Published in final edited form as:

Expert Opin Drug Metab Toxicol. 2010 January ; 6(1): 1–15. doi:10.1517/17425250903329095.

New cytochrome P450 mechanisms: implications for understanding molecular basis for drug toxicity at the level of the cytochrome

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Abstract

Importance of the field—Cytochrome (CYP) P450 is a collective name for a very large group of heme enzymes, which catalyze largely oxidative reactions, including those of pharmacological and toxicological importance. Their efficient operation requires coupling of specific electron donor and O₂ consumption and substrate hydroxylation. Many drug oxidation reactions are partially uncoupled, leading to the formation of highly toxic reactive oxygen species, which can cause unpredictable toxic effects on the cell. Rational approaches to avoid uncoupling require knowledge of the underlying mechanisms.

Areas covered in this review—In this communication, attempts have been made to bring together past as well as present information indicating that i) the P450 active site has two differently accessible allosterically interacting subsites geared for entirely different types of functionally relevant interactions; and ii) substrate binding to the specific protein residues (Site I) forming the reducible high-spin complex and product binding at L6 (Site II) of the heme iron forming inhibited low-spin complex can regulate the functional state of the enzyme during catalysis.

What the reader will gain—Since P450 enzymes catalyze a wide variety of reactions, understanding the molecular basis for their efficient operation is of interest to many fields, including rational approaches to design safer drugs, tailoring P450 for a given task (e.g., bioremediation).

Take home message—It is important to take into account that the two sub-sites function as interacting sites rather than parts of a site functioning as single site for rational approaches to P450 mechanisms. This is important especially in regard to interpretation of the observed effects of drugs, products and inhibitors on these enzymes.

Keywords

Coupling/uncoupling; Cytochrome P450; Drug toxicity; Product; Substrate

1. 1 Introduction

Cytochrome P450 (CYP) is the collective name for a large group of enzymes which are hemoproteins. The CYP which is the major site of drug metabolism catalyzes mainly hydroxylation reactions in the presence of specific electron transport system and molecular oxygen. These are phase I reactions for preparing the drug for elimination. Therefore the CYP plays important role in maintaining therapeutic level of a drug in the body. Paradoxically, most adverse effects of drugs are attributable to the CYP enzymes. The adverse effects can result in

more than one way for example, from drug-drug interactions when one drug inhibits the metabolism of another drug increasing the drug to toxic levels. In addition, the drug induced toxicity can also result from reactive oxygen species generated during drug metabolism, due to uncoupling of oxygen utilization and drug hydroxylation which is a problem with many drugs. The system is fully coupled that is, operates with full catalytic efficiency when reducing equivalents and O₂ consumed are stoichiometrically related to the hydroxy product formed (Eq. 1).

When the system is uncoupled, the reducing equivalents and oxygen consumed are deviated from normal pathway of substrate hydroxylation, resulting in the formation of one or more of the side products of oxygen reduction (H₂O, H₂O₂, O₂⁻). The latter two can be converted to the highly toxic OH radicals in the presence of reduced transition metals such as iron in P450 itself. The free radicals can cause unpredictable toxic effects on the cell. The resulting pathology is treated with drugs which they themselves are uncouplers. Therefore there is a great need for safer drugs. The drug may be considered safer if it is a good substrate for P450 and not an uncoupler.

Currently, computational techniques in combination with biochemical and biophysical techniques are being used for drug candidate selection. Although these techniques can detect the problem of uncoupling, they have not been able to offer reasonable insights for finding solution to the problem. This is not easy. However, the first step for gaining insights toward the solution requires reasonably correct understanding the molecular basis for coupling/uncoupling and drug-drug interactions. This in turn requires reasonably correct view of the active site structure-function relationships.

The present aim is to bring together published information indicating that (a), CYPs cannot be treated as single site enzymes, and that the active site has two different interacting sub sites geared for entirely different types of functionally relevant interactions, (b), how substrates as well as products interact with the two sites is important for determining the functional state of the enzyme during catalysis, (c), these interactions may have necessary features to contribute in a major way to coupling/uncoupling mechanisms., and (d), product may play significant role in these mechanisms.

2.1 Current Approaches to Coupling/Uncoupling

All our current approaches to P450 mechanisms, including those of coupling/uncoupling are based on the assumption that P450 is like a single-site enzyme in which substrate and product compete for the same site. Therefore it is assumed that at sufficiently high substrate concentrations usually used in biochemical experiments, product interactions can be neglected. Therefore, all approaches have been focused on substrate binding only. In addition, all approaches to coupling/uncoupling have been aimed at investigating mechanisms of uncoupling. In regard to what causes uncoupling, it has been proposed [1,2] that primary cause is probably the presence of extra solvent, in addition to the distal ligand (water), around the di-oxygen bond undergoing cleavage. Solvent access to the oxygen-bound site during catalysis would provide a source of protons that could facilitate di-oxygen dissociation as hydrogen peroxide and/or di-oxygen cleavage and reduction to water [1]. This proposal was based on the X-ray data on CYP101, showing residual water in the active site of P450cam bound to uncoupler substrates, but not in the active site of P450cam bound to the natural substrate camphor, which undergoes fully coupled hydroxylation [1,2]. It is well known that protic environment can destabilize oxyheme complexes. Then one can expect that protecting the

oxygen activating machinery from unrestricted bulk water access is important for preventing uncoupling. However, uncoupling could not be related to any of the substrate binding parameters alone [1,3]. In addition, there is reasonable evidence indicating that possibility of product interactions with the active site becoming involved cannot be ignored. The X-ray and spectroscopic data have shown that the product 5-exo-OH camphor can bind through its -OH group to P450cam forming Fe-O bond with appreciable strength, resulting in six coordinated low spin complex [4]. This is consistent with the abilities of certain amines and -OH compounds such as products of P450-catalyzed reactions to serve as excellent L_6 ligands forming low spin six-coordinated inhibited complexes [5,6]. Product interactions can be envisioned to play important role in preventing uncoupling in more than one way for example, by blocking water access, and keeping the enzyme non functional until the next substrate binds (see section 7.0) Therefore there are several ways a P450 system can be envisioned to become uncoupled. For example, premature release of the product from L_6 before next substrate can bind, and pseudo- substrate effects of the product [3,7, and 8]. Since uncoupling can result in more than one way, it is difficult to be always certain of the observed cause and effect relationships.

