytic rate constants have been assumed the same for all binding intermediates (Kenworthy et al., 2001), leading to the attribution of catalytic cooperativity to the binding cooperativity.

4. Structural origins of cooperativity

Because of cooperative properties (Shou et al., 1994; Ueng et al., 1997; Korzekwa et al., 1998; Szklarz & Halpert, 1998) and efficient metabolism of large antibiotics and other drugs (Guengerich, 1999), there was little doubt in ability of several cytochromes P450 to simultaneously bind multiple substrate molecules of moderate size. High resolution X-ray crystallography provides an indispensable source of the detailed information about the possible modes of packing of two substrates or inhibitors in the binding pocket and of their accessibility to the iron-oxygen catalytically active intermediates. Currently, there are 8 structures available of 6 different cytochromes P450 with two or three substrate molecules bound to one protein molecule, Figure 3. For CYP245 (Makino et al., 2007) and CYP2B4 (Zhao et al., 2006), where three substrate or inhibitor molecules are well resolved as bound to a single enzyme molecule (Figure 3G and 3H), the physiological significance of this binding is not clear and may be a crystallographic artifact (Zhao et al., 2006; Makino et al., 2007).

The X-ray structures of CYP107 in 2000 provided the first case of simultaneous binding of two substrate molecules in the distal binding pocket of cytochrome P450 (Cupp-Vickery, Anderson, & Hatziris, 2000). The packing of androstenedione and 9-aminophenanthrene was very similar, with one molecule close to the heme and to the I-helix, and another on top of the first (Figure 3A and 3B). All other structures reveal the same pattern with only one substrate molecule in the vicinity of the heme iron, and another with no access to the heme (Figure 3A – 3F). Thus, there is no precedent of simultaneous access of two molecules to the catalytic ferryl-oxo intermediate, although sequential access of two molecules bound within the distal heme pocket is possible. An X-ray structure of cytochrome P450 with two different ligand molecules bound has yet to be resolved, and no unambiguous structural assignment of allosteric sites in cytochromes P450 have been done until now.

Another source of cooperativity in cytochromes P450 can involve coupling between oligomerization of enzymes and substrate binding. In several X-ray crystal structures of cytochromes P450, the ligand molecules have been resolved as bound between two monomers in what can be considered as a dimeric unit. Such interaction between two P450 monomers as mediated by fatty acid molecules was reported in X-ray crystal structures of substrate free CYP2C8 (1PQ2.PDB (Schoch et al., 2004)) and CYP2C8 with 9-cis retinoic acid bound (2NNH.PDB (Schoch et al., 2008)), Figure 4A where two palmitate molecules are bound between two monomers of CYP2C8, with the F–G loop positioned at the dimer interface. Another example of similar dimer interface interactions with ligands can be seen in three

structures of CYP2R1 (with vitamin D3 bound, 3C6G.PDB, (Strushkevich et al., 2008), with vitamin D2 bound, 3CZH.PDB, and with the product, α -hydroxy vitamin D2, 3DL9.PDB). In these structures two cyclodextrin molecules are well resolved between two monomers of CYP2R1, while the dimer interface involves parts of the F and G helices and F–G loop, as shown in Figure 4B.

Interestingly, in the crystal structure of CYP3A4 the substrate progesterone is bound at the remote site between the F' and G' helices (Williams et al., 2004), as shown in Figure 4C. The molecule of bound substrate occupies the central part of the contact interface between molecules A and B in another crystal structure of CYP3A4, in which two molecules of the inhibitor ketoconazole are bound in the distal binding pocket (2V0M.PDB), Figure 3E. Apparently, if dimerization of CYP3A4 can take place with the same protein – protein interface as for CYP2C8 and CYP2R1, the binding of progesterone or other steroid hormones can interfere with monomer-dimer equilibrium and thus appear as a source of cooperativity in this system. There is also an example of the contact between ‘open’ and ‘closed’ conformations of two monomers of substrate-free CYP107L1 in an asymmetric unit (2BVJ.pdb, (Sherman et al., 2006)), in which the F–G loop of the ‘open’ monomer is in contact with the N-terminus of the H-helix. (See also Section 8 for discussion of other examples of cooperativity originating from coupling of substrate binding with formation of oligomers of cytochromes P450).

5. Quantifying ligand binding in the cytochromes P450

CYP107

CYP107 is the only monomeric soluble P450, in which binding cooperativity has been systematically studied. The kinetics of CYP107 catalyzed hydroxylation of the native substrate, 6-deoxyerythronolide B, does not deviate from Michaelis-Menten mechanism (Kim, Kim, & Han, 2001), consistent with the X-ray structure demonstrating that only one large substrate molecule can fit to the binding site distal to the heme (Cupp-Vickery & Poulos, 1995). Cupp-Vickery and coauthors have successfully crystallized and solved the structures of CYP107 in the presence of two model substrates, androstenedione and 9-aminophenanthrene (9-AP) (Cupp-Vickery et al., 2000). Both structures provided the first direct confirmation of the ability of cytochrome P450 to bind two molecules of the substrate close to each other in the binding pocket, Figure 3a and 3b. Spectral binding studies showed that binding of both substrates was cooperative with Hill coefficients $n_H = 1.31$ for androstenedione, and $n_H = 1.38$ for 9-AP. Similar, or even higher, positive cooperativity was documented for the metabolism of several non-native substrates by CYP107 supported by peroxides (Xiang, Tschirret-Guth, & Ortiz de Montellano, 2000; Khan et al., 2002). For instance, cooperative properties of the WT CYP107, point mutant A245T and two double mutants G91A/A245T and I174F/A245T have been documented in (Khan & Halpert, 2002) using several substrates and inhibitors, including one fluorescent substrate 7-benzyloxyquinoline (7-BQ). Both spin-shift titration curves and kinetics of 7-BQ oxidation appeared sigmoidal for all three mutants with comparable half-saturation points, but with significantly higher Hill coefficients for the activity measurements (from 1.9 to 2.2) than for the spectral binding experiments (1.1 – 1.7).

Binding of 9-AP and TS to CYP107 was also studied by 2D NMR (Yoon, Campbell, & Atkins, 2004) using a uniformly labeled ^{15}N -Phe enzyme. Two distinct changes in the NMR spectra upon binding of the first and second 9-AP molecules have been interpreted as two corresponding exchange processes, intermediate and slow on the NMR time scale. They have been assigned to the dissociation rate constants of the 9-AP molecule from CYP107 complexes with one and two molecules of the substrate. This effect of slower substrate dissociation when two substrates are bound can be considered a manifestation of allosteric interaction of two substrates, also evident from the X-ray structure (Cupp-Vickery et al., 2000). No slow exchange binding mode has been observed in similar experiments with TS binding to CYP107 (Yoon et

al., 2004), which was tentatively attributed to the difference between the dissociation rates of these two substrates.

