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MOLECULAR BASIS OF P450 INHIBITION AND ACTIVATION

Implications for Drug Development and Drug Therapy

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ABSTRACT:

Three-dimensional homology models of cytochromes P450 (P450) 2B1 and 450 3A4 have been utilized along with site-directed mutagenesis to elucidate the molecular determinants of substrate specificity. Most of the key residues identified in 2B enzymes fall within five substrate recognition sites (SRSs) and have counterparts in bacterial P450 residues that regulate substrate binding or access. Docking of inhibitors into 2B models has provided a plausible explanation for changes in susceptibility to mechanism-based inactivation that accompany particular amino acid side-chain replacements. These studies provide a basis for predicting drug interactions due to P450 inhibition and for rational inhibitor design. In addition, the location of P450 3A4 residues capable of influencing homotropic stimulation by substrates and heterotropic stimulation by flavonoids has been identified. Steroid hydroxylation by the wild-type enzyme exhibits sigmoidal kinetics, indicative of positive cooperativity. Based on the 3A4 model and single-site mutants, a double mutant in SRS-2 has been constructed that exhibits normal Michaelis-Menten kinetics. Results of modeling and mutagenesis studies suggest that the substrate and effector bind at adjacent sites within a single large cavity in P450 3A4. A thorough understanding of the location and structural requirements of the substrate-binding and effector sites in cytochrome P450 3A4 should prove valuable in rationalizing and predicting interactions among the multitude of drugs and other compounds that bind to the enzyme.

There is considerable interest in the function of cytochromes P450 (P450)\(^1\) because of their involvement in drug metabolism as well as carcinogen bioactivation. Based on their structure, P450 enzymes are classified into gene families and subfamilies, with members of the same family exhibiting at least 40% amino acid sequence identity, and the members of the same subfamily >55% identity (Nelson et al., 1996). The main xenobiotic metabolizing enzymes belong to families 1 through 4 (Wrighton and Stevens, 1992). Many forms of P450 display broad substrate specificities, but individual isoforms often exhibit strict regio- and stereospecificity toward a particular compound. The elucidation of the structural basis for such specificity is of great importance in understanding enzyme function and mechanism. It may also help to predict the possible metabolic fate of drugs and carcinogens, as well as provide a foundation for the rational design of drugs and inhibitors.

In recent years, site-directed mutagenesis has become an important tool to study the structure-function relationships of mammalian cytochromes P450. The concept of substrate recognition sites (SRSs) was introduced by Gotoh (Gotoh, 1992) for the P450 2 family, based on the alignment of mammalian P450s with a bacterial enzyme, P450cam. This was the only enzyme at the time for which the crystal structure was solved. The SRS concept has provided an excellent guide in exploring the basis for P450 specificity, and a number of key amino acid residues responsible for substrate specificity in various mammalian P450s have been determined (von Wachenfeldt and Johnson, 1995). However, further advances in understanding enzyme function require information about the three-dimensional (3D) structure. To date, four bacterial P450 structures solved by X-ray crystallography have been published: namely, P450cam (Poulos et al., 1985 and 1987), P450 BM-3 (Ravichandran et al., 1993), P450terp (Hasemann et al., 1994) and P450eryF (Cupp-Vickery and Poulos, 1995). Therefore, molecular models of various mammalian enzymes have been constructed in order to explain substrate specificity and to relate enzyme function to structure. However, because of inaccuracies inherent in homology modeling, all modeling predictions must be verified experimentally. A recent review summarizes the state of the art in the field of homology modeling with an emphasis on the utilization of models in conjunction with site-directed mutagenesis studies to investigate mammalian P450s (Szklarz and Halpert, 1997). This article will describe our recent studies in which models of cytochromes P450 2B1 and 3A4 have been utilized to study enzyme function. The models have been used to identify key residues, to explain changes in regio- and stereospecificity of substrate oxidation upon site-directed mutagenesis, and to aid in the analysis of P450 inhibition and activation.
Homology Modeling of Mammalian Cytochromes P450