While untimely unrestricted water access to the oxygen activating machinery may cause uncoupling, timely regulated water access is necessary for proton delivery required for O-O bond heterolysis, which is required for generation of the hydroxylating species. This aspect has been investigated by site directed mutagenesis. Thr252 and Asp251 have been initial targets for mutagenesis. The mutation of Thr252 to a non hydroxyl amino acid such as Ala or Gly converts the monooxygenase to an oxidase. That is, T252A mutation strikingly reduces product formation whereas NADH oxidation remains the same as in the wild type enzyme, indicating the mutant is mostly uncoupled [9,10]. The D251N mutant decreases both product formation and NADH oxidation to essentially the same extent [11,12]. These results implicated the two residues Thr252 and Asp251 in proton delivery machinery required for oxygen activation. However Thr252 is not the direct proton donor since replacement of the Thr252 OH group with an OCH₃ group, does not block activity [13]. Asp251 also is viewed as being directly involved in the proton shuttle mechanism [14]. However, structures of the oxy complexes in T252A and D251N mutants provide different picture [15]. Based on the X-ray structures, Thr252 has been envisioned as H-bond acceptor in stabilizing the Fe(III)-OOH intermediate rather than as a key side chain in organizing active site waters. The role of the Asp251 is viewed as playing more of a structural role rather than a direct catalytic role in proton delivery [15]. These roles of Thr252 and Asp251 have been reviewed [16]. More recently, based on investigation of the mutants of Gly248 bearing bulky residues in order to dehydrate the P450 distal pocket, it has been proposed that maintaining the number and location of the active site water molecules is important for effective proton mediated oxygen activation in CYPs [17]. It appears that based on the changing roles of Thr252 and Asp251, it may not be always easy to attach the observed effects to the mutated residue or residues.

3.1 CYPs Cannot be Considered as Simple Single-site Enzymes

Although different aspects of the mechanism may not be revealed by different CYPs by any given technique and under given set off experimental conditions, all CYPs may be expected to share a common reaction cycle in regard to certain basic requirements for functioning. These are, the CYP must be in the five coordinated high spin state to undergo reduction, and the L_6 position of the iron free for O₂ binding to the reduced enzyme. In the P450 catalytic cycle, there are two one electron transfer steps. Substrate binding converts the substrate-free low spin (E^{LS}) to the substrate-bound high spin state (E^{HS}) which undergoes first electron reduction and starts the first cycle. Then O₂ binds to the reduced enzyme forming the ternary complex Sub-Fe⁺⁺ O₂ with the substrate and O₂ bound to their respective sites. Although CYPs' may differ in regard to the hydroxylating species, the ternary complex is the starting point for

forming the hydroxylating species in all CYPs. Therefore CYP active site is best described as having two different sub-sites geared for entirely different types of functionally relevant interactions. The two sites are the substrate binding site, the specific protein residues (will be referred to as Site I) and the L_6 position of the iron (Site II) to which O_2 binds upon reduction. In the ferric enzyme, products and certain inhibitors can bind to Site II forming inhibited complexes as also indicated earlier (Section 2.1). Thus the two-site behavior of the active site is reflected not only in binding of substrate and O_2 to the reduced enzyme, but also in binding of substrates, products and inhibitors to the ferric enzyme. Since binding of substrates and inhibitors to the two sites form functionally different complexes, the two sites cannot be considered as parts of a site functioning as a single site. The single-site view of the CYP active site may be the reason we have not been able to arrive at a reasonable mechanism for coupling/uncoupling. Therefore it appears that we need a paradigm change from single-site view of the CYP active site to two different sub-sites in binding of substrates, products and inhibitors. This change is the basis for new P450 mechanisms.

4. 1 Crystallographic Picture of Site I and Site II

Fig. 1A & Fig. 1B [18] and Fig. 1C [4] provides a crystallographic picture of what is meant by Site I and Site II. As indicated by X-ray structure of camphor-CYP101 complex (Not shown), the Tyr96 donates an hydrogen bond to the camphor carbonyl group. The Tyr96 is among the residues of the access channel (Site I) which make major contacts with camphor [19-21]. The inhibitor metyrapone is bound to the camphor binding site (Site I) as well as to Site II (L_6) (Fig. 1B). The Tyr96 donates an hydrogen bond to a N atom of metyrapone, which is similar to the camphor carbonyl group-Tyr 96 hydrogen bond. In addition, the inhibitor through its N_2 atom, is ligated to the L_6 of the iron the Site II (18). On the other hand, the 1-Phenyl imidazole (Fig. 1A) is bound to Site II forming N-Fe bond, but it is not bound to Site I. That Site I is free is also indicated by the temperature factor of this region (F and G helices and F/G loop) being the same as that in the free enzyme, which has a water strongly ligated at Site II [18]. Therefore as seen in the X-ray structures, metyrapone as well as the exogenous product 5-exo-OHcamphor may be considered to be bound to both Site I and Site II whereas the imidazole is seen bound only to Site II.

The Site I and Site II complexes are spectroscopically distinguishable. The high spin Site I complexes are five coordinated, and the Site II complexes are low spin and six coordinated. Formation of these two types of complexes results in characteristic difference spectra designated as Type I and Type II. In terms of Site I and Site II, the Type I complex is the same as Site I complex. The Type II complex is Site II complex with or without binding to Site I. Therefore the terminology Site I and Site II can distinguish between the two types of Type II complexes which is an important consideration for the present communication.

