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Allosteric P450 mechanisms: multiple binding sites, multiple conformers, or both?

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Abstract

According to the initial hypothesis on the mechanisms of cooperativity in drug-metabolizing cytochromes P450, a loose fit of a single substrate molecule in the P450 active site results in a requirement for the binding of multiple ligand molecules for efficient catalysis. Although simultaneous occupancy of the active site by multiple ligands is now well established, there is increasing evidence that the mechanistic basis of cooperativity also involves an important ligand-induced conformational transition. Moreover, recent studies demonstrate that the conformational heterogeneity of the enzyme is stabilized by ligand-dependent interactions of several P450 molecules. Application of the concept of an oligomeric allosteric enzyme to microsomal cytochromes P450 in combination with a general paradigm of multiple ligand occupancy of the active site provides an excellent explanation for complex manifestations of the atypical kinetic behavior of the enzyme.

Keywords

allostery; conformational dynamics; cooperativity; cytochrome P450; drug-drug interactions; protein-protein interactions

1 Introduction

The first observations of cooperative (“non-Michaelis-Menten”) behavior in microsomal monooxygenation date back to the late 1970’s – early 1980’s [1,2]. However, close attention to the apparent allostery in cytochromes P450 was prompted by observations of the activating effect of α-naphthoflavone (ANF) and demonstration of prominent cooperative behavior of cytochrome P450 3A4 (CYP3A4), the principal P450 enzyme in humans [3–8]. The practical interest in cooperativity stems from the potential importance in *in vitro-in vivo* extrapolations, drug-drug interactions, and adverse drug effects [9–11]. Practical significance of the cooperative behavior of cytochromes P450 is supported by a number of observations obtained *in vivo* and in cell culture studies [12–19]. To date instances of cooperativity have been reported for various microsomal cytochromes P450, including CYP1A2, CYP2A6, CYP2B4, CYP2B6, CYP2C9, CYP2D6, and CYP3A4, [1,18,20–25]. The growing number of P450 enzymes known to possess cooperative behavior suggests that this feature it is not an oddity of CYP3A4 and a few other species, but rather represents a common characteristics of a wide variety of microsomal drug-metabolizing cytochromes P450.

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2. “Allosterism” or “atypical kinetics”? Basic mechanistic concepts

2.1. Multiple binding sites within one pocket. A static “space-filling” model

Initial hypotheses to explain the origin of cooperativity were based on the presumption that due to their broad substrate specificity, some microsomal drug metabolizing cytochromes P450 possess a large substrate-binding pocket that can accommodate more than one ligand simultaneously [21,26–33]. Therefore, a loose fit of a single substrate molecule was hypothesized to require the binding of a second ligand for efficient catalysis [27,34]. Soon after these initial publications set the stage for rigorous studies of P450 cooperativity, the first X-ray structures showing two substrate molecules in a P450 binding pocket were reported by Cupp-Vickery and co-authors [35]. This work was performed on cytochrome P450eryF, an enzyme from the actinobacterium *Saccaropolyspora erthrea* that is involved in biosynthesis of the macrolide antibiotic erythromycin. The fact that two molecules of androstenedione or 9-aminophenanthrene bind in the cavity normally occupied by the natural substrate 6-deoxyerythronolide B prompted the authors to postulate that cooperativity in P450 involves no “major conformational changes” [35]. According to this concept the homo- and heterotropic cooperativity in cytochromes P450 does not represent true allosteric behavior, i.e. an effector-induced conformational transition in the enzyme and/or alteration of its oligomeric structure.

The current literature provides strong lines of evidence indicating the presence of two substrate-binding loci in the active site of CYP3A4 [31,32,36–41], including an X-ray structure of the complex of the enzyme with two molecules of ketokonazole [42]. However, attempts to apply a static model with multiple binding sites to interpret additive activating effects of different effectors or lack of competition between substrates possessing cooperativity [9,29,31–33,43–47] gave rise to a set of complex and mutually incompatible models suggesting the presence of three or even more substrate-selective binding sites in one molecule of the enzyme [43,45, 48–53].