Homology Modeling Methods. In homology modeling, a 3D model of the protein is constructed based on its amino acid sequence and the crystal structure of one or more reference proteins. The model can be built using molecular replacement or consensus methods. In both methods, the first step involves a sequence alignment between the protein to be modeled and the template(s). In regions of low homology, secondary structure predictions, as well as additional information, such as site-directed mutagenesis data (Szklarz et al., 1995), can be invaluable. In the next step, the structurally conserved regions (SCRs) of the modeled protein are determined. When the model is built using molecular replacement methods, the coordinates for SCRs are copied directly from those of the reference protein(s). The coordinates for the variable regions, such as loops, are calculated or obtained from those for the similar loops in known protein structures. The initial model is then refined using minimization and molecular dynamics methods.

A consensus method, in contrast to the molecular replacement method described above, is based on distance geometry calculations and requires at least two reference proteins as templates. The first step in modeling also involves a sequence alignment and the determination of SCRs, but the coordinates of the model are weighted averages of the coordinates of all reference proteins within SCRs. The coordinates for loops are calculated simultaneously, and more than one model structure can be obtained.

Limitations of Homology Modeling. The basic assumption in homology modeling is that a modeled protein resembles the structure(s) used as templates; therefore, the choice of the template(s) is of crucial importance. The final 3D homology structure is highly dependent upon the modeling procedure and is influenced by a number of factors. The most important of them is sequence alignment, which may lead to differences in the location of some residues and thus result in different models. For example, in P450 2B1 models, the identity of active site residues depended upon the alignment (Szklarz et al., 1994). Thus the accuracy of the alignment is of crucial importance for the final structure. Another important factor is the choice of the modeling method. In molecular replacement methods, the coordinates of the SCRs are identical to those of a given reference protein, while in consensus models, they are averages of those of several templates. In the case of cytochromes P450, a model based on structures of several known enzymes should be more accurate than one based on the crystal structure of only a single protein, especially in view of the low sequence identity between mammalian and bacterial P450s. Moreover, the choice of coordinates for loops can alter the location of key residues, as shown in the case of P450 2B1 models (Szklarz et al., 1994).

The geometry of the final model is also dependent upon the forcefield used. A forcefield contains atomic parameters and energy terms utilized to calculate the energy of the molecule, and these may be different in different forcefields. A variety of forcefields have been developed to model proteins, and of these AMBER and CVFF have been used successfully in P450 modeling as well as molecular dynamics studies of crystallized P450s (Paulsen et al., 1996). Additional parameters are required in order to describe the heme moiety, such as those to be used with the CVFF forcefield (Paulsen and Ornstein, 1991 and 1992). Finally, the choice of the refinement method, such as different minimization algorithms, or final minimization with or without water, may also influence the 3D structure of the model.

Docking of Substrates/Inhibitors Into the Active Site of P450 Models. Docking of enzyme substrates or inhibitors into the active site of the homology model can help to explain enzyme-substrate interactions as well as the role of particular residues in catalysis. An important issue is the orientation of the substrate bound in the active site. Several choices are possible: (1) docking a compound based solely on steric considerations, (2) docking a compound based on steric considerations but orienting the site of metabolism toward heme and ferryl oxygen, and (3) docking a compound in a reactive (productive) binding orientation. The first choice can be appropriate for the docking of competitive inhibitors that are not metabolized by the enzyme. It may also reflect the preferred binding orientation of the substrate in the initial enzyme-substrate complex. Approach 2, based on the orientation of camphor in the crystallized P450cam, has frequently been used in docking P450 substrates. That orientation may also reflect the enzyme-substrate complex. In contrast, approach 3 represents an orientation of the substrate that is necessary for the first oxidative event in the P450 catalytic cycle. Thus the substrate is oriented in the active site to allow for the initial hydrogen or electron abstraction. The latter approach has been successfully utilized to interpret changes in regiospecificity of substrate oxidation and susceptibility to inactivation upon residue replacement by site-directed mutagenesis (e.g. Szklarz et al., 1995; Kent et al., 1997; Kobayashi et al., 1998). Additional methods, such as molecular dynamics and evaluation of enzyme-substrate interaction energies, may further increase our understanding of P450 catalysis and the motion of the substrate in the active site.

