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A Novel Type of Allosteric Regulation: Functional Cooperativity in Monomeric Proteins

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Abstract

Cooperative functional properties and allosteric regulation in cytochromes P450 play an important role in xenobiotic metabolism and define one of the main mechanisms of drugdrug interactions. Recent experimental results suggest that ability to bind simultaneously two or more small organic molecules can be the essential feature of cytochrome P450 fold, and often results in rich and complex pattern of allosteric behavior. Manifestations of non-Michaelis kinetics include homotropic and heterotropic activation and inhibition effects depending on the stoichiometric ratios of substrate and effector, changes in the regio- and stereospecificity of catalytic transformations, and often give rise to the clinically important drug – drug interactions. In addition, functional response of P450 systems is modulated by the presence of specific and non-specific effector molecules, metal ions, membrane incorporation, formation of homo- and hetero oligomers and interactions with the protein redox partners. In this article we briefly overview the main factors contributing to the allosteric effects in cytochromes P450 with the main focus on the sources of cooperative behavior in xenobiotic metabolizing monomeric heme enzymes with their conformational flexibility and extremely broad substrate specificity. The novel mechanism of functional cooperativity in P450 enzymes does not require substantial binding cooperativity, rather it implies the presence of one or more binding sites with higher affinity than the single catalytically active site in the vicinity of the heme iron.

Keywords

Cytochrome P450; Cooperativity; Allosteric effects; Heterotropic interactions; Drug – drug interactions; Substrate specificity

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Epigraph

SJG: Let’s talk about the ideas that were gradually forming for the concept of allosterism.

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> JW: They had really begun earlier. The paper with David Allen was published in 1951. But it was during my first trip to Japan that I really had a clear vision of conformational changes as giving rise to an entire array of functional properties.
>
> SJG: And this idea just came to you?
>
> JW: It came to me as I was walking in Kyoto in a Zen garden
>
> *Conversations with Jeffries Wyman. Interview by Stanley J. Gill* [1]

Introduction

Most of the key biological processes, such as formation of native biomolecules and their functional assemblies, as well as cellular signaling and regulation, are highly cooperative [2-4]. Cooperativity and allostery are observed as a result of a mutual perturbation of functional properties of a biological macromolecule interacting with two or more ligands [2]. This feature is commonly used in living systems to improve sensitivity to external chemical perturbation and to amplify the response of receptors, transport molecules [5-7], or xenobiotic metabolizing systems, including cytochromes P450 [8-11]. Homotropic and heterotropic cooperative effects in cytohromes P450 are commonly known as one of the main sources of drug – drug interactions [12-18]. We recently reviewed cooperativity concepts applied to cytochromes P450 [19]. However, many recent results suggest an update and refinement of the earlier conclusions on the molecular interactions giving rise to the cooperative functional response of cytochromes P450.

In biochemistry and biophysics, cooperativity is typically defined as the mutual perturbation of interactions of the ligand with a macromolecule (protein or DNA) at different binding sites [20]. Various aspects of cooperative effects in macromolecular binding have been thoroughly described in several excellent books [2, 21-25]. Positive cooperativity is defined as the increase of the binding affinity at one site when other site is also occupied, and negative cooperativity is manifested if the second binding event is disfavored. Cooperative enzymes typically display a sigmoid plot of the reaction rate against substrate concentration, and an experimentally observed sigmoid dependence of activity on the substrate concentration is often interpreted as indication of cooperative binding, but this is not always true. Deviations from the simple Langmuir or Michaelis-Menten hyperbolic dependence of the enzyme properties on the substrate concentration can suggest the presence of more than one binding site and simultaneous interactions with several substrates and / or effectors. Importantly, sigmoidal steady-state kinetics is also possible in monomeric enzymes which bind only one substrate molecule at the single catalytic site [26]. An example of such system is represented by the human glucokinase, where slow conformational rearrangements between the states with low activity and high activity upon substrate binding explains significant functional cooperativity [27], as reviewed in this issue by Larion and Miller [28].

The word ‘allosteric’ was introduced by Monod and Changeux 50 years ago, as described in [29], while the idea of thermodynamic coupling between binding and conformational changes in macromolecules “…which gives rise to the entire array of functional properties …” [1] have been put forward by Jeffries Wyman as early as in 1951 [30]. His analysis of the cooperative binding of oxygen and carbon monoxide to the tetrameric human hemoglobin, and of the pH dependence of cooperativity known as Bohr effect, successfully attributed all observed effects to the large scale pH-dependent conformational equilibrium of hemoglobin tetramer between two well defined states with substantially different affinities. This work was the first seminal study which suggested the concept of the thermodynamic linkage between functional properties of macromolecules and their conformational and dissociative equilibria, which was developed further in 60s and 70s as reviewed [20, 31-36].

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In the simplest case, the overall binding of the small ligand to the macromolecule with N identical and non-interacting binding sites is described by the Langmuir isotherm (Equation 1), which does not depend on N:

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

As shown on the Figure 1, the shape of the binding isotherm plotted as the full line, is the same for N = 1,2,3. However, concentrations of the binding intermediates (dashed lines) are not identical. As seen from Equations 2 and 3, the binding isotherms can be represented as $Y = y_1/2 + y_2$ for two-site macromolecule, and $Y = y_1/3 + 2•y_2/3 + y_3$ for three sites, where y1, y2, and y3 are fractions of intermediates with one, two or three ligands bound.

$$Y=\frac{\frac{[S]}{K_1}+\frac{2[S]^2}{K_1K_2}}{2\left(1+\frac{[S]}{K_1}+\frac{[S]^2}{K_1K_2}\right)}=\frac{1}{2}y_1+y_2 \quad (2)$$

$$Y=\frac{\frac{[S]}{K_1}+\frac{2[S]^2}{K_1K_2}+\frac{3[S]^3}{K_1K_2K_3}}{3\left(1+\frac{[S]}{K_1}+\frac{[S]^2}{K_1K_2}+\frac{[S]^3}{K_1K_2K_3}\right)}=\frac{1}{3}y_1+\frac{2}{3}y_2+y_3 \quad (3)$$

The multipliers 1/2 and 1 for two-site binding (equation 2), and 1/3, 2/3 and 1 for three-site binding (equation 3) yield the fractional contribution of corresponding binding intermediates to the overall binding isotherm, as they represent the fractions of occupied sites for each macromolecule. For non-cooperative binding to the macromolecule with N identical sites, the population of jth intermediate reaches it's maximum value at the same time when the average saturation reaches j/N [37].

In case of highly cooperative binding, the population of all binding intermediates except the last is low because of the large increase of the binding affinity when macromolecule approaches saturation. This results in the change of the shape of binding isotherm from convex (hyperbolic, non-cooperative Langmuir isotherm, Figure 1) to concave, or "sigmoidal", as shown in Figure 2 for dimeric hemoglobin HbI from *Scapharca inaequivalvis* [38] and tetrameric human hemoglobin [39].

Importantly, the same result can be observed for the functional response of macromolecule even in the case of non-cooperative binding, if the fractional contributions of the binding intermediates into the overall observed macromolecular function is different because of the different activity at the different levels of saturation. For the molecule with three binding sites the overall response can be expressed as the linear combination of contributions from each binding intermediates as shown in equation (3a):

$$Y_a=\frac{a_0+a_1\frac{[S]}{K_1}+a_2\frac{[S]^2}{K_1K_2}+a_3\frac{[S]^3}{K_1K_2K_3}}{1+\frac{[S]}{K_1}+\frac{[S]^2}{K_1K_2}+\frac{[S]^3}{K_1K_2K_3}}=a_0y_0+a_1y_1+a_2y_2+a_3y_3 \quad (3a)$$

Here the shape of the overall observed response Y_a (typically measured as a spectral signal or activity) as a function of the substrate concentration, depends not only on the stepwise dissociation constants K_i, (i=1,2,3), but also on the fractional amplitudes a_i. For instance, if

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the signal is high only at full saturation, and all amplitudes are small compared to a_3, the shape of Y_a as the function of S will be sigmoidal with a highly cooperative functional response, even if the binding is non-cooperative.

Such model free qualitative analysis of these equations illuminates two limiting cases with alternative sources of the observed cooperative properties of the system. One source of the observed functional cooperativity may be the presence of significant binding cooperativity, giving rise to a substantial deviation of the binding intermediates distribution from their non-cooperative reference state. Alternatively, functional cooperativity can be the result of difference in the functional properties of binding intermediates even in the system with non-cooperative binding. The first case is realized in such textbook examples of cooperativity as human hemoglobin (see Figure 2). The second case is the main source of cooperative functional properties of cytochromes P450, as will be discussed later in this review (Figure 4 below). Examples of the spectrally silent, or functionally silent binding of the first substrate molecule with higher affinity, and strong functional response generated by the subsequent binding of one more substrate molecule have been described for CYP3A4 [40-43], CYP1A2 [44], CYP2C9 [45], CYP107 [46-47].