5.1 The CYPs have Two Ferric States Differing in Spin and Coordination States of the Heme Iron and Their Abilities to Undergo Reduction

The majority of substrate-free ferric CYPs are low spin six coordinated with a water ligated at Site II keeping the enzyme in the resting state - the nonfunctional state (E^{LS}). Substrate binding to Site I releases the water from Site II, converting the enzyme to the reducible five coordinated high spin ferric state (E^{HS}) [19,20] - the functional state. Substrate is hydroxylated by interacting with the hydroxylating species at Site II. Since certain -OH compounds can serve as excellent Site II ligands, products when formed may be expected to be bound or to bind at Site II. Consistent with this expectation and as indicated earlier, is the X-ray and spectroscopic data on 5-exo-OHcamphor-CYP101 complex, showing that the product through its -OH group binds to Site II (L_6) forming Fe-O bond resulting in low spin six coordinated inhibited complex [4]. Then the substrate binding to Site I and the product binding to Site II can regulate the

functional state of the enzyme during catalysis by determining when to turn on and turn off the enzyme.

CYP101 is the first enzyme to be crystallized [19,20]. Therefore our structural insights into structure-function relationships of CYPs have been based on the structural information on CYP101. But CYP101 is a soluble microbial enzyme. What we are interested in regard to drug toxicity are interactions of drugs with mammalian xenobiotic metabolizing CYPs which are membrane-bound. More recently, X'ray structures of several membrane-bound CYPs with the membrane anchor region removed are available [21-24]. Although CYP101 is soluble and the mammalian CYPs are membrane-bound, the overall fold is conserved with the exception of the membrane anchor region [19-22]. However, any drug metabolizing CYP can oxidize variety of structurally different substrates. Therefore it appears that, while overall structure is conserved it is also flexible. Of particular importance are the F and G helices and the F/G loop which are flexible. These regions undergo open/close motions and they are probably involved in substrate access [19]. These regions may be considered as constituents of the substrate binding site - Site I.

6.1 Inhibitory Allosteric Interactions Between the Differently Accessible Site I and Site II

The X'ray and spectroscopic data on CYP101 [19,20] and CYP102 [25] have shown that binding of substrate to the more accessible Site I releases the water ligated at Site II the L_6 position of the buried heme iron. Substrate releases the L_6 water despite its inability to bind to this site which is indicative of inhibitory allosteric effect of Site I binding on Site II ligation. The release of L_6 water results in conversion of the six coordinated substrate-free low spin enzyme (E^{LS}) to the five coordinated high spin complex (E^{HS}). The resulting difference spectrum (Fig. 2) is characterized by absorption maximum around 385-392 nm, a minimum close to 420 nm, and an isosbestic point around 403-407 nm. When the substrate-bound fraction of the CYP is completely converted to the high spin state, the peak to trough ratio of the Type I difference spectrum with respect to the isosbestic point is usually around 0.85-0.9. The inhibitory effect of Site I binding on Site II ligation which is reflected as the static Type I spectrum can be readily observed by the routinely used static spectroscopy. However, to observe inhibitory effect of Site II ligation on Site I binding, requires measuring of Site I binding before and after Site II re-ligation to form the final six coordinated Type II complex. This is possible because formation of the Type II complex is apparently a two-step process as was observable for binding of product to CYP2B4, by Temperature-jump relaxation spectroscopy. This is explained as follows.

6.2 Evidence Showing that Type II Binding is Apparently a Two-step Process

As indicated earlier, in P450 enzymes, the heme is buried in the active site cavity, therefore Site II would not be expected to be directly accessible to exogenous ligands. Since Site I would be much more accessible, one would expect that an exogenous ligand which forms six-coordinated low spin complex may first bind to Site I, releasing the native L_6 ligand from the buried iron, through the inhibitory effects of Site I binding on Site II ligation. This would result in the formation of five-coordinated high spin intermediate observable as transient Type I difference spectrum. Whether or not one can observe the transient high spin intermediate, would depend upon the magnitude of the time difference between a ligand binding to Site I, and Site II re-ligation, and the time-scale of the technique. In the present case, the Transient Type I difference spectrum (TDS) was observable for the binding of the substrate benzphetamine (not shown) as well as for its demethylation product DesBnz (also referred to as NorBnz) to CYP2B4 by the Temperature-jump relaxation technique. The TDS spectra were constructed from the amplitudes of the bi-exponential relaxation curves obtained at different

wavelengths at constant product concentration as previously described in greater detail [26]. The fast phase reflects formation of the transient Type I complex, and the slow phase represents the Site II re-ligation to form the final Type II complex within 20 milliseconds. This is the final form observable by the static difference spectroscopy.

In Fig. 3, the Panel I shows absolute static spectra of CYP2B4 in the absence (curve 1) and in the presence of the product DesBnz (Curve 2). The curve 3 is the DesBNZ-induced static difference spectrum which has zero absorption in the high spin region (385 to 390 nm). This product forms the low spin Site II complex. It is interesting that based on theoretical free energy calculations, it has been suggested that hetero atom of DesBNZ binds to Site II as strongly as water [27]. In panel III, curves A and B show the initial Type I and the final Type II difference spectra within 20 msec. It may be seen that the transient Type I spectrum closely resembles the static Type I difference spectrum of camphor-CYP101 complex (Fig. 2) which is characteristic of the purely high spin complex,. This suggests all of the product-bound CYP2B4 is converted to the transient high spin intermediate. The panel II shows kinetically derived Type I and Type II difference spectra by applying SVD Global fit analysis to the bi-exponential curves obtained at different wavelengths as described below. In panel IV, the kinetic curves A, B and C and the lag period in appearance of C are typical of consecutive irreversible reactions such as $A \rightarrow B \rightarrow C$. The rate constant for $A \rightarrow B$ was $\sim 690 \text{ sec}^{-1}$ and for $B \rightarrow C$ was $\sim 120 \text{ sec}^{-1}$.

The SVD stands for singular value decomposition. The technique is used to deconvolute overlapping spectral data. The SVD uses all of the spectral and kinetic information to define transitions occurring in time, in terms of difference spectra and kinetic constants. In the present experiments, plot of 10 kinetic traces were assembled into 3D format (difference spectra versus time). Two exponentials were required to fit the kinetics. The data set were analyzed by the global fit program as follows: A. Collected multiwavelength data for each kinetic data point; B. Applied SVD to the 3D data set. This showed the number of kinetic processes; C. Fitted the kinetics using all the wavelengths (Global fit); D. Constructed spectra on the basis of two exponential mechanisms. Among many two exponential mechanisms tested only $A \rightarrow B \rightarrow C$ gave expected spectra shown in Fig. 3, Panel II. The B becomes visible on an expanded scale as previously shown [26].