Application of Homology Models to Study P450 Function

Identification of Key Amino Acid Residues. With a 3D P450 model, the location of amino acid residues of interest can readily be visualized. Key residues should be present in the active site and be able to interact with a substrate. Docking of the substrate in the active site of the model should also make it possible to determine the prevalent enzyme-substrate interactions. Moreover, the model should be in agreement with experimental results.

In a consensus model of P450 2B1 (Szklarz et al., 1995), key residues 114, 206, 209, 290, 302, 363, 367, 477, 478, and 480 constituted part of the active site, while other residues studied were farther from the active site, consistent with all-site-directed mutagenesis data for cytochromes P450 2B. Moreover, key amino acids were shown to be able to interact with the substrate androstenedione when it was docked in a 16α- or 16β-binding orientation. The analysis of enzyme-substrate interactions indicated that hydrophobic interactions are mainly responsible for the binding of steroids in P450 2B1 (Szklarz et al., 1995). Generally, the identity of residues that contact the substrate depends upon the structure of the compound and on the particular orientation it may assume in the active site. Recent studies on the stoichiometry of 7-ethoxycoumarin metabolism by P450 2B1 wild-type and five site-directed mutants provided firm biochemical evidence that residues 206, 363, and 478 comprise part of the substrate binding site of the enzyme and are able to interact with this substrate (Fang et al., 1997).

Docking of a substrate into the active site of the enzyme model also reveals previously unidentified residues that may affect enzymatic activity. These predictions have to be corroborated experimentally by site-directed mutagenesis. The experiment thus provides a means to verify the model and may lead to the construction of a more accurate P450 model. A model of P450 2B1 based on the crystal structure of P450cam (Szklarz et al., 1994) suggested that, in addition to key residues known at the time, amino acids such as Tyr-111, Leu-209, Ile-477, and Ile-480 may interact with androstenedione and thus affect activity. Since these residues had not been studied previously, single mutants at these and other positions were constructed by site-directed mutagenesis, expressed in Escherichia coli, and their activities eval-
is then compared with that of the wild-type enzyme. In general, the product. The binding of the substrate in the active site of the mutant and docking of the substrate in an orientation leading to the expected replacement in the model of a given amino acid to mimic the mutant, structural basis of enzyme function. The usual procedure involves the use of 3D molecular models, which may also give some insights into stereospecificity of substrate oxidation upon residue replacement is amino acid residues, but the interpretation of changes in regio- and site-directed mutagenesis can pinpoint key residues essential for progesterone oxidation,2 as predicted by the model.

Figure 1 shows the key residues of P450 3A4 identified to date. Interpreting Alterations in Substrate Specificity Upon Site-Directed Mutagenesis. Site-directed mutagenesis can pinpoint key amino acid residues, but the interpretation of changes in regio- and stereospecificity of substrate oxidation upon residue replacement is uncertain. However, these difficulties can be overcome with the help of 3D molecular models, which may also give some insights into structural basis of enzyme function. The usual procedure involves the replacement in the model of a given amino acid to mimic the mutant, and docking of the substrate in an orientation leading to the expected product. The binding of the substrate in the active site of the mutant is then compared with that of the wild-type enzyme. In general, the loss of activity upon mutation of a key residue can be a result of (1) van der Waals overlaps that hinder substrate binding, or (2) increased substrate mobility when enzyme-substrate interactions become too weak. In contrast, an increase in activity can be related to the stabilization of a given binding orientation through an increase in van der Waals contacts and decreased substrate mobility. Changes in substrate mobility may alter the coupling efficiency of the mutant.3