There is evidence that the homotropic cooperativity in CYP3A4 involves more than two substrate binding events in some instances [54–56]. In particular, results obtained in the laboratory of S.G. Sligar, who used CYP3A4-containing Nanodiscs to mimic the membrane environment of the enzyme and prevent its aggregation, suggest that the formation of the high-spin complex of CYP3A4 with testosterone requires the binding of three molecules of this substrate to the enzyme [40,41]. However, there is important evidence that this additional substrate-binding site may be located distant from the CYP3A4 heme and outside of the main substrate-binding pocket [55–57].

The presumption that the atypical kinetic behavior of cytochromes P450 does not involve any alteration of enzyme structure impeded initially the use of the term “allostery”. However, a turning point occurred with the publication of Atkins and co-authors on the hypothesis of “nested allosterism” [58], where a marriage of the static “space filling” model with a classical allosteric concept was proposed as the most plausible scenario explaining the complex cases of P450 cooperativity. The increasing appearance of the term “allostery” in subsequent publications [59–61] reflects a change in our understanding of the problem. Thus, the major trend in the current research on cooperativity in CYP3A4 is to consider the involvement of conformational flexibility of the enzyme in its mechanism [55,58,61–65]. This trend is consistent with observations of conformational flexibility of several other P450 enzymes, notably CYP2B4 [66] and CYP2C9 [67,68].

2.2 Cytochrome P450 as an allosteric enzyme: the role of effector-induced conformational transitions

Among the first evidence of the involvement of effector-induced conformational changes in P450 cooperativity was the finding that the addition of Mg^{2+} abolishes the cooperativity of pyrene oxidation by CYP3A4 and also alters the regioselectivity of modification of the porphyrin ring by phenyldiazene [69]. Other early evidence was the profound effect of ionic strength on the cooperativity and the amplitude of the substrate-induced spin shift in the interactions of P450eryF with 1-pyrenebutanol (1-PB). Since the effect of ionic strength was greatly diminished by chemical modification of Cys-154 or its substitution with isoleucine, we hypothesized that the cooperativity mechanism may involve an effector-induced reorganization of the structure of a charge-pairing bundle among helices C, D, E, and G, which surround Cys-154 [60,63].

Support for the involvement of an effector-induced conformational transition in CYP3A4 in the mechanism of action of ANF was obtained in our recent study employing site-directed incorporation of a fluorescent probe into α-helix A (at Cys-64) of a cysteine-depleted mutant of CYP3A4. This research revealed an ANF binding site distinct from those involved in the ANF-induced spin shift and showed that ANF binding to this newly discovered site results in a transition that affects the conformation of helix A [55]. Additional support for conformational transitions in CYP3A4 caused by allosteric ligands has been provided by a recently solved crystal structure of ketoconazole-bound CYP3A4, which revealed two ligand molecules in the active site and demonstrated significant differences in conformation compared with the ligand-free structure [42]. Although the indications of ligand-induced transitions in cytochromes P450 do not contradict the basic premise of the “space-filling” model, they also suggest that P450 cooperativity is considerably more complex and more relevant to the classical concept of enzyme allostery than was initially realized.

Important information on the conformational dynamics of CYP3A4 and P450eryF in substrate binding was derived from pressure-perturbation studies of the substrate-induced spin shift [70,71]. Our studies with CYP3A4 showed that, in contrast to a non-allosteric substrate bromocriptine (BCT), binding of 1-PB or testosterone to the enzyme results in unusual stabilization of the substrate-bound high-spin state of the heme protein at high hydrostatic pressures. This finding reveals an apparent decrease in the accessibility and hydration of the active site. Similar behavior was recently observed in P450eryF interactions with Fluorol-7GA (F7GA), a novel fluorescent allosteric ligand [71]. Decreased accessibility of CYP3A4 heme to small ligands caused by the enzyme interactions with testosterone was also demonstrated in the studies of the kinetics of autooxidation of CYP3A4 incorporated into Nanodiscs and its interactions with cyanide and carbon monoxide [72].