The kinetic K_D derived from the fast phase of the bi-exponential relaxation curves obtained at different DesBNZ concentrations is the K_D^{APP} for Site I binding before Site II ligation. The static K_D^{APP} which is determined by the static spectroscopy gives the K_D^{APP} after Site II ligation. The kinetic and static constants are compared in Table I. In regard to K_D^{APP} for Type II binding what we measure by the static spectroscopy is the equilibrium binding of exogenous Type II ligand to its low affinity site - Site I, followed by Site II ligation. The static K_D may be referred to as apparent K_D^{APP} which reflects net effect of interaction between the two sites. The static K_D s' of the Camphor (Cam) and 2-Adamantanone (2-Adone) complexes of C334A-CYP101 are similar to their kinetic and transient K_D s. Both substrates essentially completely converted six-coordinated low spin to the five coordinated high spin state observable by the static spectroscopy. Therefore their static and kinetic constants are both for the high spin complexes, and their kinetic traces were essentially single exponential.

The C334ACYP101 which has only one surface residue altered preserves the essential features of the wild type (wt) enzyme, including full catalytic efficiency, while avoiding propensity to dimerize upon freezing and thawing, which has been observed with the wild type enzyme [28,29]. This single mutation also avoids or minimizes aggregation, which makes it easier to handle [30]. In addition it avoids having to take into account protein-protein interactions in mechanistic studies. Then the C334A P450cam with its natural substrate camphor and product

is considered as an ideal system to further investigate substrate and product interactions with the active site.

7.1 The Two-site Behavior of CYP Active Site is also Reflected in Binding of the Substrate to CYP101 in the Presence of the Product (Implications for Coupling/Uncoupling) : [26]

The uncoupling of oxygen activation from substrate hydroxylation was first observed with CYPC21 [34], when a non-hydroxylatable substrate (now referred as pseudo-substrate) was capable of eliciting low- to high-spin state transition, stimulating NADPH oxidation and O₂ consumption in the 4e oxidase 2:1 stoichiometry as opposed to the 1:1 stoichiometry of the fully coupled system (Eq. 1). Since then, it has been recognized that a P450 system can become uncoupled in more than one way. There are multiple activated oxygen species in CYP catalysis [31]. Unrestricted water access destabilizing the activated oxygen intermediates (Fe⁺⁺O₂, Fe⁺⁺⁺OOH & Fe^VO) has been proposed as one of the causes [1,2,32 rev]. In another instance, uncoupling observed during benzphetamine metabolism by CYP2B4 has been attributed totally to pseudo-substrate effects of the product desmethyl benzphetamine (DesBNZ) [8].

The question is, how does a fully coupled system avoid all the different ways of uncoupling? For now, the questions are: how does a fully coupled system avoid the two ways of uncoupling i.e. water access, and pseudo-substrate effects of the product? It is expected that the answer lies in the two-site model for substrate (S) binding to P450 (E) in the presence of the product (P) involving the ternary complex ESP, in which the S has much higher affinity to EP than P has to ES, i.e. $K_3 > K_4$. These relative affinities allow the dissociation of the ESP predominantly to ES^{HS} + P and not EP^{LS} + S (Fig. 4, Table II.). Since the substrate (S) binds to Site I and does not bind to Site II, and the same molecule of the exogenous product (P) binds to both sites, the S can directly release P from Site I, and the only way the S can release P from Site II is via the ternary complex ESP, through the inhibitory effect of Site I binding on Site II ligation. This suggests that the release of the product from Site II is under the control of the next substrate. Such a scenario may prevent product dissociating from Site II before next substrate binds, thereby keeping the system non-functional until the next substrate binds. This can also prevent pseudo-substrate effects of the product. This is because the substrate binds to Site I releasing the product from this site before the product is released from Site II. This prevents the formation of the high spin reducible Site I complex of the product. In addition, in this model, S or P or S+P are always present in the active site cavity during turnover. The presence of the S or P has been observed to substantially protect preformed CYP101-Fe⁺⁺O₂ complex [33], which could be by blocking water access. Furthermore, bypassing E^{LS} (Fig. 4) which has L₆ and 5 other waters sequestered in the active site cavity [19] avoids structures which require water access from becoming involved during turnover. The final proof for the role of ESP requires its demonstration during turnover. However the relative values of the substrate (S) binding to EP (K_3) being sufficiently greater than the product (P) binding to ES (K_4) (Table II) indicates that ESP has the potential to serve as an important intermediate in the cycle for replenishing ES^{HS} bypassing E^{LS} (Fig. 4). If K_3 were = or < than K_4 the ESP would be useless. In addition, replenishing ES^{HS} via ESP bypassing E^{LS} is consistent with existence of several substrate-free high spin P450s that are also functionally fully competent, despite the absence of the substrate-free low spin species (E^{LS}) [32 rev]. This indicates that E^{LS} may not be required for catalysis.

There is no obvious role for E^{LS} in the catalytic cycle. The catalytic cycle shown in Fig. 4 requires that EP^{LS} formed remain as such until the next molecule of the S binds at site I releasing the product (P) from Site II via the ternary complex ESP. The P binding to Site II may even be irreversible as suggested by the SVD analysis of the Temperature-jump relaxation data on

binding of DesBNZ the demethylation product of benzphetamine to CYP2B4 (Fig. 3, Panel 4). As also indicated earlier, strong binding of this product is also suggested by theoretical analysis showing that the hetero atom of DesBNZ binds at L₆ (Site II) as strongly as water [27].