Davydov et al. first demonstrated the presence of the high affinity binding site in CYP107 using 1-pyrene butanol (1-PB) as a model substrate (Davydov et al., 2002). Studies revealed highly cooperative binding of 1-PB with a Hill coefficient $n_H = 2.3$ and $S_{50} = 12.4 \mu\text{M}$, when monitored by the changes of optical absorption spectra caused by the spin shift, while the FRET from 1-PB to heme revealed another binding process with $K_d = 2.15 \mu\text{M}$. This observation again shows that in case of multiple substrate molecules binding to cytochromes P450, the first binding event very often does not perturb the spin state of the heme iron. Later Davydov et al. reported a detailed study of 1-PB binding to the CYP107 labeled with fluorophores at the C93 residue (S93C mutant CYP107) using FRET from 1-PB to these labels (Davydov et al., 2005). Results directly confirmed the presence of two binding sites with an approximately 8-fold difference in affinities ($K_{d1} = 1.2 \mu\text{M}$, $K_{d2} = 9.4 \mu\text{M}$), and revealed no positive cooperativity in substrate binding. However, titration with the same substrate, 1-PB, monitored by the spin shift consistently showed positive cooperativity with Hill coefficients from 1.4 to 2.4 for different mutants. These experiments directly confirmed that the apparent cooperativity in spin shift titration of CYP107 with 1-PB was caused by the lack of the spectral signal from the first binding event, as suggested earlier (Davydov et al., 2002). The same group has shown that the amplitude of the spin shift transition caused by 1-PB binding significantly increases, while S_{50} and cooperativity both decrease at high ionic strength, indicating an important role of electrostatic interactions in the spin equilibria of CYP107 with one and two molecules of 1-PB bound (Davydov et al., 2004). Simultaneous analysis of the ionic strength effects and 1-PB binding on the spin state of mutants (Davydov et al., 2005) revealed an increase of the affinity of the second binding site caused by binding of the first 1-PB molecule, and thus the presence of a positive cooperative interaction between the binding sites. In the recent study (Davydov, Davydova, & Halpert, 2008a) Davydov et al. used new fluorescent substrate, laser dye Fluorol-7GA (F7GA), to improve the sensitivity and selectivity of FRET experiments. Pressure perturbation of FRET and spin equilibria at different substrate concentrations allowed for the resolution of three binding processes and the allosteric effects caused by conformational changes in enzyme-substrate complexes.

The binding of several ligands to CYP107 was also studied by isothermal titration calorimetry (ITC) (Muralidhara & Halpert, 2007; Muralidhara, Negi, & Halpert, 2007). This method does not rely on the assignment of spectral changes and allows direct monitoring of formation of protein-ligand complexes by measuring heat effects of stepwise mixing of two solutions. The results of their work reveals very similar dissociation constants for the two-step sequential binding, with slightly lower affinity for the second binding event, and do not indicate any substantial positive cooperativity for binding of 1-PB ($K_{d1} = 10 \mu\text{M}$, $K_{d2} = 24 \mu\text{M}$), 9-AP ($K_{d1} = 5 \mu\text{M}$, $K_{d2} = 11 \mu\text{M}$), or ANF ($K_{d1} = 12 \mu\text{M}$, $K_{d2} = 27 \mu\text{M}$) to CYP107 (Muralidhara et al., 2007). Despite several fold difference between stepwise dissociation constants derived for CYP107 from ITC experiments (Muralidhara & Halpert, 2007; Muralidhara et al., 2007) and from spectroscopic studies (Davydov et al., 2002; Davydov et al., 2005; Muralidhara et al., 2007; Davydov et al., 2008a), these results consistently reveal the absence of positive binding cooperativity in CYP107, and the presence of allosteric linkages between binding events. The presence of interaction between two ligands in CYP107 was also supported by heterotropic cooperative effects of androstenedione and flavones on the binding 1-BQ and 9-AP monitored by the spin-state changes (Khan, Liu, & Halpert, 2003).

CYP3A4

CYP3A4 is arguably the most studied of all cytochromes P450. This enzyme is one of the most abundant cytochromes P450 in the human liver, where it catalyzes more than a dozen of

different chemical reactions and plays a major role in the metabolism of hundreds of substrates of very different size and chemical structure (Guengerich, 2005), including 37% of the most commonly sold drugs (Zanger et al., 2008). In many cases this P450 exhibits non-Langmuir binding and non-Michaelis kinetics with homotropic and heterotropic cooperative features. In several review articles Guengerich (Guengerich, 1999; Guengerich, 2005) provided comprehensive documentation of all aspects of CYP3A4 mechanisms, including a summary of the current understanding of its cooperative properties. Recent review of the substrate binding to cytochromes P450 (Isin & Guengerich, 2008) gives a useful summary of the main results obtained in equilibrium and kinetic studies of ligands binding to CYP3A4. Cooperative properties of CYP3A4 and the main concepts of allosteric phenomena in cytochromes P450 have been reviewed by Atkins (Atkins, 2005; Atkins, 2006) and Davydov and Halpert (Davydov & Halpert, 2008).

Positive cooperativity was observed in CYP3A4 binding experiments when monitored by optical absorption (spin shift spectral titration experiments) using the following substrates: testosterone (TS) (Harlow & Halpert, 1998; Hosea, Miller, & Guengerich, 2000; Baas, Denisov, & Sligar, 2004; Cameron et al., 2005; Jushchyshyn et al., 2005; Roberts, Campbell, & Atkins, 2005; Isin & Guengerich, 2006; Lampe & Atkins, 2006; Davydov et al., 2007; Denisov et al., 2007a; Denisov et al., 2007b; Fernando et al., 2007; Roberts & Atkins, 2007; Tsalkova et al., 2007), progesterone (Harlow & Halpert, 1998), pyrene-butanole (1-PB) (Fernando et al., 2006; Davydov et al., 2007; Davydov et al., 2008b), 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC) (Davydov et al., 2008b), ANF (Domanski et al., 2000; Hosea et al., 2000; Fernando et al., 2007; Nath et al., 2007; Tsalkova et al., 2007), 7-benzyloxyquinoline (7-BQ) (Kapelyukh et al., 2008), flavone (Isin & Guengerich, 2006), and Nile Red (Nath et al., 2008a). No binding cooperativity was detected with other substrates and inhibitors, such as bromocriptine (Fernando et al., 2006; Isin & Guengerich, 2006), erythromycin, oligopeptide inhibitors (Hosea et al., 2000), ketoconazole, midazolam (Isin 2006), acetaminophen (Cameron et al., 2007). Cooperativity of the spin-shift curves was usually characterized by fitting to the Hill equation. For all substrates, which show sigmoidal spin-shift curves, the Hill coefficients are, in most cases, lower than 2.0 consistent with the generally accepted view of two or three substrate molecules binding simultaneously to one CYP3A4 molecule.