Structural basis of cooperativity in P450

Simultaneous binding of several substrate molecules to one monomeric P450 heme enzyme in P450 systems is required to explain the homotropic and heterotropic effects, which has been observed in equilibrium titration studies and in steady-state kinetics of the substrate turnover. Visualization of multiple substrates binding modes can be realized via X-ray structure determination, where several substrate molecules, inhibitors, or small molecule detergents are well resolved in the main substrate binding pocket or at the tentative peripheral binding sites. Figure 3 illustrates fours examples of this situation, and other examples can be found in [19].

In the case of cytochrome P450, positioning of the substrate molecule in the immediate vicinity of the catalytically active heme – oxygen complex and in the proper orientation with respect to the ferryl-oxo intermediate is necessary for efficient and regiospecific catalysis. This functionally efficient positioning can be stabilized by conformational changes of the substrate binding pocket caused by interactions of the enzyme with other small molecules, which appear in such cases as allosteric effectors. In addition, in many cytochromes P450, the volume of the substrate binding cavity is large enough to accommodate two or more molecules of the same substrate, while binding of the second one can improve packing and define the stereospecific preference of catalytic transformation. The resulting packing and regiospecific catalysis has also been shown to depend on the nature of effector molecule [48-50] and on the interactions with the redox partner.

If the binding site is not involved in catalytic action, this effect is often termed “allosteric”. On binding an allosteric effector molecule, the catalytic activity of an enzyme towards the substrate may be enhanced, in which case the effector is an activator, or reduced, as in the case of an inhibitor. A classic example of the allosteric activation in P450 system is the K^+ stabilizing effect on the conformation of B-B’ helices in P450cam, which results in higher affinity to the substrate camphor and almost complete spin shift upon camphor binding in the presence of 100 mM K^+ cation [51-52]. A structural interpretation was recently provided through X-ray crystallography of CYP101 in the presence and in the absence of KCl [53]. Without K^+, the B-B’ loop is partially disordered, and the F-G helices move to open the main substrate entrance pathway. The presence of K^+ alone is not enough to shift the structure towards the closed state, while in the presence of the substrate the closed conformation is the most favorable. This is consistent with traditional models of allosteric

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regulation, which are generally interpreted following the Monod – Wyman - Changeux paradigm, which is based on the existence of several conformational states (active and inactive) with a different affinity of these states for the ligand (substrate, effector, etc.) [54]. For the cytochromes p450 such concepts have been developed by D.R. Davydov and J.R. Halpert [55-56]. In their work, emphasis is made on the role of conformational equilibria of the heme enzyme molecules, which are coupled with changes in the oligomerization state of the enzyme.

Many studies of P450 enzymes suggest the presence of yet another mechanism of allosteric interactions between substrates, or substrates and effectors. This mechanism is essentially different from the traditional paradigm of allostery in two fundamental aspects; (i) it involves direct contact or interactions between two or more substrate molecules; and (ii) it does not necessary require (albeit may involve) any specific conformational changes of the protein which mediate affinity modulation caused by effector binding to the remote site. It also does not involve significant changes in the affinity of the enzyme for the substrate caused by the effector binding, so that the cooperativity free energy ($\Delta\Delta G$) is close to zero. At the same time, this mechanism falls under definition of functional allostery because the dose - response function deviates from a simple Langmuir (Michaelis) behavior as a result of the effector binding to the same enzyme molecule. For instance, resolution of the stepwise binding constants for three molecules of testosterone to CYP3A4 revealed almost non-cooperative binding [40], despite significant cooperativity derived from the spectral titration and product formation curves. Similar result was obtained for homotropic titration with alpha-naphthoflavone (ANF[1]) [57]. Binding isotherm for ANF calculated with the stepwise binding constants evaluated using global analysis has the Hill coefficient 1.19, indicating low intrinsic binding cooperativity, while the spin shift titration curve and product formation have much higher cooperativity [57]. These results require new conceptual approach for interpretation of cooperativity and allosteric properties, unlike the traditional paradigm of cooperativity in biological macromolecules. For the human hemoglobin, the function and all observed properties are directly proportional to populations of intermediates, and the spectral response is the same from each binding event. Contrary, for some receptors it was shown that ligand binding and populations of intermediates are non-cooperative or even negatively cooperative [58], but the response is positively cooperative [59]. The available structural and functional information for the cytochromes P450 suggests the presence of some new and unusual features, which allow the substantial functional cooperativity without significant binding cooperativity and extremely broad specificity with respect to the small hydrophobic substrates, in combination with the true allosteric effects specific for some isozymes but absent in others.

Cooperativity in P450 catalytic cycle

In most cases, cytochromes P450 are assumed to be fully functional as monomeric heme enzymes, although there is evidence for a significant role of aggregation and formation of homo- and hetero-oligomers, especially for the membrane bound eukaryotic enzymes [55-56, 60-61]. The importance of cytochrome P450 oligomerization for their cooperative and allosteric functional properties is briefly discussed further in this review. In addition, the interaction of the P450 heme enzyme with its redox partners can be also modulated by the presence of substrates, effectors, and by interactions with other cytochrome P450 molecules [56, 60, 62-65].

The commonly accepted catalytic cycle shown in the Scheme 1, includes all important intermediates of P450 catalysis. In the substrate free resting state **[1]** the heme iron Fe^{3+}

[1]Abbreviations: ANF, alpha-naphthoflavone; MDZ, midazolam; TST, testosterone;

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usually is coordinated to water or hydroxide and is predominantly in the low spin state (S=1/2), although in some cytochromes P450 it can be mostly in the high spin state (S=5/2) even in the absence of substrates [66]. In the former case, substrate binding **[2]** displaces water from the 6th coordination position, lowering the ligand field, shifting the spin state to the high spin (S=5/2) and lifting the midpoint potential [67-69]. As a result of these changes, cytochromes P450 saturated with substrates are usually reduced much faster than in the substrate-free state, due to a positive shift of the redox potential by 80 - 130 mV [67-70]. In addition, faster electron transfer in the presence of substrate may be partly due to the lower reorganization energy [71-72].

One-electron reduction of the heme iron to the ferrous state **[3]** is necessary for oxygen binding and formation of and oxyferrous complex **[4]**, which has predominantly ferric-superoxo character. One more reduction step results in peroxo-ferric complex **[5a]**, which is sequentially protonated to form hydroperoxo-ferric complex **[5b]** and, after heterolytic scission of the O-O bond, the main catalytic species termed Compound I is formed **[6]**. Most reactions driven by P450 enzymes are catalyzed by this species via oxidative transformation of the substrate (hydroxylation, epoxidation, N-demethylation, etc.). In order to afford the direct contact with the catalytically active oxygen atom, the substrate molecule has to be positioned in the immediate vicinity of the ferryl-oxo moiety. At the same time, such substrate binding close to the iron atom in the resting state of the enzyme will result in destabilization of the 6th axial ligand, water, and in the shift of the spin state necessary for the efficient reduction, as described earlier. Thus, positioning of the substrate molecule close to the heme iron is mandatory for both the efficient transition of the cytochrome P450 from the resting state **[1]** through the oxygen binding and activation steps **[2]** → **[3]** and **[3]** → **[4]**, and for the committed catalytic step leading to the product formation **[6]** → **[7]**.

Modulation of the rate of the first electron transfer by the substrate binding represents an important feature of the overall steady-state kinetics of many cytochromes P450, whereas both substrate binding close to the heme iron and the concomitant spin shift are necessary for the efficient catalytic turnover of P450 system. In many cases a strong correlation exists between the spin shift caused by the substrate binding and the rate of the product formation [40]. A clear example of critical dependence of the first electron transfer to the heme upon the substrate binding is represented by CYP102, in which reduction and formation of Fe-CO complex was undetectable in the absence of substrate [67, 73-74]. This coupling of the substrate binding and accelerated reduction of cytochromes P450 allows for the screening of potential substrates by monitoring NAD(P)H consumption in the reconstituted systems [75]. In other cases, substrate binding does not produce a substantial spin shift, but acceleration of NAD(P)H consumption indicates that this step indeed is important for functional turnover. In some sense, catalytically competent binding of the substrate may be considered as an allosteric effector of functional interaction with redox partner.

Accordingly, more complex behavior and different scenarios can be expected in case when several substrate molecules can bind simultaneously to the single monomeric cytochrome P450. A great variety of binding configurations can be envisioned, with the binding sites well separated or partially overlapping, depending on the specific cytochrome p450 and substrate size and structure [76-77]. Several examples of the X-ray structures of different cytochromes P450 with two or three substrate molecules are represented in Figure 3. As shown in Figure 3, because of the steric restrictions only one substrate molecule at any given moment of time can be placed at the catalytic site within contact distance to the active oxygen species (see Figure 3). In case of substrate binding at the sites remote from the heme iron, neither spin shift, nor product formation is expected to occur. Finally, if binding to the remote site(s) is stronger, the substrate molecules will first saturate these high affinity sites before binding at the catalytically competent position near heme iron. In such case, the

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functional dependence of the spectral signal (spin shift titration) and of the steady-state rate of product formation on the substrate concentration display significant deviations from the Michaelis-Menten or Langmuir hyperbolic non-cooperative shape and will yield a sigmoidal behavior, reflecting the features of cooperative system.