Our latest studies on the effect of hydrostatic pressure on the interactions of P450eryF with F7GA monitored with time-resolved fluorescence spectroscopy showed that the increase in hydrostatic pressure results in increased affinity of the enzyme for F7GA. This behavior is evidence of a considerable decrease in the molar volume of the system upon the binding of the effector molecule(s) of F7GA to the enzyme. When considered together with a prominent increase in the cooperativity of CYP3A4 [70] and P450eryF [71] at elevated pressures, this observation suggests that the allosteric mechanisms involve a substrate-induced conformational transition that is accompanied by a decrease in protein hydration.

Additional evidence of a substrate-induced conformational transition in CYP3A4 was presented by Isin and Guengerich [56], who compared the kinetics of CYP3A4 interactions with BCT monitored by quenching of substrate fluorescence with the kinetics registered by the spin shift. Based on these and other findings the authors proposed a three-step binding model, where rapid formation of an initial ("encounter") complex is followed by its

reorganization with apparent relocation of the substrate molecule closer to the heme. This reorganization induces a subsequent slow conformational transition, which is necessary for a full-amplitude substrate- induced spin shift to occur [56]. Therefore, a multi-step mechanism involving a substrate-induced conformational transition is hypothesized even for the binding of BCT, which does not show any allosteric behavior. A similar mechanism was also suggested for the interactions of. CYP3A4 with the allosteric substrates testosterone and ANF.

The transient character of the initially formed enzyme-substrate complex is also revealed in our studies of the interactions of bacterial P450eryF with F7GA using time-resolved fluorescence spectroscopy, pressure perturbation and Job's titration [71,73]. Our results also suggest that the formation of a 1:1 enzyme-substrate complex, where the substrate molecule is bound close to the heme, involves two consecutive substrate binding events. To explain this observation we proposed a model shown in Fig. 1. Here the binding of a second substrate molecule to an initially formed 1:1 enzyme-substrate complex induces a conformational transition, which causes a release of the first bound substrate molecule from the apparent peripheral site [71]. The states similar to the complexes a - d (Fig. 1) may be also involved in the enzyme interactions with its natural substrate 7-DEB. The instances of homotropic cooperativity observed in the interactions of P450eryF with F7GA, 1-PB or testosterone may result from a juxtaposition of this mechanism with the ability of the enzyme to bind two molecules of these small substrates in one binding pocket (complex d, Fig. 1). Our latest results suggest that the above model is also applicable to the interactions of F7GA with CYP3A4[1].

3. Conformational heterogeneity of cytochromes P450: relevance of “Non-equilibrating” conformers to P450 cooperativity

3.1. Hypothesis on the involvement of functional heterogeneity of CYP3A4 in the cooperativity mechanisms

In parallel with the early studies elaborating the concept of “multiple binding sites in the same pocket”, a series of publications from the laboratory of F. K. Friedman suggested an alternative hypothesis on the mechanisms of heterotropic cooperativity in CYP3A4. These studies, which examined the effect of substrates on the kinetics of CO rebinding after flash photolysis [74–76], prompted the authors to propose that CYP3A4 both in solution and in the membrane is distributed between two pools with different substrate specificity and functional properties. The ability of allosteric effectors, such as ANF, to modulate the partitioning of CYP3A4 between the conformers was hypothesized to be a keystone of heterotropic activation. One main reason why this model did not gain widespread acceptance was the lack of physicochemical basis for the divergence of the CYP3A4 pool into two “non-equilibrating” sub-populations. As we will demonstrate below, the combination of this model with that of multiple binding sites may provide the most viable explanation for complex allosteric behavior of CYP3A4. Furthermore, the distribution of the P450 pool between two “non-equilibrating” conformers can be readily explained by taking into account protein-protein interactions of the enzyme and its possible oligomerization.