More detailed multistep kinetic binding models developed by Isin and Guengerich (Isin & Guengerich, 2006; Isin & Guengerich, 2007, 2008) revealed that the fast initial binding of substrates and inhibitors to CYP3A4 is followed by slower conformational rearrangement of the enzyme-substrate complex. The fast phase, with the rates comparable to the diffusion limited formation of encounter complex (second order rates $\sim 4 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$), can be detected by changes in fluorescence of the ligand while the slower phase (first order rates $\sim 10 \text{ s}^{-1}$) has been monitored by the spin shift changes using optical absorption in Soret region. Notably the results obtained from the kinetics of TS binding to CYP3A4 (Isin & Guengerich, 2006) could be fitted to the two-site binding scheme with negligible spin shift at the high-affinity binding site and all changes of the spin state attributed to binding of the second TS molecule to CYP3A4, consistent with other equilibrium and kinetic evidence (Denisov et al., 2007a; Denisov et al., 2007b).

In general, few attempts have been made to use more detailed and mechanistically relevant two-site or three-site equations in spectral studies of ligand binding, like the one given by equation (3a), or similar to those developed by Korzekwa and Shou (Korzekwa et al., 1998; Shou et al., 1999) for steady-state kinetics (Scheme 4 and Scheme 5). The main difficulty with such approach is the strong correlation of the spectral amplitudes of the spin shifts caused by the first and the second binding with corresponding binding constants, which makes the simultaneous resolution of all these unknown parameters almost impossible (Baas et al.,

2004). However, these parameters can be resolved if additional information about separate binding events is available from other experiments performed under identical conditions and the results are analyzed simultaneously. For example, independent measurement of the binding constant for the high affinity site in 1-PB binding to CYP107 (Davydov et al., 2005), and to CYP3A4 (Fernando et al., 2006) by FRET allowed Davydov and coworkers to determine the second binding constant and assign the spin shift signal observed in optical absorption spectra to the second binding event. An example of the global analysis of multiple experimental data sets was given in our study of the cooperative properties of TS binding and metabolism by monomeric CYP3A4 incorporated in Nanodiscs (Denisov et al., 2007a), see also a recent review (Sligar & Denisov, 2007).

NMR relaxation methods have also been used to detect interactions between the substrate midazolam and effectors ANF and TS in CYP3A4 binding pocket (Cameron et al., 2005). The different effect on the ratio of two products, 1'- and 4'-hydroxymidazolam could be attributed to the changes in positioning of midazolam with respect to the catalytically active ferryl-oxo intermediate, caused by the presence of these effectors in the enzyme binding pocket. The effect of caffeine on binding and metabolism of acetaminophen (APAP) by CYP3A4 was also studied in (Cameron et al., 2007) by spin shift titration, NMR T_1 paramagnetic relaxation measurements and kinetics of oxidation of APAP, and the similar conclusions on the mutual positioning and orientation of two ligands in the binding pocket have been made.

The binding of two molecules of pyrene in the active site of CYP3A4 was directly confirmed by excimer fluorescence (Dabrowski et al., 2002). Pyrene is oxidized by CYP3A4 with high cooperativity ($n_H = 1.7$, (Schrag & Wienkers, 2000)), which was attributed to simultaneous binding of two pyrene molecules and the predominant product formation from this binding intermediate.

6. Homotropic cooperativity in steady-state kinetics of substrate metabolism CYP3A4

Sigmoidal kinetics in steady-state turnover have been observed with rabbit isozymes of CYP3 group in 80s (Ingelman-Sundberg & Johansson, 1980; Johnson et al., 1983; Johnson et al., 1988; Schwab et al., 1988), before CYP3A4 was first isolated and purified (Guengerich, 2005). Since 1994, when the first systematic study of heterotropic cooperative effects in CYP3A4 was published (Shou et al., 1994), this cytochrome P450 provided multiple examples of homotropic and heterotropic interactions between substrates, inhibitors, and effectors, with many important observations of such drug-drug interactions *in vitro* and *in vivo* (Tang & Stearns, 2001; Shou, 2004; Atkins, 2005; Galetin et al., 2005; Guengerich, 2005; Houston & Galetin, 2005; Atkins, 2006; Niwa et al., 2008a). The list of substrates for which sigmoidal kinetics was observed in microsomes (Carr et al., 2006) includes aflatoxin B1, amitriptyline, carbamazepine, diazepam, estradiol, nifedipine, progesterone, testosterone (Table 3 cited work).

Guengerich and his group systematically studied cooperative effects in CYP3A4 using spectral binding and activity assays with several substrates, such as aflatoxin B (Ueng et al., 1995; Ueng et al., 1997), progesterone (Ueng et al., 1997), testosterone (Ueng et al., 1997; Hosea et al., 2000; Isin & Guengerich, 2006), 17 β -estradiol (Hosea et al., 2000), selectively deuterated testosterone (Krauser & Guengerich, 2005), ANF (Hosea et al., 2000), flavone (Isin & Guengerich, 2006) and some others. Cooperativity was characterized using the Hill equation, and for all substrates Hill coefficients in the range 1.1 – 2.0 were detected. Higher Hill coefficients ($n_H = 1.8 - 3.6$) characterized the oxidation of aflatoxin. In order to explain cooperative effects in binding and steady-state turnover, several different models have been proposed and discussed, such as models with two ligand molecules (substrate and/or effector)

that can bind within one large binding pocket distal to the heme, or alternatively, to two separate binding sites, one of which plays a role of allosteric regulator (Ueng et al., 1997). More complex heterotropic effects observed in the subsequent work (Hosea et al., 2000), provided evidence in favor of simultaneous binding of three ligand molecules, two of which can appear in the immediate vicinity of the heme iron atom while the third may bind at the remote regulatory site.

Heterotropic effects were probed by Phe and Trp scanning mutagenesis, with progesterone, testosterone, 7-benzyloxy-4-(trifluoromethyl)coumarin (7-BFC), and ANF also suggested the presence of at least three binding sites in CYP3A4 (Domanski et al., 2001). High homotropic cooperativity was observed for the ANF oxidation ($S_{50} = 14 \mu\text{M}$, $n_H = 2.5$) with stimulation in the presence of progesterone only at low ANF concentrations. ANF also significantly stimulated reactions of hydroxylation of progesterone and testosterone by decreasing S_{50} without significant changes in V_{max} , and of debenzoylation of 7-BQ by increasing V_{max} with no effect on S_{50} . These heterotropic effects have been diminished in several mutants as compared to the wild type CYP3A4. Comparison of the mutual effects of these four substrates allowed the authors to suggest the presence of three partially overlapping sites with significant specificity with respect to steroid hydroxylation and 7-BQ debenzoylation, and with moderate correlation of both to the ANF oxidation.