Moreover, even in case of several binding sites with the same affinity, i.e. for the purely non-cooperative stochastic binding, a pronounced functional cooperativity will be observed, if only one of these sites is catalytically active. The only condition for such *functional* cooperativity with no *binding* cooperativity is the presence of catalytically non-competent sites with the same or higher affinity for substrate than the affinity of catalytic site. To the best of our knowledge, there is no other example of monomeric enzyme with such mechanism of functional cooperativity as is displayed in the eukaryotic cytochromes P450. This may be due to the fact that in most enzymes the substrate binding is highly specific, with affinity of catalytic site significantly higher than that of other sites, when they are present, and the overall functional response in such cases follows simple hyperbolic dependence on the substrate concentration, typical for non-cooperative enzymes. This is also true for the specific cytochromes P450, such as bacterial CYP101, or mammalian CYP19, CYP17, and other enzymes involved in steroid hormone biosynthesis, with substrate affinities in the range $10^{-8} – 10^{-6}$ M. On the contrary, in the xenobiotic metabolizing cytochromes P450, such as CYP3A4, with their extremely broad substrate specificity, binding to the catalytic site is relatively weak, with dissociation constants in $10^{-6} – 10^{-4}$ M range. This is comparable to the general non-specific binding of hydrophobic compounds and drugs to the human serum albumin [78-79] and thus corresponding to the levels of these drugs in blood plasma.

As a result, the allosteric response from P450 systems is based not on the highly specific conformational perturbations of the active site caused by effector binding at another well defined allosteric site, as it is usually happens in other classical types of allosteric enzymes [7]. Although there are examples of the similar well defined and highly specific allosteric effects in cytochromes P450, such as the potassium ion effect in CYP101 [51], in most cases cooperative or allosteric functional response in P450 enzymes are based on different mechanism, which is not common for other enzymes and combines very broad substrate specificity with the pronounced activation triggered by various effectors in the relatively narrow concentration range. In addition, this allosteric P450 mechanism is realized in a monomeric heme enzyme with the single catalytic site, as opposed to most other allosteric enzymes and proteins, where the key feature for the cooperative response is oligomerization.

The non-hyperbolic functional response in cytochromes P450 is based on the presence of several binding sites for the substrates and / or effectors, and on the lower affinity of the catalytically active site, as compared to one or more other sites on the macromolecule of the enzyme. In such cases, high affinity sites are saturated first during titration, with no spectral response from the catalytically active heme iron site, and no product formation in the functionally reconstituted system. Only when the substrate is bound at the active site in the immediate vicinity of the heme, this can be detected by spectral methods (Type I spin shift), by increasing NAD(P)H consumption and by appearance of the product, all these functional properties clearly indicating non-hyperbolic, or cooperative response. In the presence of effector molecules (other than target substrate), the binding of effectors to the remote non-active sites will efficiently increase the total substrate concentration and accelerate metabolism of the substrate. Of course, the overall result can vary for different substrates and effectors, which may perturb the structure of the heme enzyme (thus acting as classical allosteric effectors), binding thermodynamics (in case of true homotropic or heterotropic binding cooperativity) and kinetics, changing the rates of substrate binding to, and product release from the catalytically active site. These allosteric effects can be present as well and

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may play an important role in overall functional response of any given combination of particular cytochrome P450 and one or more substrates. However, even without such site-specific and substrate-specific allosteric contributions, the general structural features and flexibility of P450 fold and ability of these enzymes to simultaneously bind several small or medium sized molecules with similar or higher affinity at the remote sites than at the catalytically active site, makes these enzymes so powerful and indispensable tools for the first line of chemical defense in a variety of organisms.

At the present time, there is little information available on the positions and affinity of secondary (non-catalytic) binding sites of substrates or effectors in P450 enzymes, although their existence is confirmed by various indirect experimental data, as well as some modeling studies. A second binding site for the endogenous substrate camphor was suggested to present in CYP101A1 (P450cam) between F-helix and beta-5 loop [80]. Two alternative sites for camphor binding have been tentatively identified in the open form of the X-ray structure of CYP101D2 [81], one at the substrate access channel, and another on the outside of the molecule near the F-helix, at the similar cavity as in CYP101A1. In CYP3A4 crystallized in the presence of the substrate progesterone, a peripheral binding site for steroid molecule was identified between F' and G' helices on the surface of the molecule [82]. Some of the residues which form this site have been previously implied to play an important role in cooperative properties of CYP3A4 based on results of point mutations in this region [76]. Further site-specific binding studies using labeled CYP3A4 and monitored by fluorescence spectroscopy [83] also indicated the presence of multiple binding sites for ANF. Other examples of binding of small organic molecules outside of the substrate binding pocket, can be visualized from the X-ray structures of CYP2C8 [84], CYP2R1 [85], CYP24A1 [86], and CYP2B4 [87].

Another important aspect of allosteric functional modulation of cytochromes P450 can be their interactions with the membrane. Consistent conclusions coming out of different experimental and theoretical studies indicate that significant part of F'-G' loop is embedded into the membrane bilayer interacting with the lipid polar head groups [86, 88-92]. Our recent work [93], in which a series of all-atom molecular dynamics simulation of the CYP3A4 incorporated at different depth of insertion into the model POPC bilayer found the equilibrium position of this cytochrome P450, also indicates that the external 'progesterone binding' site is involved in contact with the membrane.

Figure 5 shows the progesterone binding site as a cyan colored surface at the membrane – water interface. Interaction of the F'-G' helices and connecting loops with the membrane should be also important because it may significantly change the dynamics of the open – close movement of the F-G helical bundle, which is considered to provide the main pathway for the substrate binding in cytochromes P450 [86, 94-99]. However, the role of the membrane in the allosteric properties of eukaryotic cytochromes P450 is yet unexplored and needs further insight from both theoretical and experimental sides.

Analysis of homotropic cooperativity

Cooperative binding of substrates to the cytochromes P450 is usually indirectly derived from cooperative functional behavior, such as the spin shift measured in titration experiments, or the rate of product formation as a function of substrate concentration. In those cases when binding was measured directly, by such methods as isothermal titration calorimetry (e.g. CYP107A1 [100]), the binding constants for the first and second steps were similar, that is. no binding cooperativity is present, despite cooperative spin shift titration curves and turnover. Several other studies which used fluorescent assay to measure model substrate binding to CYP3A4 [42, 83, 101], CYP1A2 [44] and to CYP107 [46, 102]

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also concluded that the first binding happens with higher affinity, i.e. no positive cooperativity in binding. The main problem for the analysis of the spin shift titration data, or turnover rates as function of substrate concentration, in terms of stepwise binding constants is their strong correlation with the partial functional amplitudes (contribution of corresponding binding intermediate to the overall spin shift or product formation) [103]. However, both binding constants and partial functional amplitudes can be resolved by simultaneous analysis of several different experimental data sets (spin shift titration, NADPH consumption rate, and product formation) obtained under identical conditions to ensure the same set of binding constants in all three data sets. Using this 'global analysis approach' we were able to resolve the full set of stepwise binding constants for CYP3A4 with testosterone [40] and ANF [57] as substrates. In both cases up to three molecules of each substrate could bind to one CYP3A4 monomer, and little or no cooperativity (less than 0.5 kcal/mol of positive interaction energy) was found. Therefore, significant homotropic cooperativity observed in the Type I titration experiments and steady state kinetics of metabolism of these substrates by CYP3A4 is mostly the result of an alternative mechanism of functional cooperativity. This mechanism is based on the significant difference of the functional response from the binding intermediates with different stoichiometry, i.e from the enzyme with one, two, or three substrate molecules bound. Substantial cooperativity of binding, which is usually based on highly specific structural and chemical features of the protein – ligand pair, is not required in this case. Such mechanism allows for a highly cooperative response to the very broad class of small and medium size hydrophobic substrates, i.e. substantial cooperativity with very broad substrate specificity (or selectivity), which is especially important for the hepatic xenobiotic metabolizing cytochromes P450.

The main features of the mechanism of functional cooperativity in CYP3A4, (and likely in other hepatic xenobiotic metabolizing cytochromes P450) are the following. Each monomeric heme enzyme can bind two or more substrate molecules, which are distributed between several possible binding sites positioned either near, or far from the heme iron, and possibly in multiple orientations. First binding event almost does not cause any spin shift (less than 20% with testosterone (TST) [40], and 4% with (ANF) [57]), and no product is formed. Most likely, the first binding happens mainly at the remote from the heme site, possibly at the progesterone binding site between F' and G' helices, however minor fraction of the substrates can appear at the vicinity of the heme iron, which may explain small but detectable spin shift observed with TST. When the second substrate binds, two substrate molecules can pack into the active site, so that the product formation rate reaches maximum, as well as the spin shift (98% for testosterone) and NADPH consumption rate [40]. Three substrates are turned over with the same rate, but coupling is better. In case of ANF, the situation is qualitatively similar with minor variations in the properties of the second binding intermediate [57]. Comparison of two plots shown in Figure 4 for TST and CYP3A4 highlights the non-cooperative binding for all three binding intermediates, and their different contribution to the overall functional properties because of their different partial functional amplitudes (spin shift, NADPH oxidation rates, steady-state turnover rates).