3.2 Evidence of persistent conformational heterogeneity in microsomal cytochromes P450

The question of persistent conformational heterogeneity of microsomal cytochromes P450 has a long history, with the first reports dating from the mid-1980’s. The early evidence of heterogeneity was obtained in the studies of the kinetics of P450 reduction by artificial donors, such as dithionite [77] or eosin radical [78] and the kinetics of C0-recombination with P450 (Fe^{2+}) [79,80]. An important indication of divergence of the pool of microsomal cytochromes

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P450 into two non-interconverting sub-populations is the differences in the kinetics of NADPH- and dithionite-dependent reduction between the high- and low-spin states of the heme protein, which are otherwise expected to exist in rapid equilibrium [81–83]. This striking inconsistency demonstrated for CYP2C11 [84], CYP2B4 [77], and most recently for CYP3A4 [64,65], suggests that the term "spin equilibrium" is inapplicable to the pool of CYP3A4 taken as a whole, but that there are several stable sub-populations of the enzyme with different position of spin equilibrium. Further evidence includes the heterogeneity in the resonance Raman spectral properties [85], sensitivity to high hydrostatic pressures [86–90], and the effect of cytochrome b_5 [91]. Most of these experiments were done with CYP2B4 and CYP3A4, although some of the studies involve cytochrome P450scc [85,89], and CYP2E1 [90]. Although most of the indications of P450 heterogeneity were obtained with the purified heme protein in solution, there are also a number of observations of functional heterogeneity of cytochromes P450 incorporated into liposomal and recombinant microsomal membranes [64,74,77,80,88, 92].

3.3. Conformational heterogeneity and P450-P450 interactions

Formation of homo- and hetero-oligomers of P450 in microsomal and model membranes is well documented. Starting with the early work of Cherry and co-authors [93,94], measurements of rotational diffusion have been widely used to determine the effect of various factors on the degree of aggregation of P450 in the membranes [93,95–100]. In most cases, 30 to 70% of membranous P450s forms oligomers with decreased rotational mobility [93–95,99,101]. Formation of CYP2C2 oligomers in the membranes of living cells has also been demonstrated by fluorescence resonance energy transfer microscopy [102]. Besides homo-oligomerization of individual P450 species, formation of hetero-oligomers was shown for CYP1A1 and CYP3A4 [103], CYP2B4 and CYP1A2 [104], as well as for mitochondrial CYP11A1 and CYP11B [105]. P450 oligomerization in membranes was shown to decrease upon its interactions with CPR [95,106,107] or cytochrome b_5 [107], as well as by reduction of the P450 heme [101]. In contrast, interaction of P450 with substrates [20,108] as well as peroxidation of the membrane lipids [96,109] results in increased aggregation of the enzyme. These observations show that the degree of P450 oligomerization is a critical determinant of the properties of the microsomal monooxygenase and may play an important role in regulatory mechanisms of physiological relevance.

It is important to note that the manifestations of functional heterogeneity discussed above may be abolished by monomerization of cytochromes P450 or solubilization of the membranes by addition of detergent [77,79,86–88,110] or incorporation of the heme-protein into Nanodiscs [64]. Therefore, we hypothesize that the functional heterogeneity of microsomal cytochromes P450, such as CYP2B4 and CYP3A4 may be caused by oligomerization of the enzyme, which gives rise to important differences between subunits in their ability to interact with substrates, the accessibility of the heme pocket, and sensitivity to pressure-induced inactivation.

3.4. "Non-equilibrating conformers" and CYP3A4 cooperativity

Despite the abundance of data documenting conformational heterogeneity of CYP3A4, the direct indications of its involvement in allosteric mechanisms remain sparse. The initial evidence presented by Koley and co-authors concerns the correlation between the activating effect of ANF on the metabolism of benzo[α]pyrene and the distribution of CYP3A4 between two fractions with different rate constants of CO-complex recombination [75]. A similar correlation has been demonstrated for allosteric inhibition of nifidipine metabolism by quinidine [76].

Further studies in our laboratory provided several pieces of evidence that ANF causes redistribution of the CYP3A4 pool. Thus, kinetics of CYP3A4 reduction in solution and in

proteoliposomes by dithionite was found to obey a three-exponential equation with the amplitudes of the fastest and the slowest phases being modulated by substrates. While the fastest phase reflects the reduction of the substrate-free low-spin heme protein, the slowest phase represents the reduction of the high-spin enzyme. The non-allosteric substrate BCT was shown to increase the amplitude of the slow phase at the expense of the fast one, while the middle phase remains unaffected [64]. In contrast, pre-incubation of the enzyme with ANF renders the dithionite-reduction kinetics monophasic [111]. Importantly, the middle phase, the amplitude of which is unaffected by BCT, also disappears upon monomerization of the heme protein by incorporation into Nanodiscs or into liposomes with a high lipid-to-protein ratio [64].