Apparent cooperativity can be observed in binding studies as a result of the presence of the residual substrate or inhibitor molecule at the substrate binding pocket of the cytochrome P450 after purification. In such a case, the direct substrate binding experiment will turn out to be a competitive replacement titration and may reveal apparent cooperativity. For instance, it was observed that the cytochrome P450BioI (CYP107H) was purified from *E. coli* in a mixed-spin state because of palmitic acid bound in the active site (Lawson et al., 2004). Titration of such an enzyme preparation with econazole and ketoconazole produced sigmoidal binding curves at pH 7.0, but turned to the usual hyperbolic shape with much tighter binding when palmitate was removed, either at pH 9.5, or by treatment with organic solvents. As a result, the enzyme almost completely shifted to the low-spin state, suggesting that at pH 7.0 palmitate blocks azole binding. Intentional usage of the bulk competitive ligand with the goal of determining the number of binding sites in a competitive titration experiment was described (Fisher, 1970). Recently this method was applied by Roberts and coworkers with CYP3A4 (Kapelyukh et al., 2008) to determine the number of bound substrate molecules. They implemented a modification of the traditional competitive binding approach by using a competitive replacement of large inhibitor molecule bound to CYP3A4 by the smaller substrate molecules to determine the number of binding sites, which become accessible simultaneously after dissociation of inhibitor. In experiments with several inhibitors and 7-benzyloxyquinoline (7-BQ) as a substrate they observed Hill coefficients as high as 3.7 with purified CYP3A4 in reconstituted system and up to 5 with human liver microsomes. As shown in the “ligand exclusion” model (Fisher, 1970), this means that at least five molecules of 7-BQ can bind to CYP3A4 simultaneously to displace a bulk bromocriptine molecule and make product formation possible in the presence of this inhibitor. This large number may be an overestimate because of other possible factors involved, such as redistribution of 7-BQ into the lipid phase of reconstitution mixture or microsomes, and aggregation of CYP3A4. With no inhibitors, CYP3A4 exhibited only a moderate cooperativity in the same reaction of 7-BQ debenzoylation with Hill coefficient of 1.7.

CYP1A2

Cooperativity of pyrene hydroxylation by rabbit CYP1A2 was studied by Guengerich and coworkers (Sohl et al., 2008). Very high Hill coefficients ($n_H = 3.6 \pm 0.6$ for pyrene hydroxylation, and $n_H = 3.0 \pm 1.5$ for benzo[a]pyrene hydroxylation) were observed in a

reconstituted system with CYP1A2 purified from rabbit liver microsomes. Detailed kinetic analysis and comparison of several possible reaction schemes attributed observed sigmoidal kinetics to the predominant contribution of the enzyme complex with two substrate molecules and negligible product formation from the first binding intermediate. These conclusions are consistent with results of the earlier study by Miller and Guengerich (Miller & Guengerich, 2001). Using different substrates they showed at least two binding sites in rabbit CYP1A2 with 8 – 300-fold different affinities, although both binding intermediates had comparable catalytic rates of O-demethylation of the nitrophenyl esters used as substrates (Miller & Guengerich, 2001).

CYP2C9

With CYP2C9 the significant increase of the high-spin fraction at substrate-saturated conditions caused by addition of the heteroactivator dapsone has been documented for several substrates (flurbiprofen, ibuprofen, naproxen) (Locuson, Gannett, & Tracy, 2006). The concomitant increase in product formation rates and a significant improvement in the overall coupling of steady-state catalysis in the presence of activators, have been attributed to changes in the substrate proximity to the heme iron and lower access of water. Effector induced changes to the substrate orientation in the binding pocket was detected by NMR relaxation methods (Hummel et al., 2004). Metabolism of flurbiprofene by CYP2C9 *in vitro* was significantly activated by dapsone in microsomes, but only a minor effect was observed *in vivo* (Hutzler et al., 2001a). Homotropic cooperativity ($n_H = 1.4$) of diclofenac hydroxylation by CYP2C9 was observed in (Konecny et al., 2007). Heterotropic effects in CYP2C9 have been reviewed (Egnell et al., 2003; Tracy & Hummel, 2004; Tracy, 2006), also see Section 7. It is worth noting that the action of heterotropic effectors may be different for different mutants, as documented for CYP2C9 (Hummel et al., 2005). Results may also depend on subtle variations of the effector structure, as was shown by different effect of dapsone and 1-hydroxydapsone on flurbiprofene 4'-hydroxylation (Hutzler et al., 2003). Another example of such variations have been reported in CYP2B1, where no cooperativity have been observed for TS hydroxylation and 7-benzyloxyresorufin (7-BR) oxidation, but oxidation of 7-ethoxy-4-fluoromethyl-coumarine (7-EFC) was cooperative (Scott et al., 2002). The same was true for several single-point mutant proteins with mutations at the F-G loop.

Other Cytochromes P450

Cooperative effects in binding and steady-state kinetics have been also observed in several other cytochromes P450. Interactions of CYP2E1 with two molecules of substrate *p*-nitrophenol or with one substrate and one inhibitor molecule results in non-Michaelis kinetics with low rates of product formation at high substrate concentrations (Collom et al., 2008). Binding of two substrate molecules by CYP2A6 and CYP2E1 was also inferred by the intramolecular isotope effects of hydroxylation of several aromatic substrates (Harrelson et al., 2007). Later, oxidation of *m*-xylene (cooperative) and *p*-xylene (non-cooperative), were studied using selectively deuterated substrates (Harrelson et al., 2008). With *m*-xylene, substrate dependence of the product distribution, intramolecular isotope effects, and heterotropic effects of *p*-xylene on the oxidation of *m*-xylene, all indicate the direct interaction of at least two substrate molecules bound to the enzyme. Cooperative *O*-deethylation of 7-ethoxycoumarin ($n_H = 1.7$) and autoactivation was documented for the Q172H mutant of CYP2B6, while the native protein exhibited normal hyperbolic kinetics (Ariyoshi et al., 2001) (although the N-terminal truncated CYP2B6 also exhibited cooperativity in this reaction in reconstituted system (Spatzenegger et al., 2003)). Interestingly, this mutant enzyme corresponds to the single nucleotide polymorphism which appears at a high frequency in the Japanese population. Cooperativity was also observed for CYP2B6 in human liver microsomes with testosterone and 7-ethoxy-4-trifluoromethylcoumarin, but not with several other substrates (Ekins et al., 1998) (see also (Ekins & Wrighton, 1999) for the review of

autoactivation of CYP2B6, as well as CYP2E1 with 7-ethoxy-4-trifluoromethylcoumarin). Structural and kinetic aspects of mechanism of CYP2E1 and role of effector site have been thoroughly analyzed in a recent review (Miller, 2008).

Hydroxylation of carteolol by CYP2D6 was stimulated by low concentrations of haloperidol, and inhibited at high concentrations of this drug (Kudo & Odomi, 1998), contrary to the simple inhibitory effect of reduced haloperidol on the same reaction. High homotropic cooperativity of propafenone hydroxylation ($n_H = 3.5$) by CYP2D6 in SUPERSOMES (baculovirus microsomes with a single expressed P450) was also reported in (Afshar & Thormann, 2006).