Allosteric effects of the substrate binding to the remote high-affinity site in CYP3A4 are manifested in variations of other properties of this enzyme. First binding, although spectrally silent and unproductive, results in the increase of the CO geminate rebinding yield and in significant stabilization of the oxy-ferrous complex against autoxidation [41]. In addition, activation energy of autoxidation increases in the presence of substrate as compared to the substrate free CYP3A4 [104]. This means that the effects of the first substrate binding can be called allosteric, i.e. remote from the catalytic site but having significant functional implications. Binding at this allosteric site inhibits the escape of diatomic ligands from the heme pocket, both structurally, through steric occlusion of the escape pathway, and dynamically, via higher activation energy of the opening large-scale movements of the

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protein molecule which modulate kinetics of binding and dissociation of small molecules in cytochromes P450. Similar effect was described in [105], where the rate of dissociation of the model substrate Nile Red from CYP3A4 substrate binding pocket was decreased by binding of the effector ANF presumably at the same site. Such global effects of the substrate or effector binding (as opposed to the local effects represented by the spin shift and changes of the redox potential as a result of substrate binding in the immediate vicinity of the heme iron) appear to be a significant part of functional behavior of CYP3A4, and likely are the essential part of cytochrome P450 fold, as more evidences appear of the strong influence of interactions with substrates and inhibitors at the remote sites of the enzyme on their activity [87].

Heterotropic cooperativity

Heterotropic interactions between the substrate and other small molecule effector have been described in other P450 systems, and are sometimes used intentionally to favor catalytic transformations of non-native substrates. In CYP102, the presence of perfluorinated fatty acids greatly improved the ability of the enzyme to hydroxylate the gaseous hydrocarbons, such as butane, propane, ethane, and even methane [50, 106]. Similar approach termed 'decoy substrate' was successfully used in peroxygenase CYP152A1 to significantly improve the efficiency of catalytic oxidation of styrene and ethylbenzene by addition of the short-chain fatty acids [107-108].

In CYP3A4 many examples of heterotropic interactions between different substrates have been described, and various mechanisms proposed to explain these effects [15, 109-113]. Changes of the region-specific ratio in midazolam (MDZ) metabolism (4-OH / 1-OH) *in vivo* and *in vitro* in the presence of fluconazole have been attributed to allosteric effects [114-115]. Interestingly, the results of the similar control experiments in vitro were different, showing no increase in 4-OH formation, consistent with the non-specific inhibitory effect of fluconazole, expected from this Type II inhibitor. The effect observed in vivo may be a result of multiple complexes of CYP3A4 existing in vivo and the difference in affinity of MDZ binding to oligomers of CYP3A4 in different orientations. It is possible also that in as crowded and complex environment as it is in the ER membranes for CYP3A4, interactions of the heme protein with fluconazole at alternative sites, which do not involve direct coordination to heme iron, can affect the substrate binding equilibria or other steps in the catalytic cycle and modify the product distribution in the overall steady-state kinetics of MDZ metabolism by CYP3A4. Such observations may be also due to the indirect perturbation of the CYP3A4 functional properties caused by interactions of the effector molecule

Detailed analysis of heterotropic effects when two different substrate / effector molecules can bind at the same or partially overlapping sites on one CYP3A4 is complicated by the various possible scenarios depending on the affinities and concentrations of each substrate. If substrate A is present at higher concentration, it may be considered as a competitive inhibitor with respect to metabolism of another substrate B, while at the lower or intermediate concentration the same substrate A can appear as an allosteric effector activating metabolism of the substrate B via binding to the remote non-productive site and effectively increasing the total concentration of substrates for a given cytochrome P450. Clearly, activation or competitive inhibition in such system depends in general on the site-specific binding constants for both substrates (which are usually not known) and not only on their absolute concentrations, but on their stoichiometric ratio in solution. Deconvolution of the results of the standard experiments, when activity with respect to the substrate B is measured in the presence of some constant concentration of the substrate (effector) A is almost impossible to achieve, because the stoichiometric ratio [A]/[B] also changes in

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course of titration with one substrate while keeping constant concentration of another. Explicit two-dimensional data sets can be used for such exhaustive analysis based on corresponding 2D binding schemes [116-118].

We have recently described more simple and faster approach, based on the properties of the two-dimensional spin-shift titration surfaces [119] or analogous two-dimensional data sets obtained by measuring activity using the mixture of two substrates [57]. The presence of positive (activating) or negative (inhibiting) heterotropic interactions in the mixture of two substrates simultaneously interacting with CYP3A4 can be revealed by comparison of the results of one titration experiment (Type I spectral binding or product formation as the function of substrate concentration) using the mixture of two substrate at the constant stoichiometric ratio with the results of two experiments of the same type (spin shift or product formation) performed with each substrate separately. Results of these two homotropic titration experiments are fitted with the Hill equation to determine the mid-point of titration for each substrate. Stoichiometric ratio of concentrations of two substrates for the mixed titration experiment is then selected so that the ratio of concentrations of substrates A and B is the same as the ratio of corresponding midpoint concentrations, K_A and K_B. The midpoint of the mixed titration curve (heterotropic experiment) is then compared to the average of those obtained in two homotropic experiments [119]. As seen from Figure 6, deviation of the midpoint determined in the mixed titration experiment from the straight line indicates the presence and the sign of heterotropic cooperativity in the mixture of two substrates. In the absence of heterotropic cooperativity, the midpoint of the mixed titration experiment will be on the black straight line.

Analysis of the Type 1 mixed titration experiments of CYP3A4 with TST and ANF [119] revealed the absence of the heterotropic cooperativity in substrate binding and concomitant spin shift in this pair of substrates. This result was partly unexpected, due to the well established role of ANF as an effector in CYP3A4 metabolism of TST and to the earlier conclusion that ANF decreases cooperativity of the spin shift titration curves in CYP3A4 [120]. However, as we have directly shown in [119], this conclusion was based on the titration experiments with ANF concentration maintained at the constant level, so that stoichiometric ratio between two substrates was changing in the broad range along the titration curve, and the competitive binding between two substrates was gradually increasing. As the midpoints of the mixed titration experiments at the constant ANF / TST ratios all appear on the straight lines, the results of these experiments can be represented by linear combination of the homotropic titration curves with no additional effect caused by simultaneous binding of two different substrates.

Finally, we have applied the global analysis method to the question of heterotropic interactions between TST and ANF [57] using the mixed titration approach first developed in [119]. No direct heterotropic interactions between TST and ANF have been revealed, which means that functional cooperativity, at least for this pair of substrates, is also the result of spectrally and functionally silent binding of both substrates to the first site. This confirmed the suggested role of this site in the functional allosteric properties of cytochromes P450, which does not involve any cooperative effects in binding, but rather serves as a modulator of the large-scale dynamics of P450 molecule and affects the dissociation kinetics of the small ligands and possibly substrate and product from the catalytic site.

Allosteric regulation in P450: K^+ as an allosteric regulator in CYP101

An interesting example of true allosteric interactions in cytochromes P450 have been described more than 40 years ago by J.A. Peterson [52]. He systematically studied the

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pronounced stimulating effect of monovalent cations on the native substrate camphor binding to the cytochrome P450 CYP101 from *Pseudomonas putida*. Metal cations such as K^+, Na^+, Cs^+, Li^+ all promoted substrate binding and functionally important shift of the spin state of heme iron Fe^{3+} from low (S=1/2) spin to the high (S=5/2) spin.

Position of the K^+ binding site and the mode of allosteric effect of the monovalent cation binding have remained unresolved for many years. Appearance of the first high resolution crystal structures of CYP101 [121] did not provide an unambiguous answer, because the specifically bound K^+ ion have not been identified. Mutations of Tyr96 revealed an essential role played by this residue in the substrate binding specificity and also supported it's involvement into the allosteric regulation of the camphor hydroxylation by the metal cations [51]. Later, binding of five monovalent cations and coupling with the camphor binding to CYP101 have been systematically studied by Douzou and coworkers [122]. K^+ binds with highest affinity, K_d = 12 mM, whereas K_d for Na^+ is 24 mM, and for Li^+ 37 mM. Linear free energy relationship has been demonstrated between the free energies of camphor binding and metal cation binding, indicating the presence of allosteric interaction between two binding sites. Mutations at the Glu84 positions also affect both the spin state and substrate binding affinity [123].