Another recent observation concerns the kinetics of NADPH-dependent reduction of CYP3A4 by BMR, which was used as a soluble substitute for the membrane-bound mammalian cytochrome P450 reductase (CPR). In the absence of substrates only about 50% of CYP3A4 was able to accept electrons from BMR. Whereas the high-spin fraction was completely reducible, the reducibility of the low-spin fraction did not exceed 42%. Among four substrates tested (testosterone, 1-PB, BCT, or ANF) only ANF is capable of increasing the reducibility of the low-spin fraction to 75%. Therefore, an increase in the reducibile pool of the enzyme in the presence of ANF may represent an important element of the mechanism of action of this heterotropic activator [65].

Another clue to the role of subunit interactions in CYP3A4 cooperativity is given by our recent studies with the CYP3A4 mutant L211F/D214E/F304W. In contrast to the wild type CYP3A4, the mutant exhibits no cooperativity in the interactions with 1-PB or in progesterone or testosterone 6β-hydroxylation. The most remarkable and unexpected finding with this mutant was that the loss of cooperativity is observed only in preparations containing substantial apoprotein (i.e., heme-depleted enzyme). Removal of this contaminating apoprotein restored the cooperativity and resulted in an important increase in the content of the high spin state in the substrate-free enzyme [112]. Therefore, the loss of cooperativity in this mutant may be attributed to an alteration of subunit interactions in the oligomer by L211F/D214E/F304W substitutions, so that co-oligomeriztion of apo-protein with the mutant holo-enzyme resulted in a conformational change that displaces the spin equilibrium and eliminates the cooperativity.

The above observations unequivocally demonstrate effector-modulated functional heterogeneity of CYP3A4 in solution and in the membrane; they also provide important support for a critical role of P450 oligomerization in CYP3A4 allosteric mechanisms. Based on these findings it seems doubtful that one can understand P450 allostery by considering just a single molecule of P450, as the mechanisms appear to involve several interacting molecules of the enzyme. However, further development of this concept requires more extensive studies on the mechanisms of P450 oligomerization in the membrane and its effect on P450 cooperativity.

4. Merging the concepts: An approach to a generalized view on P450 cooperativity

Recent advances demonstrate that CYP3A4 cooperativity involves binding of multiple (at least 2) substrate molecules in the binding pocket and represents a case of true allostery characterized by effector-induced conformational transitions. At the same time, a large body of experimental data shows the presence of functionally distinct subpopulations of CYP3A4 both in solution and in the membrane. The modulation of the distribution of the enzyme between these conformers by allosteric effectors such as ANF suggests that the CYP3A4 heterogeneity caused by the association of the enzyme into oligomers is tightly related to the cooperativity mechanisms. If we allow that substrate-induced conformational transitions may affect protein-

protein interactions of the enzyme and, in particular, P450-P450 interactions, a simple and logically relevant picture of modulatory events begins to emerge.

Modulation of P450 conformation by substrate binding, which is now well recognized [42, 55,63,64], may be necessary to synchronize the electron flow to the enzyme with substrate binding. There is substantial evidence of substrate-dependent modulation of the affinity of microsomal cytochromes P450 for CPR and cytochrome b_5 [113–117]. In addition, substrate-induced changes in water accessibility and hydration of the active site [41,64,70,71], may be important in the control of P450 uncoupling [118,119]. Synchronization of water access to the active site with substrate binding and the redox state of the enzyme appears to play an important role in preventing futile cycling of the enzyme and production of reactive oxygen species, such as hydrogen peroxide and superoxide anion-radical. A mechanism of this kind is operative for bacterial P450cam [120,121].