In addition, several soluble cytochromes P450 also exhibited cooperative behavior with selected substrates. Cooperative binding of the natural substrate flaviolone to CYP158A2 ($n_H = 1.6$) has been monitored by the Type I spectral titration in (Zhao et al., 2005). Two flaviolone molecules are seen in the X-ray structure of the substrate bound CYP158A2 (Figure 3C), with one molecule almost parallel to the heme close to the iron atom, and another above the first. A rare example of cooperative epoxidation by soluble bacterial cytochrome P450, cryptophycine P450 epoxidase P450crpE, have been described in (Ding et al., 2008). The binding of cryptophycine and four analogs monitored by the Type I spin shift spectral titration revealed high cooperativity with Hill coefficients between 1.53 – 2.4, and apparent spectral binding constants within 1 – 2 μ M range. However, all Michaelis-Menten plots (v as a function of $[S]$) were linear up to the limit of solubility of the substrates, 100 μ M. The authors attribute this difference to the rate-limiting equilibration of the enzyme-substrate complex, as compared to the relatively efficient catalytic step. For the mutant CYP102, heterotropic and homotropic cooperativity in catalytic reactions have been described in (van Vugt-Lussenburg et al., 2006). Kinetic isotope effects for palmitate ω -hydroxylation in the presence of laurate indicated the presence of the second substrate in the binding pocket (Rock et al., 2003). Sigmoidal kinetics of indole metabolism with the formation of indigo was observed in (Huang et al., 2007). Cooperative hydroxylation of dodecanoic acid ($n_H = 2.3$) was reported for CYP52A1 from *Candida albicans* (Kim et al., 2007), but no further analysis of the source of this cooperativity was described.

Packing of two substrate molecules into the binding pocket was the source of unusual cooperativity in oxidation of 4,6-dimethyldibenzothiophene (Torres & Aburto, 2005) and several other polycyclic aromatic hydrocarbons (Aburto et al., 2008) by chloroperoxidase ($n_H > 2$). Analysis of kinetics attributed the cooperative effects in this system to the preferential binding of stacked π - π dimers of aromatic substrates, compared to the weaker binding of monomeric substrates. Results of these works show that aggregation of substrate may also be a source of sigmoid kinetics even in enzymes which usually exhibit normal Michaelis-Menten behavior.

7. Analysis of heterotropic cooperativity

With the understanding that P450s can simultaneously interact with multiple substrate molecules, and also a variety of substrates the door is open to heterotropic interactions where complex kinetic behavior arises from the interplay between the different components of the system, and larger number of possible enzyme-substrate complexes (Tracy & Hummel, 2004; Atkins, 2005). Several mammalian isoforms of P450s, most notably CYP3A4/5, and also CYP1A2, and CYP2C9, show changes in their kinetic behavior in the presence of a secondary substrate or an effector molecule (Brown et al., 2006; Niwa et al., 2008a). It has also become apparent that this behavior is not limited to mammalian P450s, as demonstrated with bacterial P450s CYP107 (Khan et al., 2003) and CYP102 (Li et al., 2005), and human UGT2B7 (Uchaipichat et al., 2008).

Similar to homotropic cooperativity, heterotropic cooperativity can either be positive (stimulatory) or negative (inhibitory), in its ability to influence enzyme behavior. However, because heterotropic effects are viewed as changes to K_m , V_{max} , or V_{max}/K_m , relative to the single substrate system, heterotropic cooperativity can appear to diminish homotropic cooperativity resulting in seemingly typical hyperbolic activity curves as is the case with ANF's effect on both TST and estradiol hydroxylation (Ueng et al., 1997) and progesterone hydroxylation (Harlow & Halpert, 1998) in CYP3A4. Additionally, in the P450 literature heterotropic cooperativity is typically discussed in terms of activation as the tendency is to explain observed inhibition with competitive, noncompetitive, or uncompetitive models (Tang & Stearns, 2001; Niwa et al., 2008a).

Korzekwa et al. (Korzekwa et al., 1998) considered using complete and simplified kinetic schemes for a two binding site model with either two catalytic sites, or one catalytic and one effector site (Figure 4 and Figure 5 in (Korzekwa et al., 1998)). The complete model of heterotropic cooperativity with six different binding intermediates (E, ES, EB, ESS, ESB, and EBB) and three catalytic rate constants (metabolism from ES, ESB, and ESS) is too complex to analyze when considering the kinetic behavior of two catalytic sites. Using the rapid equilibrium assumption (Segel, 1975), the equation for positive heterotropic cooperativity was derived for the following scheme, when substrate is metabolized at one site, while another site is treated as an effector:

$$v = V_{max} \frac{[S]}{K_m \left(1 + \frac{[B]}{K_B}\right) \left(1 + \frac{\beta[B]}{\alpha K_B}\right)^{-1} + [S] \left(1 + \frac{[B]}{\alpha K_B}\right) \left(1 + \frac{\beta[B]}{\alpha K_B}\right)^{-1}} \quad (7)$$

Here K_m is the apparent Michaelis constant for the product formation from the [ES] binding intermediate, α and β – cooperativity coefficients for the binding enhancement and activation caused by effector B. Other equations, which include competitive binding and possible metabolism of another substrate molecule at the second site (Korzekwa et al., 1998), or simultaneous binding of two substrates and one effector (Kenworthy et al., 2001), have been also derived and reviewed (Tracy, 2006).

Such analysis allows for the estimation of the kinetic parameters, K_m and V_{max} , and the interaction parameters α and β , for the substrate but only a binding constant for the effector. In the case where both of the interacting molecules are enzymatic substrates these methods does not take into account the full extent of heterotropic interactions as the changes of the kinetic parameters of only one of the substrates at a time are compared in the presence and absence of the other. This effectively divides the heterotropic interactions into that of two separate substrate-effector pairs while neglecting that the two substrates can simultaneously modify each other's activities. In addition, K_m determined in steady-state kinetic experiments is not always equal to the equilibrium dissociation constant K_d for substrate binding, and may also depend on the rates of other binding events shown in Scheme 6.

Several allosteric mechanisms have been suggested to give rise to the phenomena of heterotropic cooperativity (Atkins, 2006). Structural studies of CYP3A4 indicate a large, plastic active site which could accommodate multiple substrate molecules, Figure 3E (Ekroos & Sjogren, 2006), while others suggest the existence of a peripheral binding pocket (Williams et al., 2004). The abilities of some effectors to direct the regioselectivity of the substrate reaction, such as in CYP3A4 where TST can increase midazolam 4-hydroxylation while inhibiting the 1'-hydroxylation reaction (Wang et al., 2000), along with CYP2C9 where dapsone can orient the substrate flurbiprofen in the active site (Hummel et al., 2004), also appear to support the active site accommodating multiple substrate molecules. High pressure

spectroscopy with CYP3A4 led to the observation of distinct enzyme conformers which could play a role in the mechanism of cooperativity (Davydov et al., 2003).