Position of the potassium cation binding site has been determined based on the X-ray structures of the ferric and ferrous CYP101 with various substrates and ligands [124-129]. In agreement with earlier predictions [121], K^+ is coordinated at the end of B-B' loop with Glu94 and Tyr96 also involved. High resolution NMR study of the effect of K^+ on the conformation of CYP101 revealed the significant stabilization of the B-B' loop and B' helix in the presence of potassium or it's analog thallium [130]. In addition, NMR results indicate that in the presence of K^+ conformation of carbonmonoxy ferrous CYP101 is perturbed to some extent similar to the perturbation caused by Pdx binding. This fact suggests a new stimulating allosteric effect of K^+ binding on the second electron transfer and reduction of the oxy-complex in CYP101 [130]. The structural origin of potassium allosteric effect on the camphor binding can be visualized from the X-ray structures resolved by Lee and Goodin [53]. Comparison of the structures of CYP101 with and without substrate camphor and at high and low concentration of K^+ cofactor (Figure 7) reveals the structural features responsible for the allosteric coupling of the substrate binding and metal binding in this cytochrome P450. In the presence of K^+ the affinity of the enzyme CYP101 for the camphor increases by the factor of 10 [51]. Affinity of the CYP101 for the K^+ binding is low with K_d ~ 10 mM [53], suggesting that the allosteric metal binding site at the B' helix has low specificity, and K^+ can be replaced by Na^+ or other metal ion.

Other studies suggest that many P450 enzymes could have auxiliary metal ions analogous to that observed in the P450cam and analogous to that observed in the present case, these auxiliary metal ions may play important functional roles in activation and stabilization of these vital enzymes. Complex pattern of stabilization and destabilization effects of Ca^{2+} and Mg^{2+} on CYP101 [131] have been attributed to their competitive binding to the K^+ sit but much less favorable coupling to the camphor binding. On the other hand, Ca^{2+} and Mg^{2+} have been reported to enhance catalysis in the membrane bound eukaryotic cytochromes P450, those involved in steroid biosynthesis [132] as well as xenobiotic metabolizing enzymes [10, 133]. Also, Ca^{2+} was reported to stimulate NADPH supported reduction of cyt b5 by CPR in microsomal preparations [134].

Tentative allosteric site in CYP3A4

In the case of the human CYP3A4, our work demonstrated that the first binding site is spectrally silent, no product is formed [40], but it stabilizes oxy-complex by slowing the

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autoxidation, and CO-photolysis also indicates restriction of the diatomic ligand escape [41]. Similar results have been reported for CYP3A4 using Nile Red as the substrate analog [105]. Results of several studies by Davydov and Halpert suggest that the high-affinity sites in CYP107A1 and CYP3A4 between F' and G' helices may be responsible for these allosteric effects [42, 46, 101, 135]. Interestingly, this site is close to, although does not coincide with the progesterone binding site first identified in the X-ray structure of CYP3A4 [82]. Earlier studies also indicated that activity and cooperativity of CYP3A4 is significantly perturbed by mutations in this part of the protein [76, 120, 136]. In these works there have been collected multiple albeit indirect evidences in favor of at least three binding sites for the substrates of moderate size (200 – 300 Da, such as steroids or flavones) on one CYP3A4 monomer.

Position of the tentative allosteric site in CYP3A4 and possibly by analogy in other mammalian cytochromes P450 between F- and G- helices (or F' and G' helices) may explain their unpredictable variability in response to the various effectors and conditions. When cytochrome P450 is incorporated into the lipid bilayer, this site is likely to be just at the membrane surface and may interact with the lipid head groups or other components of the native membrane, such as steroids or fatty acids. Two latter cases are represented by the X-ray structures of CYP3A4 with progesterone [82] and CYP2C8 with palmytate [84] bound at this site. Such interactions can modulate functional properties of these and other cytochromes P450 and change their response to a different extent as compared to the various solubilization mixtures containing detergents and lipids. In particular, the binding of small molecule effectors at this site should be perturbed to a different extent by the membrane incorporation in one case, and by detergent solubilization in another. In addition, the same site of the P450 molecule was proposed to be a part of monomer-monomer interface in P450 homo- and heterodimers [61, 84]. Finally, binding of effector molecules to, or interactions with bilayer at this site is expected to perturb the large scale 'open – close' movement of P450 molecule, which is critically important for the substrate binding and product release [96, 137-138]. This perturbation can be due to the restricting of the sliding motion of F- and G- helices, as described in [138], or alternatively binding of the effector between F- and G-helices can directly block the tentative channel 4 [99] or shift the conformational equilibrium in favor of the closed state. An example of such modulation of the audoxidation kinetics and the CO geminate rebinding amplitude in CYP3A4 with one TST bound [41] may be explained by such perturbation of the open-close equilibrium.

Cooperativity in oligomers of cytochromes P450

Formation of oligomers and interactions between monomers of cytochromes P450 can also be a source of cooperativity, if it is coupled with the substrate binding. Such effects are known for various types of enzymes (see [23] for review) and have been suggested as the source of cooperative effects in several P450 systems [55-56, 139]. In the case of the soluble CYP130 [139], the isothermal titration experiments reveal significant enhancement of the second substrate binding, with 12-fold decrease of the dissociation constant from 36 uM for the first substrate binding to 3 uM for the second one. Crosslinking with glutaraldehyde confirmed dimerization of the enzyme at high substrate concentration, although dimers were not stable enough to show up in the size exclusion chromatography or native gel electrophoresis [139]. Thus, the low affinity binding of the substrate tothe monomers have been followed by dimerization with much higher affinity of the dimers to the second binding event. This coupling of the substrate binding and dimerization is attributed to the higher propensity to form a dimer of the 'closed' form of CYP130, which has higher affinity for the substrate, while the low affinity open form exists as a monomer in solution. In this case, dimerization appears as an alternative way to stabilize the high affinity 'closed' form of P450 molecule, which is performed by K^+ binding to the allosteric metal cation binding site

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in CYP101 [51, 53]. Importantly, the same B-C loop, which is stabilized by K^+ binding in CYP101, is involved in the monomer – monomer interface in the CYP130 dimer crystal structure, as well as in the other examples of cytochromes P450 crystallized as dimers, CYP154C1, and the one from *Thermus Thermophilus*, 1WIY.

A special case is the CYP102A1 from *B. Megaterium*, a fusion protein with the heme domain and flavoprotein domain appearing sequentially in a single polypeptide chain, which exists in solution as a functional dimer [140-142]. In this dimer electron transfer from FAD to FMN, and / or from FMN to the heme [143] are utilized across the monomer – monomer interface. Although multiple examples of cooperative ligand binding and turnover of some substrates have been described for the wild-type and mutant CYP102A1, as well as for similar CYP102A2 and CYP102A3 from *B. subtilis* [144-145], none of these studies suggested involvement of the dimer dissociation. Instead, in many cases cooperativity is due to the simultaneous binding of two substrate molecules to one or both heme domains in a dimer. This model is strongly supported by the decoy substrate mechanism recently reported for CYP102A1, in which the rate of propane hydroxylation increased greatly in the presence of perfluorooctanoate [106]. Binding of perfluorinated fatty acids which appear as non-metabolized analogs of CYP102 substrates, blocks the distal part of the substrate binding pocket, stabilizes binding and enhances hydroxylation of smaller hydrocarbons, such as propane, ethane, and even methane [106].

However, oligomerization of soluble bacterial cytochromes P450 remains an unusual feature of only several systems. Mostly formation of oligomers has been inferred as an important aspect of structural organization in the native membrane bound state of the eukaryotic P450 enzymes [55-56, 60]. Possible functional implications of cytochromes P450 oligomerization in the membrane have been described in experimental reconstituted liposomal bilayers [56, 60, 101, 146-148], in detergent solubilized systems [55, 146, 149] and in the native membranes [55-56, 61]. Formation of oligomers can be an important source of cooperative effects in P450 systems, because of possible limited access of substrates to some monomers, different modes of interactions with reductases, and potential redistribution of the high affinity binding sites among the catalytically active and inactive ones. However, the lack of information on the mutual affinity of different P450 to form oligomers, and on the effect of such interactions on their functional properties, makes it impossible to claim any general conclusion on the importance of the crowding and formation of homo – and hetero-oligomers of cytochromes P450 in the native ER membranes.