As we discussed above, promiscuous microsomal cytochromes P450 with large substrate binding sites, such as CYP3A4, appear to be capable of binding several molecules of some substrates in the active site. In the case of large substrate molecules, such as BCT or erythromycin, binding of one molecule is sufficient to induce a substrate-dependent conformational change and to form a high-spin-enriched and catalytically competent substrate complex. However, in the case of smaller substrate molecules, such as 1-PB, F7GA or testosterone, the substrate-induced change in the enzyme conformation and substrate-induced displacement of spin equilibrium (signifying the formation of a catalytically competent substrate complex) are caused by separate substrate-binding events [36,39,40,63,122]. Modulation of water accessibility of the heme pocket by multiple substrate binding events in CYP3A4 is supported by recent results of Denisov and co-authors. Studying the rates of testosterone hydroxylation and NADPH oxidation in Nanodisc-incorporated complexes of CYP3A4 with CPR these authors demonstrated an important difference in the degree of coupling among enzyme-substrate complexes of different stoichiometry [41]. Such separation of the substrate-induced conformational transition and substrate-induced spin shift between (at least) two separate binding events results in homotropic cooperativity. It is also reflected in unusual stabilization of substrate-bound high spin state at high hydrostatic pressures, which has been recently demonstrated for both CYP3A4 [70] and P450eryF [71]. Cooperativity of this kind is observed not only in membranous eukaryotic enzymes, such as CYP3A4, but also in soluble P450eryF, which has a large substrate binding pocket.

However, in contrast to P450eryF, allostery of membranous cytochromes P450 seems to be complicated by modulation of protein-protein interactions by ligand binding. Formation of the oligomers may result in unequal conformation and/or orientation of the subunits in the oligomer and limit conformational mobility of some of them. This inequality of the subunits is thought to be the most plausible reason for functional heterogeneity of the enzyme. At the same time, conformational changes induced by some substrates, such as ANF, are shown to modulate the partitioning of the enzyme conformers, apparently due to their effect on subunit interactions. A close interrelationship between the mechanisms of cooperativity and P450-P450 interactions is revealed in our recent experiments with L211F/D214E/F304W mutant of CYP3A4, where the loss of cooperativity was shown to take place only in mixed oligomers of holoenzyme with the heme-depleted protein [112].

In our recent study on the kinetics of CYP3A4 reduction by BMR [65] we proposed a model of homo- and hetrotropic cooperativity in CYP3A4, which combines all three basic premises, namely multiple substrate binding, ligand-induced conformational transitions, and ligand-modulated persistent heterogeneity (Fig 2.). In this model the substrate binding events observed in the absence of a heterotropic activator (Fig. 2, top row) are similar to those discussed above for the mechanism of homotropic cooperativity in P450eryF and CYP3A4 (Fig 1), with the

exception that some subunits in the CYP3A4 oligomer are represented by a non-functional conformer shown in blue. Interaction of the enzyme with a heterotropic activator, such as ANF, is likely to involve an additional, remote effector-binding site [54–57,123,124], which may be located at the interface of two interacting P450 molecules [124]. Binding of an effector to this site results in a rearrangement of the oligomeric structure, which eliminates the above non-functional conformer (Fig 2, bottom row). This hypothetical scheme is consistent with the observations of CYP3A4 heterogeneity and its modulation by ANF [64,65,75,88,111]. According to the this hypothesis, the complex pattern of homo- and heterotropic cooperativity observed in CYP3A4 is caused by an overlap of multiple substrate binding with ligand induced conformational changes affecting protein-protein interactions and oligomerization.

5. What may constitute a physiological function of P450 allostery (if any)?

Although the current interest in CYP3A4 cooperativity is primarily due to the practical aspects, such as its importance for drug-drug interactions, interest in the physiological significance has propelled several investigations in recent years. One attractive possibility is that the heterotropic cooperativity with such effectors as ANF may simply represent "incidental manifestations" of an allosteric regulatory mechanism that is triggered by some unknown physiological effector(s). This allosteric regulation of monooxygenase may serve to minimize its uncoupling and to coordinate P450 turnover with the function of metabolically related enzymes, such as glutathione transferases, glutathione reductase, and the enzymes of the pentose phosphate pathway. One possible candidate for this role is reduced glutathione (GSH), which is tightly related to the oxidative status of the cell and involved in the pathways of drug disposition. Our recent results on the effect of physiological concentrations of GSH on CYP3A4 cooperativity and the evidence of GSH-induced conformational changes in the enzyme [125] provide a solid support for the role of GSH as an effector of CYP3A4.