Perhaps the most well documented observations of heterotropic cooperativity are described for CYP3A4. Stimulation of P450 activity by flavonoids was observed as early as the 1980s in liver microsomes (Huang et al., 1981; Schwab et al., 1988). Although CYP3A4 is the most abundant hepatic P450 (Shimada et al., 1994), effects of other P450 isoforms cannot be excluded, particularly those of CYP3A5, which is 83% identical to CYP3A4, when working with human liver microsomes (McConn et al., 2004). Initially these effects were believed to be due to allosteric modulation, however later work began to suggest models where multiple substrate molecules could simultaneously reside within the CYP3A4 active site (Shou et al., 1994; Korzekwa et al., 1998). In a recent work (Okada et al., 2009) diverse mutual orientation of thalidomide and midazolam bound in the active sites of CYP3A5 and CYP3A4 provides rationalization for the significant difference in thalidomide effect on midazolam clearance by these two isozymes. An extensive list of CYP3A4 heterotropic interactions was recently compiled by Niwa et al. (Niwa et al., 2008a), which covers a variety of substrate reaction systems, effectors, models of parameter estimation, enzyme sources (various tissues and species), and concentrations, making direct comparison of the reported parameters difficult. Indeed, ANF is reported to competitively inhibit, have no effect, and activate TST 6 β -hydroxylation in the various microsomal systems (Emoto et al., 2001; Emoto & Iwasaki, 2006) presented.

ANF is one of the most commonly studied effectors in this system, even though it is itself a substrate for CYP3A4. As an effector it has been shown to activate a number of reactions including aflatoxin B₁ 8,9-epoxidation, 17 β -estradiol hydroxylation, and TST hydroxylation, while inhibiting aflatoxin B₁ 3 α -hydroxylation (Ueng et al., 1997). ANF has also been shown to activate progesterone hydroxylation (Schwab et al., 1988; Domanski et al., 2000), TST hydroxylation (Harlow & Halpert, 1998), estradiol hydroxylation (Kerlan et al., 1992), diazepam hydroxylation and demethylation (Andersson et al., 1994), carbamazepine epoxidation (Kerr et al., 1994), nifedipine oxidation (Emoto et al., 2001). ANF can also simultaneously activate formation of carboxylic acid and inhibit the ω -3-hydroxylated metabolites of losartan, while losartan, in turn, can inhibit the 5,6-epoxidation of ANF (Shou et al., 2001), as can progesterone (Domanski et al., 2001). Curiously, while ANF was shown to stimulate midazolam 1'-hydroxylation and inhibit 4-hydroxylation, the opposite was true for TST which had the opposite effects on the two hydroxylation products (Wang et al., 2000).

Given the numerous different types of heterotropic interactions observed with CYP3A4 attempts have been made to organize substrates into different classes to gain insight into their competition for overlapping binding sites or lack thereof (Galetin et al., 2003). One such undertaking divided ten substrates into two groups: erythromycin, cyclosporine, and TST formed one, and dextromethorphan, diazepam, midazolam, and triazolam formed the second group. However, the remaining three substrates, terfenidine, nifedipine, and benzyloxyresorufin did not fit neatly into one group or the other (Kenworthy et al., 1999), again demonstrating the difficulty of working with a diverse group of substrates displaying complex behavior.

Evidence for the ability of CYP1A2 to bind multiple substrate molecules came from binding and oxidation studies of the rabbit enzyme (Miller & Guengerich, 2001). Furthermore, in competitive binding studies 1-isopropoxy-4-nitrobenzene increased CYP1A2's apparent affinity for 1,4-phenyldiisocyanide by four fold. Although ANF has long been a known inhibitor for human CYP1A2 (Shimada et al., 1998), only recently has it also been revealed as

a substrate for rabbit CYP1A2 (Sohl et al., 2008) opening the possibility for a new branch of heterotropic interactions in this enzyme system.

CYP2C9 is one of the major drug metabolizing hepatic P450s, and can oxidize a broad variety of substrates (Rendic, 2002). Despite the identification of over 30 heteroactivators through high throughput fluorescent screening (Egnell et al., 2003) analysis of heterotropic interactions with this isoform have been largely based dapson's ability to activate hydroxylation of flurbiprofen (Korzekwa et al., 1998), and the demethylation of naproxen and piroxicam. Flurbiprofen does not appear to stimulate dapson's conversion to the hydroxylamine metabolite (Hutzler, Hauer, & Tracy, 2001b). There is also some question as to the *in vivo* relevance of this heterotropic effect (Hutzler et al., 2001a), although heterotropic activation does appear to be linked to an increased shift in heme spin equilibrium and NADPH consumption (Locuson et al., 2006). Further complicating matters with CYP2C9 is observation that different allelic variants, CYP2C9*1, CYP2C9*3, and CYP2C9*5 show distinct metabolic profiles for substrates such as flurbiprofen (Tracy et al., 2002).

It is worth noting that several other human P450s have also been indicated in heterotropic cooperativity, such as CYP2D6 (Niwa et al., 2008b), CYP2C8 (Wang & Unadkat, 1999), and CYP3A7 (Li et al., 1997; Nakamura et al., 2003). CYP3A7, the major fetal hepatic P450 (Kitada et al., 1985), has catalytic activities similar to CYP3A4/5 (Gorski et al., 1994; Gillam et al., 1997) and therefore is a likely candidate to display heterotropic cooperativity. While the mechanisms for the observations in these enzymes remain unclear, as does their *in vivo* significance, it suggests that more heterotropic interactions remain to be discovered.

8. Alternative sources of functional cooperativity in the cytochromes P450

In CYP101, Glutamate-84 mutation to lysine disturbs the K^+ -binding site and changes the camphor dissociation constant as well as the spin shift induced by substrate binding (Westlake et al., 1999), revealing cooperative interaction between K^+ and camphor binding in the wild type CYP101. A K^+ ion is coordinated between main chain oxygen atoms of Glu84 and Tyr96, and may stabilize the position of B'-helix involved in the substrate recognition, although only subtle differences in positions of these residues can be detected in comparison of the substrate free (1PHC.PDB) and substrate bound (1DZ4.PDB) structures. The effect of Mg^{2+} on the substrate binding and activity of CYP3A4 was also reported (Yamazaki et al., 1995), (Schrag & Wienkers, 2000), although the exact binding site for Mg^{2+} is not known. The allosteric role of metal cations, together with multiple examples of allosteric effectors described above, suggest the existence of a general allosteric mechanism common for the cytochrome P450 fold. A striking example of allosteric regulation of homotropic and heterotropic cooperativity of CYP3A4 by glutathione discovered in the recent work (Davydov et al., 2008b) may also reveal the possibility of such regulatory mechanism. This mechanism possibly involves the binding of ligands near substrate recognition regions formed by the F-G loop and B' helix (Pylypenko & Schlichting, 2004; Wade et al., 2004), which cause conformational and/or dynamic changes with important functional consequences. Such changes triggered by binding at the allosteric site may include changes in the equilibrium properties, i.e. the heme iron spin state (e.g. K^+ binding in CYP101 and the much more favorable spin shift observed after the second ligand binding in CYP3A4 and CYP107), or in dynamic properties (changes in autoxidation rate and geminate rebinding amplitude caused by remote binding of TST in CYP3A4 (Denisov et al., 2007b); an increase of coupling of NADPH consumption to the product formation with the binding of the third TST molecule (Denisov et al., 2007a) and the recently reported slowing down of dissociation rate of Nile Red from CYP3A4 caused by the presence of an effector ANF at the tentative allosteric site (Nath et al., 2008b).