Conclusions

Cooperativity and allosteric properties in cytochromes P450 have been known since early 80s for hepatic xenobiotic metabolizing isozymes [19]. These enzymes, especially the most abundant and well studied human CYP3A4, CYP2C8 and CYP2C9, as well as CYP1A2 from rabbit liver, are capable of binding two or more substrates and often exhibit sigmoid kinetics. In addition, their functional response can be modulated by the presence of alternative substrates or effectors, which gives rise to heterotropic cooperativity and results in drug – drug interactions mediated by cytochromes P450 *in vivo*. Such effects have deep impact on the pharmaceutical chemistry, and Phase I clinical trials of new drugs always involve screening of large chemical libraries against the most important P450 enzymes *in vitro* with the goal to detect possible adverse effects as early as possible. In addition, computer modeling of binding and metabolism is routinely used in order to predict *in silico* the metabolic pathway of new candidate drugs. This broad field of medicinal and pharmaceutical chemistry of cytochromes P450 still is far from complete understanding of all possible outcomes of simultaneous presence and metabolism of more than one drug, and yet further away from predicting such results for the array of new compounds. Many recent

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results reviewed in this article suggest that the cooperative non-Michaelis response may be more common for all cytochromes P450 than it was recognized before. Perhaps, the essential features of P450 fold include remote binding sites which can be used for the allosteric regulation of equilibrium and / or kinetic functional properties, such as substrate binding and product dissociation, stability of oxy-complex and autoxidation, as well as fluxes along other uncoupling channels, interactions with redox partners and kinetics of electron and proton transfer steps towards formation of the catalytically active iron-oxygen species. Tentatively, these sites can be suggested to include the progesterone binding site between F' and G' helices in CYP3A4, the second camphor binding site proposed between F helix and E helix in CYP101A1 [80] and in CYP101D2 [81], the K^+ binding site stabilizing B' helix in CYP101, or residues potentially involved in oligomerization of p450 monomers. Importantly, because of the similar fold of all cytochromes P450, one can expect the appearance of the same homotropic and / or heterotropic interactions as described in well studied CYP3A4, CYP2C8, etc., also in other not as thoroughly known isozymes. In addition, several mutations can drastically change the functional response in some cases and create a non-native, and sometimes cooperative, activity with unexpected substrates, as it was observed for CYP102 mutants with steroids [150]. All these facts indicate that cooperativity and allosteric regulation may be the essential feature and potentially can or should be expected to occur while exploring new P450 systems from prokaryotes as well as from eukariotes.

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Highlights

- The mechanisms of cooperativity and allosteric properties of the cytochromes P450 are reviewed.
- Cooperative functional response is not based on binding cooperativity.
- Homotropic and heterotropic effects have a common origin
- Analysis of allosteric behavior is important for human drug-drug interactions

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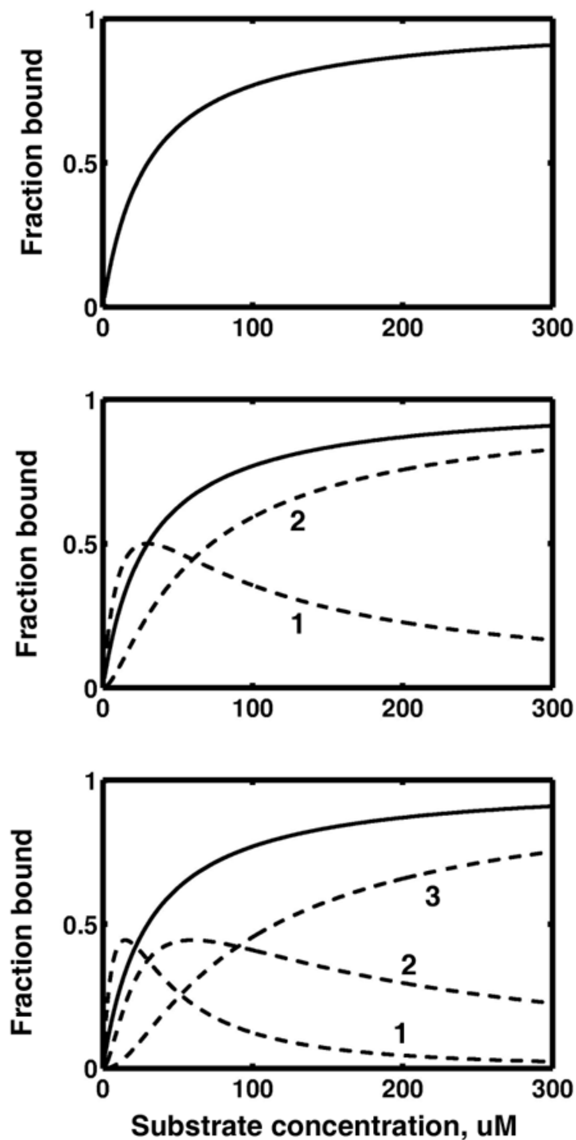

Figure 1.

Non-cooperative binding isotherms for one (top), two (middle), or three (bottom) sites without cooperativity are shown. The resulting isotherms are identical, however the difference in distributions of binding intermediates is shown as dashed curves.

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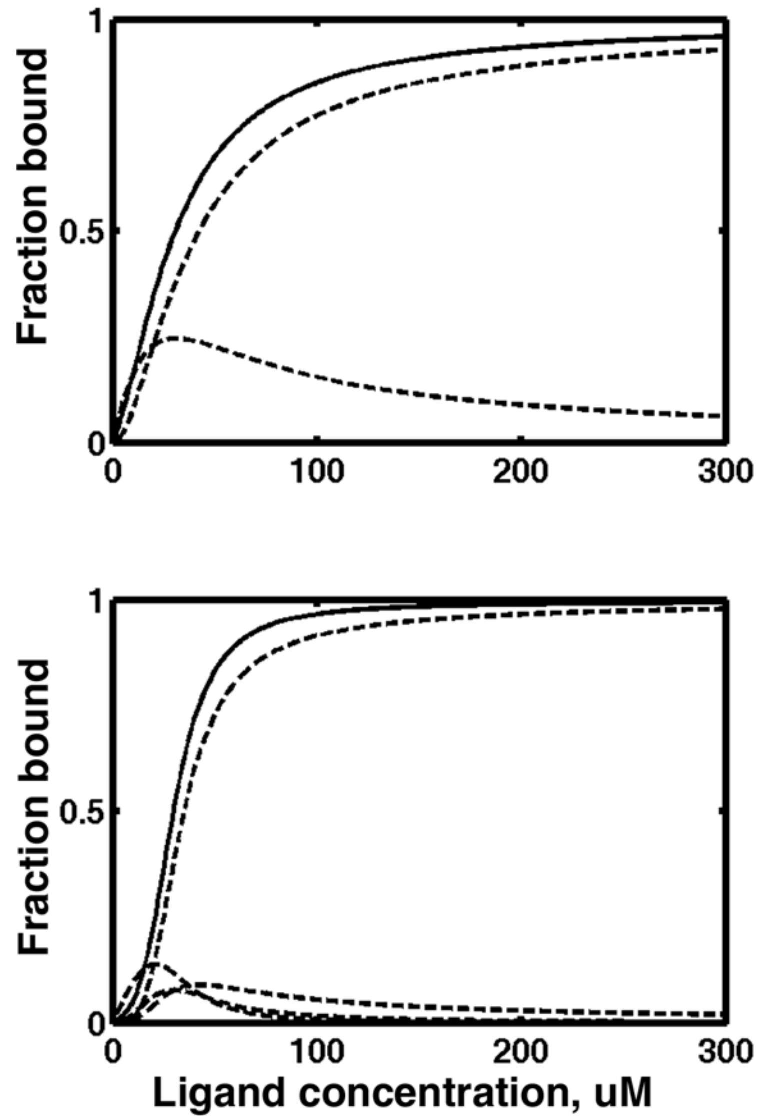

Figure 2.
Cooperative binding to a macromolecule with two (dimeric HbI from *Scapharca inaequivalvis*) [38], or four (human hemoglobin) binding sites

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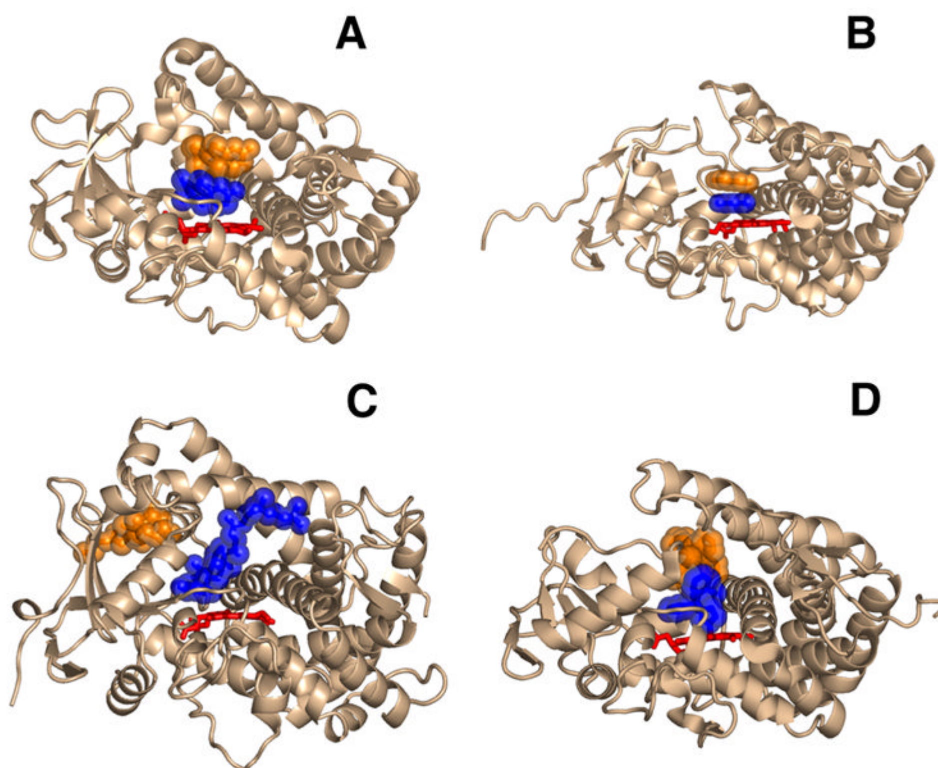

Figure 3.