Another possible physiological role of allosteric regulation of microsomal P450 may be suggested based on our hypothesis of the involvement of P450-P450 interactions in the cooperativity mechanisms. According to our recent results, conformational heterogeneity of cytochrome P450 results in incomplete reducibility of the enzyme [65]. An important fraction of the enzyme appears to be unable to accept electrons from the electron donor (BMR in our studies) without dissociation of the enzyme oligomers or their reorganization caused by allosteric effectors, such as ANF. Physiological relevance of this mechanism becomes understandable when one considers that the endoplasmic reticulum contains multiple species of cytochrome P450 with different substrate specificity. There are several reports showing clear reciprocal influence of different isoforms of P450 in reconstituted systems and microsomes on activity and other properties (see [126] for review), which prompted some authors to consider direct association between different P450 species as an important determinant of the properties of microsomal monooxygenases [103–105,126–132]. We suggest that the substrate-modulated formation of the hetero-oligomers of different P450 isozymes hinders the species lacking substrate from productive interactions with CPR, and hence diminishes unproductive electron flow and improves the coupling of the monooxygenase as a whole.

6. Expert opinion

Cytochrome P450 cooperativity, which was initially thought to be a peculiar case of pseudo-allosteric behavior caused by multiple substrate binding into a large and static binding pocket, is now understood to represent a very complex allosteric mechanism. The instances of cooperativity in microsomal cytochromes P450 reveal a complex overlay of a multiple substrate binding “space-filling” mechanism, ligand-induced changes in the enzyme conformation, and modulation of protein-protein interactions in the enzyme oligomers. The apparent physiological significance of this mechanism stretches far beyond the problem of

drug-drug interactions, which was the initial concern. Homo- and heterotropic cooperativity in microsomal P450 enzymes are likely to reveal a novel mechanism of allosteric regulation, which may be triggered by endogenous effecrtors, such as gluthatione. This regulatory mechanism may play an important role in coordinating the function of the monooxygenase with the antioxidative status of the cell, functioning of metabolically related enzymes, and cellular exposure to xenobiotics. Further studies of this mechanism require close attention to the nature of ligand-induced conformational transitions and their effect on protein-protein interactions, and P450-P450 interactions in the membrane in particular. We believe that further understanding of this mechanism will require extensive biophysical studies on membranous systems, including those with different concentration of the enzyme (different protein-to-lipid ratio) and those containing several interacting species of P450.

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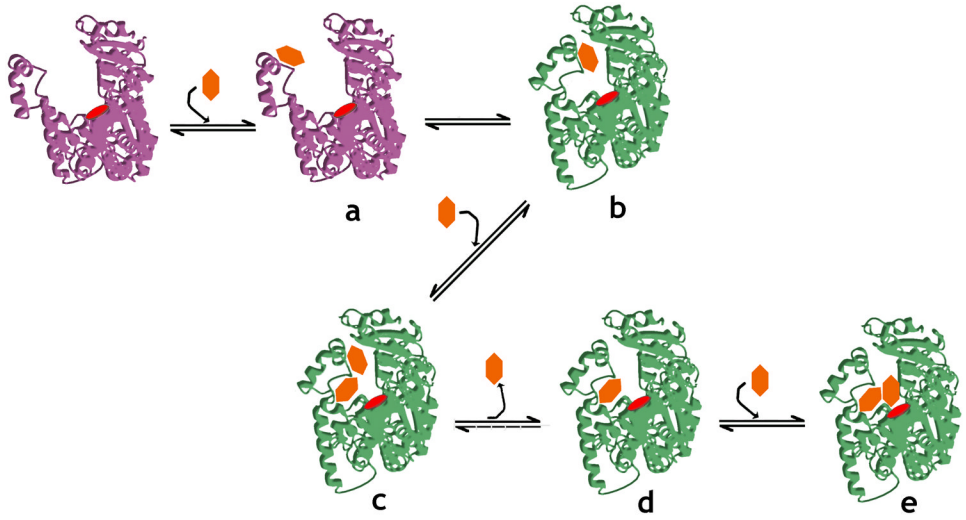

Fig. 1.