Aggregation or formation of oligomers of enzyme molecules may be another source of cooperativity (Gutheil, 1994; Davydov & Halpert, 2008). In cytochromes P450, the aggregation dependent allosteric regulation by ligand binding at the peripheral site may be due to the thermodynamic linkage of such binding and oligomerization of the enzyme (Ouellet et al., 2008), and concomitant changes in binding affinity, as well as to the modulation of interaction with redox partners (Hazai et al., 2005) and possibly of other functional properties (Davydov & Halpert, 2008). As shown in Figure 4, small ligands may bind at the protein – protein interface and shift equilibrium between monomeric and oligomeric state of cytochrome P450. Examples of such interactions have been reported in X-ray crystal structures of CYP2C8 (Schoch et al., 2004; Schoch et al., 2008) and CYP2R1 (Strushkevich 2008).

Cooperative binding of several azole antifungal drugs has been recently documented for CYP51 and CYP16A2 from *M. smegmatis* (Warrilow et al., 2008). For CYP51, analysis of Type II spectral titration curves with the Hill equation revealed the highly cooperative binding of clotrimazole, econazole, itraconazole, and miconazole ($n_H = 1.8 - 2.55$), and marginal cooperativity ($n_H = 1.2 - 1.36$) for fluconazole, ketoconazole, and voriconazole. For CYP16A2, the binding of clotrimazole was cooperative ($n_H = 1.68$), but for all other azoles almost no cooperativity was observed. Interestingly, for all above systems cooperativity could be eliminated by adding high concentrations of NaCl, with a concomitant decrease of affinity in some cases (Warrilow et al., 2008), which may indicate salt dependent aggregation of the enzyme.

Linkage of the enzyme dimerization and binding of an azole inhibitor have been recently documented for CYP130, a cytochrome P450 from *Micobacterium tuberculosis* (Ouellet et al., 2008). CYP130 with econazole bound appears as a dimer in the crystal (2UVN.PDB), and also exhibits positive cooperativity in the spectral binding of econazole ($n_H = 1.37$) and clotrimazole ($n_H = 1.95$). Results of isothermal calorimetric titration analyzed with a sequential binding model revealed 12-fold enhancement of binding affinity at the second binding step, with calorimetric dissociation constants $K_{d1} = 36 \mu\text{M}$, and $K_{d2} = 3.0 \mu\text{M}$, suggesting low affinity binding to the monomer CYP130 and subsequent formation of dimer, which can bind the second inhibitor molecule with high affinity. The presence of dimers of CYP130 in solution was confirmed by crosslinking with glutaraldehyde. Such coupling of ligand binding and oligomerization is attributed to higher propensity to form dimers of the ligand bound CYP130, which is in the ‘closed conformational state’, as compared to the substrate-free CYP130, which is predominantly in the ‘open form’ (Ouellet et al., 2008). The authors (Ouellet et al., 2008) also note that the dimer with a similar interface is found in the crystal structures of substrate-free CYP154C1 (1GWI.pdb, (Podust et al., 2003)) and of thermophilic cytochrome P450 from *T. thermophilus* (1WIY.PDB, deposited by Y. Kousumi et al., 2004). Other examples of such interactions between monomers in the crystal structures of cytochromes P450, which involve fragments of the F and G helices and the F–G loop include CYP2A6 (1Z10.pdb and 1Z11.pdb, (Yano et al., 2005)), CYP2D6 (Rowland et al., 2006), and CYP3A4 (with the F’ and G’ helices involved, 2J0C.pdb, (Ekroos & Sjogren, 2006)). As shown in Figure 4, binding of the substrate progesterone between the F’ and G’ helices (Williams et al., 2004) in the crystal structure of CYP3A4 involves the part of interface formed between molecules A and B in another crystal structure of CYP3A4, (2V0M.PDB), Figure 3E. In such system, cooperativity can be a result of coupling of substrate binding and monomer – dimer equilibrium, if monomers and dimers have different substrate affinities.

In general, ‘open’ and ‘closed’ forms of cytochromes P450 have been identified in crystal structures of many P450 isozymes, including CYP102 (Li & Poulos, 1997), CYP119 (Yano et al., 2000; Park et al., 2002), CYP101 (Hays et al., 2004), CYP2C5 (Wester et al., 2003a; Wester et al., 2003b), CYP154 (Podust et al., 2004), CYP51 (Waterman & Lepesheva, 2005), CYP158 (Zhao et al., 2005), CYP107L1 (Sherman et al., 2006), CYP2B4 (Scott et al., 2004; Zhao &

Halpert, 2007), CYP3A4 (Ekroos & Sjogren, 2006), and CYP130 (Ouellet et al., 2008), with the main difference in positions of the F and G helices together with the FG loop, and BC loop. Displacement of these fragments with respect to each other changes the volume of the substrate binding pocket distal to the heme and may open or close the substrate access channel (Pylypenko & Schlichting, 2004; Wade et al., 2004; Otyepka et al., 2007). Shift of the conformational equilibrium between these two forms with concomitant changes of ligand binding affinity and other functional properties due to formation of dimers and higher oligomers may be another source of sigmoidal behavior of spin-shift titration and steady-state kinetics (Davydov & Halpert, 2008). In general, if there is an equilibrium between “open” and “closed” forms of the protein, and they can accommodate different numbers of ligands, apparent binding cooperativity will be observed based on the shape of binding isotherm (Johnson, Tanford, & Reynolds, 1985; Rostovtseva et al., 2000).

An additional source of cooperativity may be the difference in the interactions of monomers and oligomers of the cytochromes P450 with their associated redox partners. For the membrane bound cytochromes P450, in liposomes and microsomes, this aspect have been experimentally studied by Backes and coworkers (Backes, Batie, & Cawley, 1998; Cawley et al., 2001; Kelley, Reed, & Backes, 2005; Kelley, Cheng, & Backes, 2006). The scheme with multiple complexes of monomers and dimers of CYP2E1 interacting with one or two molecules of CPR has been invoked in the global analysis of kinetics of p-nitrophenol oxidation (Jamakhandi et al., 2007). Different modes of interaction of dimers formed by cytochromes P450 with CPR have also been modeled in (Hazai et al., 2005). The allosteric role of CPR was suggested in the study of CYP2D6 (Modi et al., 1997). Using NMR relaxation only a single orientation of the bound substrate with respect to the heme was detected, while in the presence of reductase two modes have been observed, with the binding constants 25 μM and 150 μM ; similar to the values of K_m for the two products formed in NADPH supported catalysis. Notably, cumene hydroperoxide supported reaction gave only one product, consistent with the substrate binding mode expected from NMR.

9. Summary

From one perspective, a plethora of effects reported for many cytochromes P450 can be classified as “cooperative” or “allosteric” behavior. On the other hand, true binding cooperativity, i.e., a significantly tighter binding on the second or third binding event, has not been documented. Thus, all, or almost all observed deviations from Michaelis behavior in monomeric cytochrome P450 are attributed to a dramatic difference in the properties of the binding intermediates (Sligar & Denisov, 2007). The observed cases of homotropic cooperativity are due to the fact that the binding of the first substrate molecule to the enzyme is not enough for the efficient metabolism, and only after the second substrate is bound the reaction proceeds at full speed.