In order to understand product interactions with CYP, It is important to take into account the difference between binding of exogenous product and product formed in-situ. The exogenous product first binds to its low affinity, more accessible site –Site I and allosterically releases the native L₆ ligand from the buried heme iron. This forms transient Site I complex of the product which is reflected as transient Type I difference spectrum as shown by the SVD analysis of the T-jump relaxation data (Section 6.1). Then the product gains access to the buried Site II, forming the low spin inhibited complex (EP^{LS}) reflected as final low spin Type II difference spectrum. The product formed in-situ enters the active site as substrate which binds at Site I with much higher affinity. In addition, the product when formed in-situ is probably in strategic position to be bound through its –OH group at L₆. Therefore it may be expected to be bound strongly at Site II with much decreased interaction with Site I due to Site I-Site II allosterism (Sections 6.1 & 6.2). Stronger the Site II ligation of a ligand, its own binding to Site I may be expected to become weaker, finally resulting in purely ligand-Site II complex without the ligand being bound to Site I. This is likely the case even if the exogenous ligand must first bind to Site I. This is because of the inhibitory allosteric effects of Site II ligation on Site I binding (Table I).

Binding of the product DesBnz formed in situ, to CYP2B4 was studied under turnover conditions (Reductase + NADPH generating system + O₂). The results (unpublished) showed that even as low as 1uM product (calculated from extinction coefficient of the EP^{LS}) formed remains bound producing the low spin Type II difference spectrum. The Type II difference spectrum was identical to that produced by exogenous product (Fig. 3, Panel I, curve 3). However, lot more of the exogenous product was needed. The K_D for exogenous product was ~700uM (Table I). That the EP^{LS} complex formed may not dissociate is also suggested by the irreversible nature of the kinetics of conversion of transient Type I complex to Type II shown in Fig. 3, Panel 4.

On the basis of the two-site model for S and P interactions with E involving ESP, one can envision several ways in which a P450 system can become uncoupled (also see section 2.1). These include (a) improper orientation of the substrate, and (b) release of the product from Site II and exit from the cavity before the next substrate binds, which could allow unrestricted water access to the oxygen binding site. Then competition between electron transfer and restoration of the L₆ water can determine whether or not the system becomes uncoupled. The single-site view of the P450 active site has the effects of masking the unique features of the differential behavior of the two interacting sub-sites in binding of substrates and the products. This has led us into ignoring product interactions, although it has not been possible to attribute coupling / uncoupling to any of the parameters of substrate binding alone [1,3].

8.1 The Two-site Model for the CYP Active Site can also Account for the Different Classes of CYP Behavior which we have Observed in Regard to the First Electron Acceptor ES^{HS}

The different classes are as follows: (i), Low spin P450s (E^{LS}) which upon substrate binding, are converted to the reducible high spin complex (ES^{HS}), and express appreciable hydroxylation activities [e.g., 32,34,35]. With this class of CYPs, substrates elicit Type I spectral changes which are observable by the routinely used static spectroscopy; (ii), Substrate-free high spin P450s (E^{HS}), which undergo reduction even in the absence of substrates, and are functionally fully competent despite the absence of the low spin state, indicating that the

low spin E^{LS} is not required for catalysis [32 rev]; and (iii) Low spin P450s (E^{LS}) which apparently form low spin complexes with their substrates (E^{LS}) therefore, they do not exhibit the substrate-induced Type I spectral changes observable by the static spectroscopy. Nevertheless they express appreciable hydroxylation activities [36,37]. The single-site view of the CYP active site leads to the conclusion that different CYPs are different, and that some low spin CYPs need to become high spin for activity (Class i), some are already high spin (Class ii) and they do not need low spin for activity, and some others which form low spin complexes with their substrates function with only low spin (class iii). Therefore the same catalytic cycle based on the single-site view of the CYP active site [2] would not be applicable to all three classes. On the basis of the two-site model involving the ternary complex ESP, all three classes become reasonable within the frame work of the same model. In this model, the E^{HS} the starter of the cycle is replenished via the ternary complex by-passing the E^{LS} as shown in the Fig. 4. Since the E^{LS} has no obvious role in the cycle, the same cycle would be applicable to class i as well as class ii which is functionally competent in the absence of E^{LS} . Same cycle is also applicable to class iii. However, in this case, it is necessary to take into account, that the formation of a low spin complex (E^{LS}), which requires Site II (L_6) ligation is apparently a two-step process and proceeds via transient high spin intermediate (E^{HS}) (Section 6.2) [26]. The transient intermediate which is observable in the time-scale of the Temperature-jump relaxation spectroscopy has the life time compatible for the CYP to undergo reduction which can occur in the nanosecond time scale [38,39]. Then the above contradictory results observed with different classes of CYPs become understandable on the basis of differential behavior of the sub-sites within the frame work of the same model the two site model. This model also keeps in line with the basic requirements which may be considered as established. These are the requirement for the ferric CYP to be in the high spin state to undergo reduction, and the requirement for the L_6 position of the iron to be free for O_2 binding to the reduced enzyme to form the ternary complex $SubFe^{++}O_2$, which is crucial for P450 catalysis. In addition, the two-site model involving the ternary complex ESP observed with CYP101 [26], indicates the significance of the abilities of CYPs to bind more than one substrate-size molecules [40,41] in normal functioning of a fully coupled system. The abilities of CYPs to bind more than one substrate-size molecules resulting in non-Michaelis Menten kinetics have been observed with drug metabolizing CYPs, and have been referred to as CYP allostereism [42,43 revs.].

9.1 CYP Allostereism

The CYP allostereism is different from the traditional allostereism. The traditional allosteric systems have high degree of substrate specificity. The substrate specificity of drug metabolizing CYPs are extraordinarily low, resulting in nonspecific allostereism which has been referred to as CYP allostereism [42]. The CYPs can bind more than one substrate and can generate more than one product from a single substrate. The CYP allostereism may result from homotropic or heterotropic effects, resulting in atypical kinetic profiles, which are categorized as autoactivation, substrate inhibition, partial inhibition and biphasic [40]. The homotropic effects result when a drug acts as both substrate and effector. The heterotropic effects result when one drug alters the CYP interactions with another drug either activating or inhibiting product formation [44,45].

In regard to the CYP allostereism, CYP3A4 receives much attention. This is because this isoform metabolizes more than 50% of the drugs used to day. This is also the isoform involved in undesirable drug-drug interactions. This CYP has relatively large conformational dynamic active site. Therefore more than one molecule of substrate can occupy the cavity enhancing catalytic efficiency, possibly by constraining the substrate close to the reactive oxygen intermediate during catalysis [46]. It is also possible that presence of more than one substrates in the active site cavity can block unrestricted solvent access to the reactive oxygen intermediates which also can enhance catalytic efficiency [1,2]. The active site cavity is much

larger near the heme iron than in other CYPs. Therefore reactive oxygen intermediates may be more accessible to the solvent.