Structures of cytochromes P450 with two bound substrates (A, B), two detergent molecules (C), and two inhibitors (D): (A) CYP107A1 with androstenedione; (B) CYP158A2 with flaviolin; (C) CYP24A1 with CHAPS; (D) CYP113 with clotrimazole.

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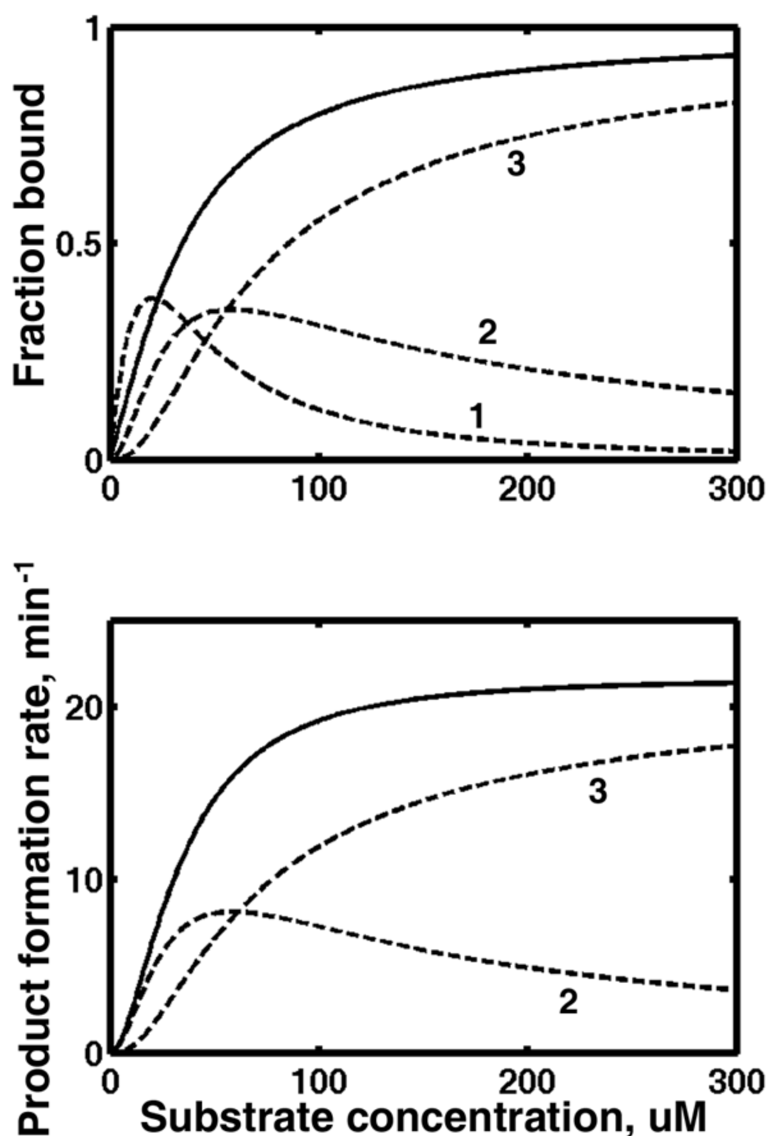

Figure 4.

Binding isotherm, i.e. fraction of occupied binding sites (full line), and populations of binding intermediates (dashed lines) calculated using stepwise binding constants for TST and CYP3A4 are shown on top figure, with corresponding overall rate of the product formation (full line) and fractional contributions from the binding intermediates with two and three TST molecules bound (dashed lines) shown at the bottom. Both are shown as the function of testosterone concentration and resolved using global analysis as described [40].

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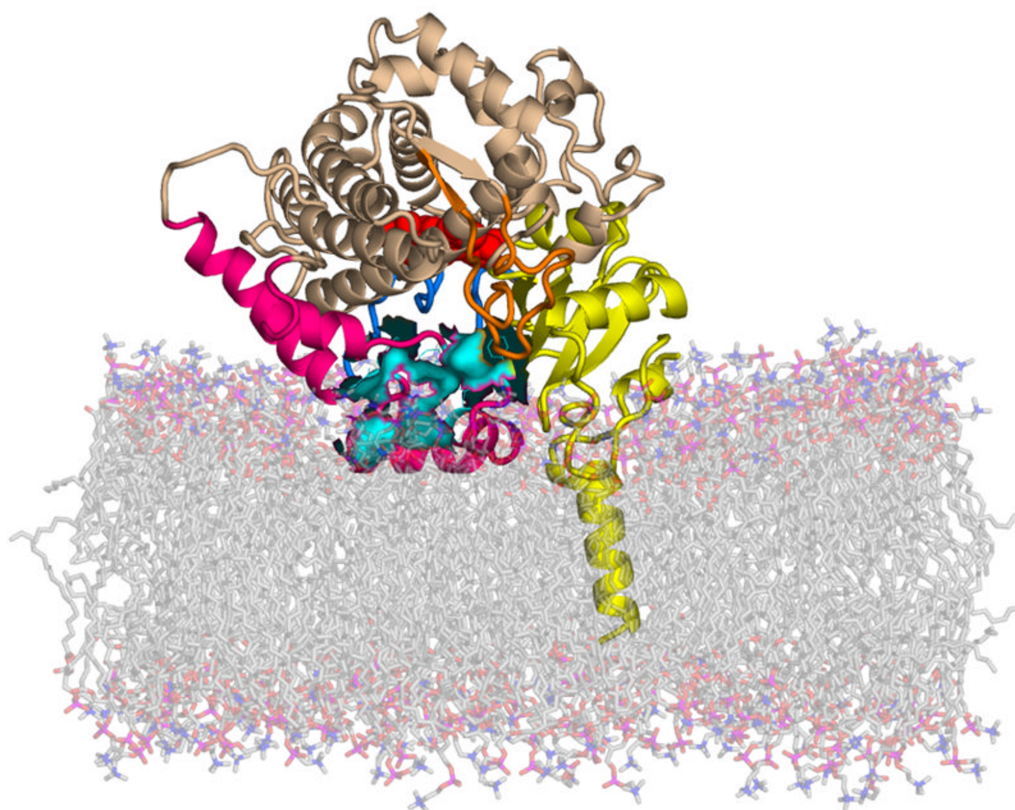

Figure 5.
Result of MD simulation of CYP3A4 equilibrated for 50 ns in the model POPC membrane bilayer [93]. Protein shown in the cartoon representation with the N-terminal beta-domain colored yellow and F-G helices in magenta. The heme shown in space filling colored red, and the progesterone binding site colored in cyan is shown at the membrane – water interface.

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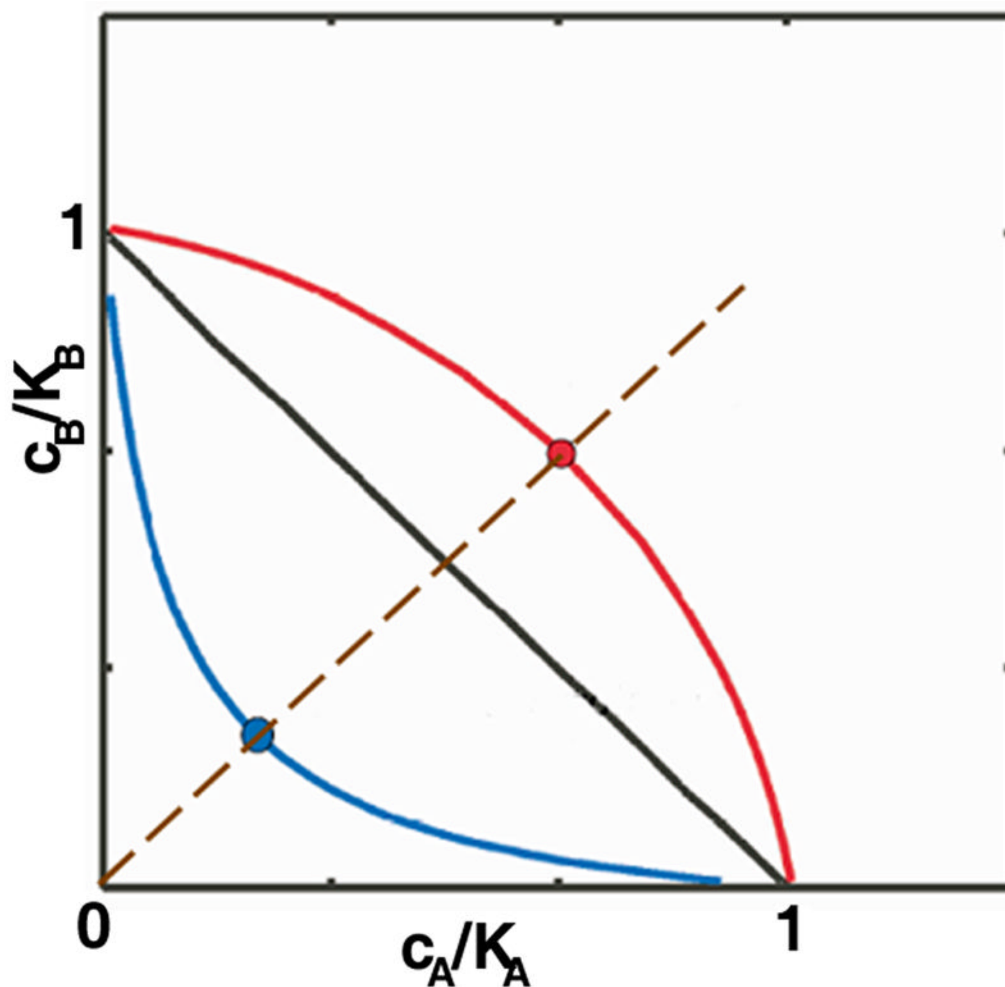