Different cytochromes P450 show similar cooperative behavior with various substrates, and in some cases different enzymes exhibit cooperativity with the same substrate. The same fold of cytochromes P450 implies common substrate binding motifs (Pylypenko & Schlichting, 2004; Wade et al., 2004; Johnson & Stout, 2005; Otyepka et al., 2007; Mansuy, 2008), and investigations reveal the presence of high-affinity spectrally silent sites in many cases. Similar observations have been made in activity studies, where cooperativity also can be attributed to the non-productive high-affinity site, and formation of product only after the second substrate molecule binds to the enzyme.

Thus, the cooperativity observed in the catalytic properties of monomeric cytochromes P450 is explained mostly by distinct differences in the functional properties of the enzyme for each of the binding intermediates, rather than by a positive cooperativity of actual substrate binding.

Direct measurements show that in most cases the second substrate binding event occurs with lower affinity than the first, with no apparent increase affinity. Thus the mechanisms of cooperativity in cytochromes P450 have much more in common with the dose-response functions, observed for some receptors, where the stepwise ligand binding is not cooperative, but the active state of the receptor is switched on only after multiple ligands are bound (Biskup, 2007). This is contrary to the classic models of cooperative proteins and enzymes, such as hemoglobin (Perutz, 1989; Ackers, 1998) or aspartate transcarbamoylase (Kantrowitz & Lipscomb, 1990; Koshland & Hamadani, 2002), where the binding affinity of the next step increases over the previous event. Other sources of cooperativity experimentally observed in cytochromes P450 may be the result of substrate dependent changes in oligomerization state of the enzyme and possible allosteric effects of small molecules and protein redox partners on the catalytic properties of cytochromes P450 (Davydov & Halpert, 2008). Given the importance of human P450 isozymes such as CYP3A4 and CYP2C9 in drug metabolism understanding how drugs act as enzyme substrates, inducers, and inhibitors is a consideration in preventing adverse drug reactions (Ogu & Maxa, 2000; Egnell, Houston, & Boyer, 2005; Brown et al., 2006; Obach et al., 2006). Understanding the complex kinetic behaviors of cytochromes P450, including cooperativity, may provide insight into mechanisms of these deleterious drug-drug interactions.

Abbreviations

1-PB, 1-pyrene butanole; 7-BQ, 7-benzoyloxyquinoline; 9-AP, 9-aminophenanthrene; ANF, α -naphthoflavone; BI, binding isotherm; FRET, Förster resonance energy transfer; ITC, isothermal titration calorimetry; K_m , Michaelis constant in Michaelis-Menten equation; S_{50} , substrate concentration corresponding to the half-saturation in the Hill equation; TST, testosterone.

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Figure 1.

(A) Binding isotherms for two-site system. Identical non-interacting sites (equation 1, black line), positive cooperativity (light gray line), negative cooperativity (equation 2, dark gray line). The ratios of stepwise binding constants $K_2/K_1 = 0.25, 2.98$, and 0.0567 (selected to provide the same Hill coefficients as shown in Figure 1B). Here cooperativity coefficients α are 1 (non-cooperative case), 11.9, and 0.227, corresponding to cooperative free energies of 0, 1.47 kcal/mole, and -0.9 kcal/mol, assuming that the difference between the two binding events is due to the cooperative interaction between two identical binding sites. (B) Binding isotherms with Hill coefficients 1.0 (black), 1.5 (light gray), and 0.7 (dark gray).

Figure 2.

(A) Hill coefficient as a function K_2/K_1 , calculated using an equation $n_H = 4 \cdot (K_2/K_1)^{1/2} / (1 + 2 \cdot (K_2/K_1)^{1/2})$. (B) Comparison of calculated binding isotherms (equation 2) with the spectral titration isotherm calculated for the case when the first binding event is 'spectrally silent', and all signal is produced by the second ligand binding. Black curves: Full line, non-cooperative binding isotherm, $K_1 = 4K_2$, $n_H = 1.0$; dashed, corresponding signal from the second binding intermediate, apparent cooperativity with $n_H = 1.3$. Gray curves: Full line, cooperative binding isotherm with $K_1 = K_2$, $n_H = 1.3$; dashed line, corresponding signal from the second binding intermediate with apparent cooperativity, $n_H = 1.48$.

Figure 3.

Structures of cytochromes P450 with two or three substrate molecules bound. All enzymes are shown in the similar orientation with hemes represented as green sticks and substrates as space filling blue mesh. (A) CYP107 with two 9-AP molecules (1EGY.PDB), cooperative spin-shift titration with $n_H=1.38$ (Cupp-Vickery et al., 2000); (B) CYP107 with two androstenedione molecules (1EUP.PDB), cooperative spin-shift titration with $n_H=1.31$ (Cupp-Vickery et al., 2000); (C) CYP158A2 with two flaviolin molecules (2D0E.PDB), cooperative spin-shift titration with $n_H=1.6$ (Zhao et al., 2005); (D) CYP158A1 with two flaviolin molecules (2NZ5.PDB), non-cooperative spin-shift titration with $n_H=1.1$ (Zhao et al., 2007); (E) CYP3A4 with two molecules of ketoconazole (2V0M.PDB), (Ekroos & Sjogren, 2006); (F) CYP2C8

with two molecules of 9-cis retinoic acid (2NNH.PDB), cooperativity was not detected in spectral binding experiments (Schoch et al., 2008); (G) CYP2B4 with three molecules of bifonazole (2BDM.PDB), binding cooperativity not reported (Zhao et al., 2006). Bifonazole binding to the two distal hydrophobic sites appears to be low affinity, because it was not detected in the ITC experiments. The functional relevance of these two additional binding sites is unclear (Zhao et al., 2006); (H) CYP245 with three molecules of chromopyrrolic acid (CPA) (2Z3U.PDB), binding cooperativity not reported (Makino et al., 2007). Binding of two additional molecules might represent a crystallographic artifact (Makino et al., 2007).

Figure 4. Dimer interfaces mediated by small molecules in X-ray crystal structures of cytochromes P450. (A) Interaction of two molecules of CYP2C8 mediated by two molecules of palmitic acid (orange sticks). Shown is the same X-ray structure as in Figure 3F (2NNH.PDB) with hemes (red sticks) and substrate 9-cis retinoic acid (magenta sticks). (B) Interaction of two molecules of CYP2R1 mediated by two molecules of cyclodextrin (orange sticks). Shown is the X-ray structure in complex with heme (red sticks) and substrate vitamin D3 (magenta sticks) (3CZH.PDB, (Strushkevich et al., 2008)). (C) Substrate progesterone (gold sticks) bound at the peripheral site of CYP3A4 (1W0F.PDB, (Williams et al., 2004)), which is shown in the similar orientation as dimers of CYP2C8 and CYP2R1.

Scheme 1.

Scheme 2.

Scheme 4.

Scheme 5.

Scheme 6.