The heterotropic effects are of particular interest in regard to drug and drug-drug interactions with CYPs. An effector drug may activate CYP-dependent metabolism of drug 1, yet inhibit or have no effect on the metabolism of drug 2 metabolized by the same CYP isoform. Also a single effector molecule may change from an activator at low concentrations to an inhibitor at higher concentrations. Thus the behavior of an effector depends on the substrate that is being metabolized, as well as the concentrations of the effector and substrate. For example, testosterone inhibits with different apparent potencies, the metabolism of terfenadine and midazolam by CYP3A4. In contrast, testosterone does not inhibit metabolism of nifedipine, but terfenadine does. Further more, testosterone itself is a substrate for CYP3A4, and its metabolism is partially inhibited by nifedipine [47-49]. Another example observed with CYP3A4 is interactions of α -naphthoflavone (α -NF) and aflatoxin B1. The α -NF activates metabolism of the aflatoxin, but the aflatoxin has no effect on the metabolism of α -NF [50]. We have not been able to understand such non-reciprocal interactions between drugs on the basis of results of CYP activity measurements. This might be because CYP allosterism takes into account only substrate interactions with one or two substrate binding sites in the active site cavity. When effects of drug 1 on the metabolism of drug 2 are studied, several interactions may become involved. These may include allosteric interactions between Site I and Site II discussed in earlier sections (Sections 6.1, 6.2 & 7.1) in connection with binding of substrates and products to CYP101 and CYP2B4. These interactions will be referred to as Site I-Site II allosterism to distinguish them from those of CYP allosterism.

9. 2 The Difference Between the CYP Allosterism and the Site I-Site II Allosterism

Allosteric interactions between Site I and Site II are probably involved in expression of overall CYP activity, since substrate binding to Site I and product binding to Site II can regulate the functional state of the enzyme. However, CYP allosterism as perceived based on the results of substrate concentration versus activity measurements, is different from the Site I-Site II allosterism. Formulation of the CYP allosterism is very complex. Therefore rapid equilibrium assumption is used to derive manageable equations. It is assumed that interchange between species is rapid relative to the catalytic steps. In addition it is assumed that product when formed is released fast relative to the oxidation rates. Therefore only substrate interactions are taken into account in the models for CYP allosterism [40]. A two-site model has also been proposed in connection with CYP allosterism. This model refers to two substrate binding sites in the active site cavity [40]. This is different from the Site I and Site II of the CYP active site (Section 3.1). A major difference between the two types of allosterisms is in regard to how the operating concentration of the first electron acceptor ES^{HS} is maintained. As implied in the CYP allosterism models, binding of substrate to its binding site converts the low spin substrate-free CYP (E^{LS}) to the high spin ES^{HS} complex, and the product when formed is released fast and the starting conformation of the enzyme (E^{LS}) is restored at the end of every cycle [40]. In the Site I-Site II allosterism, the ES^{HS} is replenished via the ternary complex ESP as explained in Section 7.1, indicating the important role product may play. The cycle (Fig. 4.) based on Site I-Site II allosterism can also account for the existence of substrate-free high spin CYPs that are functionally fully competent.

9. 3 Can we Understand the Non-Reciprocal Drug-Drug Interactions on the Basis of Site I-Site II Allosterism?

Recall that Site I and Site II of a CYP active site are differently accessible, allosterically interacting sub-sites. With any given drug substrate (S), the overall activity under given set of experimental conditions, depends on the operating concentration of the ES^{HS} complex. This

in turn depends upon the relative values of the parameters of the equilibria of the two-site model involving the ternary complex ESP as explained in section 7.1 (Fig. 4).

$$ES^{HS} = E_T K_1 S_T / [1 + K_1 S_T + K_2 P_T + K_2 K_3 S_T P_T]$$

When the effects of drug 1 on the metabolism of drug 2 by a CYP are studied, interactions that become involved are as follows: The interactions of drug 1 (S1) and its hydroxy product (P1) and interactions of drug 2 (S2) and its hydroxy product (P2) with Site I and Site II. This results in a very complex situation. However, using chemically synthesized products, determination of the parameters of the equilibria of the two-site model (Fig. 4) for drug 1 and drug 2 separately might be informative. This requires determination of the parameters for binding of (a) S1 in the presence of P1; (b) S1 in the presence of P2; (c) S2 in the presence of P2 and (d) S2 in the presence of P1. The parameters can be determined by the spectral titration technique as previously described for CYP101 [26], provided that the drug and its product can elicit characteristic spectral changes in the CYP, observable by the static spectroscopy. This is not always possible because some substrates may work through transient high spin intermediates (Section 6.1, 6.2). Then the substrate-induced spectral changes cannot be observed by the static spectroscopy. Knowledge of coupling efficiency is of great importance for understanding certain types of non-reciprocal drug-drug interactions with CYPs. For example, if drug 1 metabolism by a CYP is maximally coupled and drug 2 metabolism is only partially coupled, the presence of drug 1 might enhance coupling efficiency for drug 2, and drug 2 may not have any effect on the metabolism of drug 1 by the same CYP. That product interactions may play significant role in coupling mechanism is also suggested by a CYP allosterism experiment discussed in section 10. 1.

Summary

In summary, the two-site behavior of the CYP active site and the Site I-Site II allosterism are reflected in several aspects of CYP mechanism (a) binding of the substrate to Site I, releasing the Site II ligation resulting in the transformation of the six coordinated low spin to the five coordinated high spin state (Sections 6); (b) Binding of substrate (S) in the presence of the product (P) to CYP101 and CYP2B4 (E) involving the ternary complex ESP (Section 7.1) and (c) in Type II binding of an exogenous ligand, which is a two-step reaction occurring via transient high spin Site I complex (Sections 6.1). The resulting overall hypothesis is as follows: Strikingly different properties of the differently accessible sub-sites, and allosteric interactions between the two sites, determine the thermodynamic and kinetic parameters of substrate and product interactions with the two sub-sites. This can regulate the five coordinated high spin functional and the six coordinated low spin inhibited states during catalysis. This in turn, can determine the catalytic efficiency i.e. degree of coupling/uncoupling by determining when to turn on and turn off the enzyme during catalysis [26].