A hypothetic "three-site sequential ping-pong" mechanism explaining the homotropic cooperativity in P450eryF and CYP3A4 based on the studies of interactions of P450eryF with F7GA [71,73]. According to this mechanism the initial binding of substrate (orange hexagon) occurs to a peripheral binding site of a resting, predominantly low-spin conformation of the enzyme (magenta) with the formation of the complex (**a**). This induces a transition to the conformation with decreased water accessibility of the binding pocket (green, **b**). The binding of another substrate molecule yields the ternary complex (**c**). This complex is unstable and releases the peripherally-bound substrate molecule with the formation of the complex (**d**), where some substrate-induced spin shift is observed. Interaction of another substrate molecule with this complex results in the final ternary complex (**e**), which exhibits a full-amplitude substrate-induced spin shift. The representation of the "green" conformation of the enzyme is based on the X-ray structure of CYP3A4 [123], which was altered slightly for compactness of the image. Although the difference between this image and the image of the resting (magenta) conformation is arbitrary, it is consistent with our understanding of the nature of the substrate-induced changes in the enzyme.

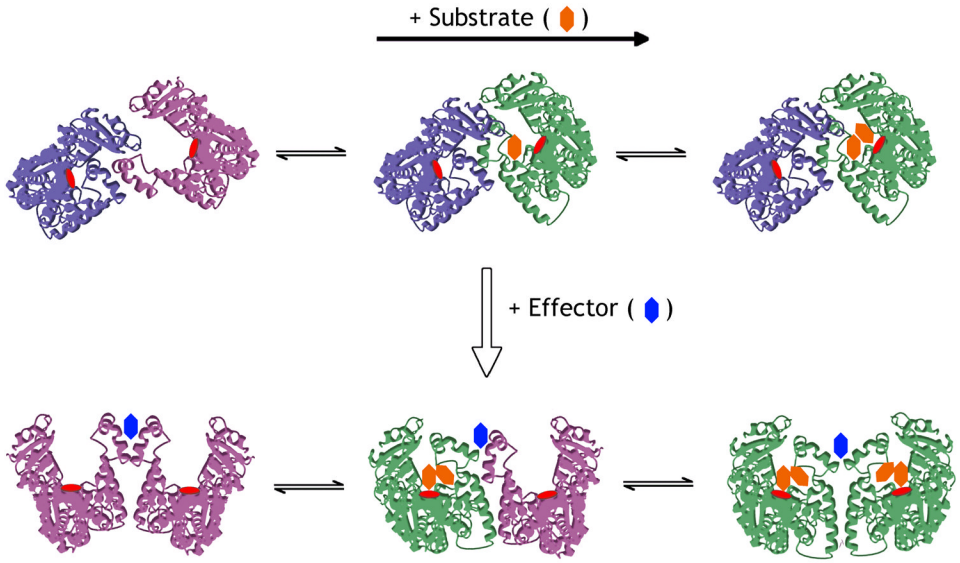

Fig. 2.

A model of CYP3A4 cooperativity combining the concepts of multiple substrate binding, ligand-induced conformational transitions, and ligand-modulated persistent heterogeneity. The scheme considers an oligomer of the enzyme, which is shown as a dimer (for simplicity). In the substrate-free state the oligomer contains subunits in two different conformations, namely a “locked”, non-functional conformation (blue) and resting, low-spin conformation (magenta), which is similar to that shown in Fig. 1. The mechanism of substrate (orange hexagon) binding in the functional subunit in the absence of a heterotropic activator is similar to that shown in Fig 1 for a monomeric enzyme. In the present figure the initial binary complex of the resting conformation is omitted for simplicity. Interaction of the enzyme with a heterotropic activator (blue hexagon) results in a rearrangement of the oligomeric structure and eliminates the non-functional conformer.