Figure 6.

Contour lines of the response surfaces are shown as the function of reduced concentrations of two substrates A and B, c_A and c_B. Mixed titration experiment at 1:1 stoichiometric ratio of reduced concentrations is shown as the dashed diagonal line. In the absence of heterotropic cooperativity, the response for any given substrate mixture can be represented as a linear combination of responses of two components, and the contour line of the midpoint (50% response) is the straight line shown in black. In case of positive heterotropic cooperativity, the mixed binding intermediates will be favorable, and the midpoint contour line (blue) is concave, while for the negative heterotropic cooperativity, the mixed substrate intermediates are unfavorable, and the midpoint 50% line is convex (red).

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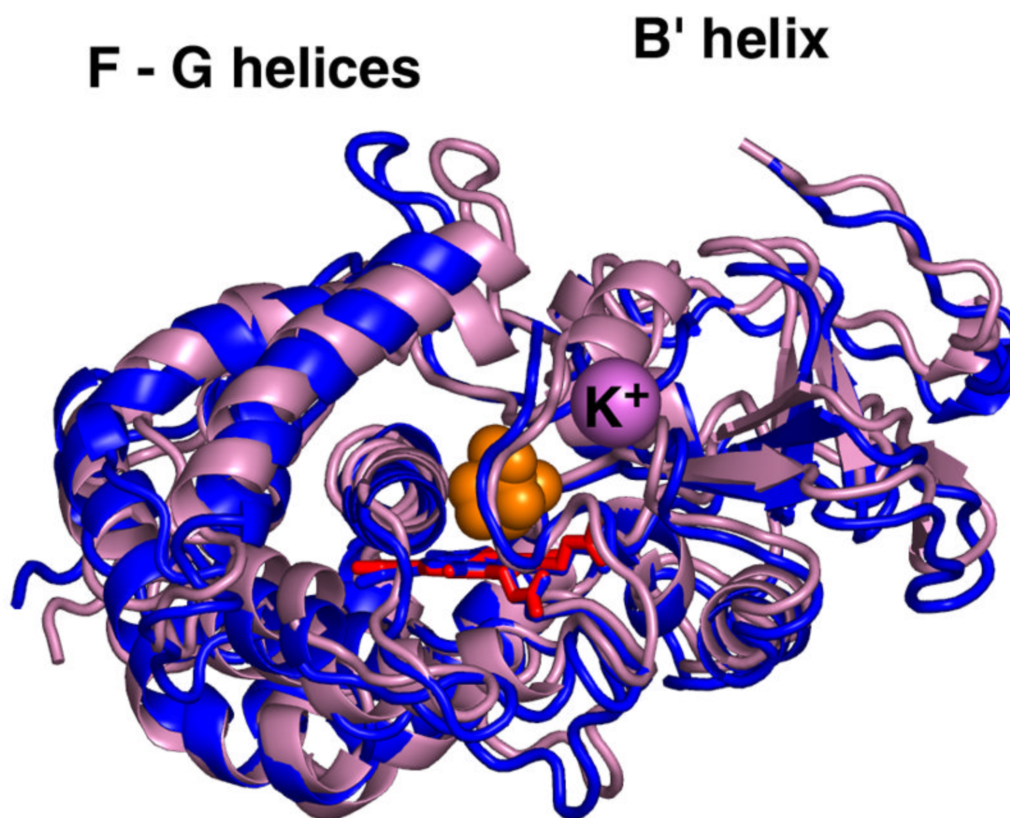

Figure 7.

Structures of the CYP101 (pink cartoon representation) saturated with camphor (orange spheres representation) and K^+ (magenta sphere) and CYP101 with no substrate and no K^+ (blue cartoons) [53] are shown superimposed in the heme frame to demonstrate the opening movement of the F-G helices and disordered B-C loop with unresolved B' helix in the absence of K^+ as discussed in [53].

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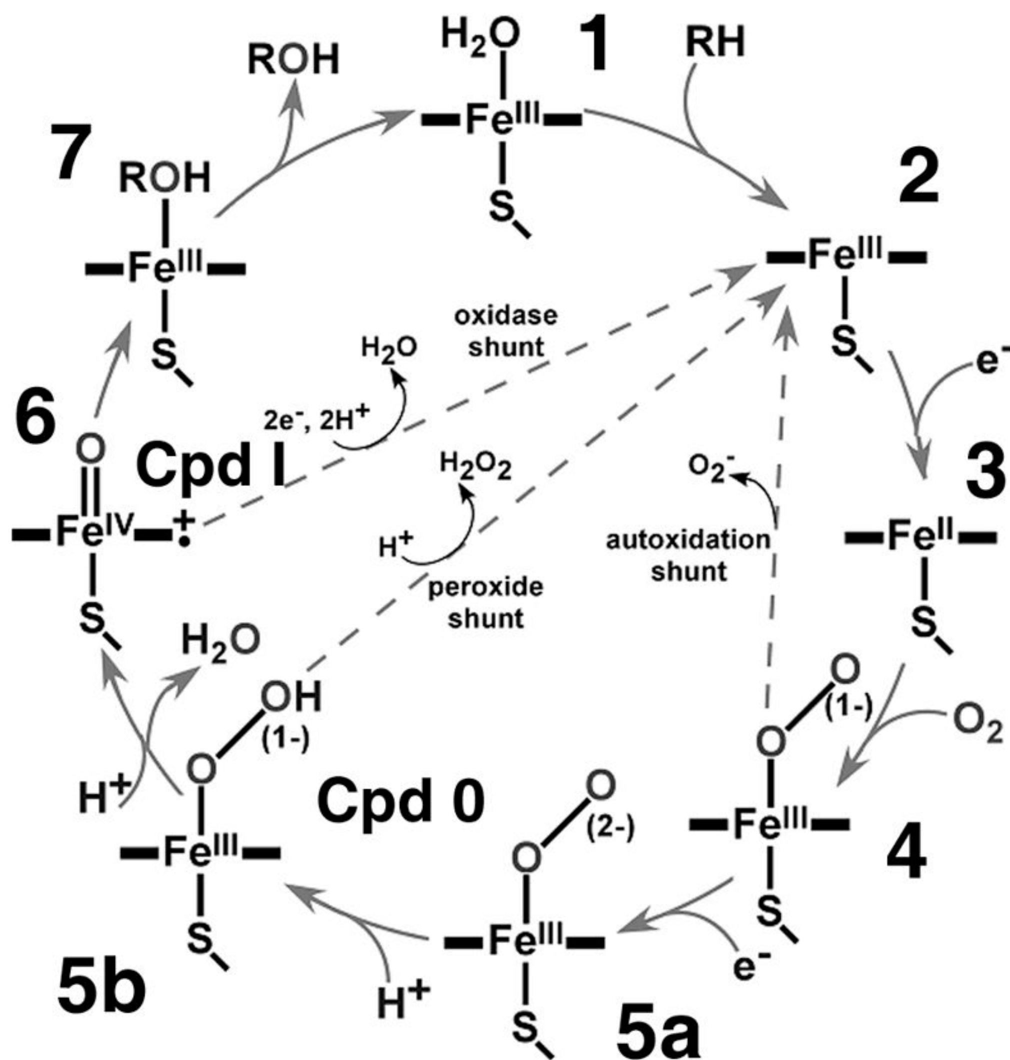

Scheme 1.
Catalytic cycle of cytochromes P450.