10.1 Expert Opinion Section

The results of CYP catalyzed drug metabolism and drug-drug interaction experiments have been observed to be too complex for understanding their mechanistic implications. One of the reasons may be the assumption that the CYP active site is like that of a single site enzyme in which product does not play any role. The single-site view cannot take into account that the active site has two differently accessible allosterically interacting sub sites (Site I and Site II) geared for entirely different types of functionally relevant interactions. In addition the single site view cannot take into account that the Site I and Site II complexes are functionally different. As also indicated earlier, substrate binding to Site I forms the functional complex and the product binding to Site II forms inhibited complex. Therefore substrate and product interactions

with the two sub-sites can determine when to turn on and turn off the enzyme during catalysis. Therefore, fine tuning of the on and off events might be important to prevent uncoupled turnover as discussed in section 7.1. In the single-site view, the two sites are considered as parts of a site functioning as a single site. This in effect masks the special features of the differential behavior of the two sub-sites of the CYP active site and the important role product can play in coupling mechanism. An example of CYP allosterism activity experiment indicating that the product can play important role in coupling mechanism is discussed as follows:

Example

The effector role of dapsone in the hydroxylation of flurbiprofen by CYP2C9 to yield 4'-hydroxy flurbiprofen [51]—This example was chosen because in addition to hydroxylation activity, coupling efficiency has been measured. In addition, the effects of products on substrate hydroxylation have been studied. The major metabolite of dapsone (N-hydroxydapsone) enhanced coupling efficiency stimulating flurbiprofen hydroxylation. This is consistent with the proposed beneficial role for product in coupling mechanism as discussed in connection with CYP101 in section 7.1.

Since the effector dapsone is also hydroxylated by CYP2C9, one would expect that dapsone would inhibit flurbiprofen hydroxylation. Contrary to this expectation, dapsone enhanced coupling efficiency stimulating the substrate hydroxylation. When hydroxylation of flurbiprofen is measured in the presence of dapsone, in addition to the interactions of the substrate and the effector, the interactions of their hydroxylated products 4'-hydroxyflurbiprofen and N-hydroxydapsone with CYP2C9 become involved. The products could bind through their -OH groups at L₆ of the iron (Site II) forming EP^{LS} complexes. This might be of significance for the observed coupling efficiency in the absence of the effector as well as for enhancing the coupling efficiency by the product of the effector. This is because the EP^{LS} complex can keep the system non-functional until the next substrate binds to Site I, releasing the product from Site II via the ternary complex ESP, replenishing ES^{HS} during turnover as discussed in connection with CYP101 in Section 7.1. Dapsone being an amine could also be a good candidate for binding at L₆ position of the iron (Site II), and play the role of the product in enhancing coupling efficiency in substrate hydroxylation. These authors [51] also entertain the possibility of products of CYP-catalyzed reactions serving as activators of CYP enzymes. These observations suggest beneficial role of Site II ligation of ferric CYPs in catalysis (See 10.2)

In regard to dapson effects, a question that needs to be addressed is as follows: Flurbiprofen elicits Type I spectral change in the CYP2C9, as it should because it is hydroxylated. However, dapsone does not elicit the Type I spectral change, yet it is hydroxylated. Good possibility exists that a substrate which does not elicit the Type I spectral change observable by the routinely used static spectroscopy, may work through transient high spin intermediate (sections 6.2 & 8.1) [26].

10.2 Structural Basis for the Beneficial Role of Site II (L₆) Ligation of the Ferric CYP in Catalysis

Based on X-ray data on CYP101, it has been proposed that flexibility of F & G helices and F/G loop (Site I) is important for substrate (S) to bind and enter the active site cavity (see Section 5.1). There is also X-ray data showing that in the substrate-bound structure, the active site is closed, and the helices and F/G loop are no longer as flexible [20]. This raises important questions as to how the next S enters the active site cavity and what makes the F/G loop become flexible again? Possibilities can be envisioned if one takes into account the effects of Site II ligation on Site I. As indicated earlier (Section 4.1), in the 1-Phenyl imidazole bound-

CYP101 forming strong N-Fe bond, the flexibility of the helices and the F/G loop is the same as in the free enzyme which has water strongly ligated at L₆ (Site II) [19,20]. In addition, in the crystal structures of another CYP-CYP119 with different types of inhibitors bound at L₆, unfolding of the C terminus of the F helix and large movement of the F/G loop were observed [54,55]. In the substrate-bound CYP101, the flexibility of the helices and the F/G loop are largely decreased, and the L₆ is not ligated. These observations suggest that Site II ligation is important to induce Site I flexibility to promote substrate binding. Yet Site II ligation can have inhibitory effect on Site I binding (Table I). A reasonable explanation is as follows: While Site I flexibility is essential for promoting substrate binding, the affinity with which the substrate binds to the flexible Site I is probably the property of the substrate in relation to the parameters of Site I–Site II allostereism. This may involve product interactions with Site II or any other Site II ligand such as an effector (Dapson discussed above) which can be released when the S binds to Site I, via ternary complex. Then product bound at Site II could induce flexibility to Site I promoting binding of the next S. One may ask why can't restoration of the L₆ water be a way to restore Site I flexibility? As also discussed earlier (Section 7.1) this can result in futile turnover. This is because one cannot be certain that when EP^{LS} dissociates to E^{HS} + P that the P's dynamics, in getting out of the active site cavity, can allow water to gain access to the ferric iron, sufficiently fast to compete with electron transfer. Since product interactions have gained little attention in the CYP field, how and when the product gets out of the cavity has not yet been adequately addressed. However, a more hydrophilic route than the substrate access channel, observed in the structure of CYP101 has been suggested as possible product exit route [19]. What ever the route may be, one may anticipate that the orientation of the product may change.

Overall, several aspects of CYP mechanisms have become reasonably understandable by taking into account that the CYP active site has two interacting sub-sites geared for entirely different types of functionally relevant interactions. The product through its ability to be bound at Site II could play more than one beneficial roles in efficient operation of the catalytic cycle (Fig. 4). These are (a) product bound at Site II may keep the system non functional preventing futile turnover until the next S binds, releasing the product (P) via the ternary complex ESP (Section 7.1), (b) product bound at Site II may induce flexibility to Site I and promote binding of the next S, and (c) the presence of the product may protect Fe⁺⁺O₂ [33], possibly by blocking water access (Section 7.1). If CYP were a simple single-site enzyme, it would have been possible to observe product inhibition of substrate hydroxylation. Product inhibition was not observable when tried with CYP73As which hydroxylates cinnamate to coumerate [52]. That it may not be possible to observe product inhibition was predicted based on substrate and product binding studies with the fully coupled CYP101 [53]. It is noteworthy that in one out of one study in which effects of products on coupling efficiency of substrate hydroxylation have been measured, the products have been found to enhance the efficiency [51].

Several aspects of CYP mechanism have become understandable by taking into account that the CYP active site has two differently accessible interacting sub sites. Therefore we may need a paradigm change from the single site view of CYP active site to that of two sub-sites. This would be important for rational approaches to CYP mechanisms, and for understanding interactions of drugs with CYPs.

Acknowledgments

This work was presented in part at the MDO conference (July 22-28, 2002) at Sapporo, Japan, in part at the P450 Conference (June 29 to July 3rd 2003) in Prague and in part at the MDO conference (July 4-9, 2004) in Mainz, Germany.

Declaration of interest: This work was supported by ONR Contract N00014-75C-0322, NIH Grant AM18545 and Research Foundation Grant 3-70580 and Harrison Dept. For Surgical Research.

References

** References important for understanding that CYP (E) active site has two differently accessible interacting sub-sites and that interactions of substrate (S) and product (P) with the two sub-sites are relevant for efficient functioning. The two sites are the substrate binding site the specific protein residues (Site I), and Site II is the L₆ position of the iron.

* Less important

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efficiency. In addition N-OHdapsone also enhanced coupling efficiency (discussed in Section 10 of the present communication).

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Abbreviations

E ^{LS}	Substrate-free Low spin CYP
E ^{HS}	The high spin CYP-substrate complex
E ^{PLS}	Low spin CYP-product complex
ESP	The ternary complex of CYP, substrate and product
BNZ	Benzphetamine
DesBnz	The demethylation product of BNZ, desmethylbenzphetamine
NorBnz	also referred to as nor benzphetamine
TDS	Transient difference spectrum

FIG. 1.

(Adapted from Fig. 2. in Ref: 19). X-ray picture of the CYP101 bound to (A). 1-Phenyl imidazole bound only to Site II; (B). Metyrapone bound to both Site I and Site II; and (C) 5-exo-OHCamphor bound to both sites [6].

FIG. 2.

Type I difference spectra of CYP101-camphor complex. The peak to trough ratio is ~ 0.87 as expected when all of the bound enzyme is converted to the high spin state. The purpose of this figure is to show the similarity of the static Type I spectrum and the transient Type I difference spectrum shown in Fig. 3.

Fig. 3.

(Adapted from Figs 7 & 8 in Ref: 26). Panel I. Static Absolute and Difference Spectra of CYP2B4 (2uM) in 0.18M K-PO4 buffer pH 7.4 + 50ugs/ml of phosphatidyl choline, + 5% glycerol, 21° C. Spectra 1 & 2 are absolute spectra in the absence and in the presence of DesBNZ (530uM). Spectrum 3 is the difference between 1 and 2; Panel II. The SVD analysis of the kinetic data on DesBNZ-CYP2B4 complex formation showing that the transient Type I difference spectrum is converted to Type II via an intermediate observable on an expanded scale [26]. Panel III. Observed Transient Difference Spectra (TDS) constructed from the amplitudes of the biexponential Temp-jump relaxation curves obtained at different wavelengths, Curve A. Transient Type I difference spectrum from fast phase amplitudes; and curve C. Type II difference spectrum from slow phase amplitudes; Panel IV. Shows the kinetics of A → B → C from SVD analysis shown in Panel II.

FIG. 4.

TABLE I
Comparison of Static and Kinetic K_D^{APP} s For Binding of Substrates and Products to CYP101 and CYP2B4

Experiments with C334ACYP101 were performed at 10-13°C jump, and those with CYP2B4 were at 18-20°C jump. Reaction medium for Cyp101 was 0.05 M K-PO4 buffer + 0.1 M KCl, and for CYP2B4 as described in the legend of Fig. 3.

	K_D^{APP} uM		
	Static	Transient	Kinetic
Cam-CYP101	4.8 ± 0.8	5 ± 0.5	6.2 ± 0.9
2 Adone-CYP101	2.9 ± 0.2	6 ± 2	2.7 ± 1.6
OHAdone-CYP101	326 ± 39	56 ± 14	42 ± 1.5
BNZ-CYP2B4	61 ± 4	ND	68 ± 10
DesBNZ-CYP2B4	701 ± 150	72 ± 27	63 ± 23

The Effects of 5exo OH Camphor on the Apparent Equilibrium Constants for Binding of Camphor and the Product to C334A CYP101

TABLE II

The reaction medium consisted of 0.05M K-PO₄ buffer pH 7.4, 0.125M KCl, 5% glycerol, 0.5 to 0.7uM C334A CYP101. The temperature was 24°C. Procedural details for performing spectral titrations by a semi micro technique, using low noise, high sensitivity dual wavelength instrument, fitted with narrow band filters, have been previously described [26]

OHcam uM	K ₁	K ₂	K ₃ uM ⁻¹	K ₄	K ₂ K ₃	K ₁ K ₄
38	.669 ± .064	.039 ± .002	.211 ± .03	.012 ± .001	.0083	.0080
66	.633 ± .029	.030 ± .004	.164 ± .024	.0078 ± .0005	.0047	.0049
100	.672 ± .018	.034 ± .002	.122 ± .011	.0063 ± .0004	.0042	.0043
133	.643 ± .013	.034 ± .002	.072 ± .008	.0039 ± .0003	.0025	.0024