In our recent studies on the metabolism of 7-alkoxycoumarins by P450 2B1 wild-type and mutant enzymes, quantitative and qualitative changes in activity were observed (Kobayashi et al., 1998). Docking of 7-ethoxycoumarin in an orientation leading to O-dealkylation showed that this substrate fits well into the 2B1 active site, with no van der Waals overlaps, and can interact with residues 363 and 478 (fig. 2). However, an increased length of the alkyl chain leads to overlaps between the substrate and the enzyme, mainly involving those two residues. This interpretation is consistent with the experimentally observed decrease in dealkylation rates. In contrast, with V363A, the highest rate of product formation was observed with 7-butoxycoumarin. This compound can be docked into the active site of the mutant in an orientation allowing its O-dealkylation (fig. 3). The Ala side chain is small enough to permit bending of the butoxy chain, which is not possible in the wild-type enzyme because of van der Waals overlaps with Val-363. At the same time, the presence of Ala at position 363 leads to increased mobility of smaller alkoxycoumarins, such as 7-ethoxy- and 7-propoxycoumarin, consistent with increased O-dealkylase activities, compared with the wild-type enzyme. In contrast to V363A, the presence of a larger Leu at position 363 does not allow for binding of 7-propoxy or 7-butoxycoumarin in an orientation leading to O-dealkylation because of significant van der Waals overlaps. However, the presence of this large residue enables binding of 7-butoxycoumarin in alternate orientations resulting in ω-1 hydroxylation of the alkoxy chain, in agreement with experimental data. Figure 4 shows 7-butoxycoumarin docked into the active site of the P450 2B1 V363L mutant in an orientation allowing for its (ω-1) hydroxylation (Kobayashi et al., 1998).

Utilization of Models in Inhibition Studies. Homology models can be of great help in the analysis of P450 inhibition or inactivation. The models can be used to explain changes in enzyme inhibition upon residue replacement, provide additional information on the mecha-

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2 Fabienne Roussel, unpublished data.

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nisms of P450 inhibition or inactivation, and aid in the design of better inhibitors.

A model of P450 2B1 has been utilized to explain changes in enzyme inactivation by N-benzyl-1-aminobenzotriazole (BBT) upon the mutation of Gly-478 to Ala (Kent et al., 1997). This single substitution prevents enzyme inactivation by the compound. BBT can be oxidized either at the 1-amino nitrogen, which results in the generation of products that inactivate the enzyme, or at the 7-benzyl carbon, which leads to the formation of a stable metabolite. Molecular modeling studies of BBT bound in the active site of P450 2B1 suggested that a mutation of Gly-478 to Ala would result in steric hindrance and thereby suppress oxidation of BBT at N-1 (fig. 5). When BBT was oriented in the 2B1 active site such that oxidation at the carbon atom could occur, no steric overlap between Ala-478 and the substrate was observed. Consequently, this orientation would be preferred by the mutant. These findings indicate that the substitution of Gly-478 with Ala altered the binding of BBT such that inactivating metabolites were no longer generated.

Homology models have not yet been used for inhibitor or inactivator design. The rational design of inhibitors relies so far mainly on small molecule models or a pharmacophore approach. However, with the increasing accuracy of homology models and their successful use for interpretation of P450 inhibition and inactivation, the stage is set for the utilization of the models for inhibitor or drug design. We can expect progress in this area in the near future.

Stimulation of P450 3A4 by α-Naphthoflavone. P450 3A4 is known to catalyze the oxidation of a number of substrates in a cooperative manner. Metabolic activities of the enzyme are also modulated by naturally occurring phenolic compounds known as flavonoids, resulting in either stimulation or inhibition of activity. An allosteric mechanism is usually invoked to explain cooperativity. Recent studies in our laboratory provided the first evidence for the location of residues that influence flavonoid stimulation of P450 3A4 (Harlow and Halpert, 1997; He et al., 1997). These residues are proposed to constitute part of the active site of the enzyme (Szklarz and Halpert, 1997; He et al., 1997). In addition to bound progesterone, up to two molecules of α-naphthoflavone (α-NF) can be fitted in the model (fig. 6). Instead of α-NF, the active site of P450 3A4 is able to accommodate a second molecule of progesterone, which may explain the homotropic enzyme stimulation observed with steroids (Harlow and Halpert, 1997).

The hypothesis that the effector binds in the active site along with the substrate has been further supported by our most recent studies (Harlow and Halpert, 1998). Residues Leu-211 and Asp-214, which were predicted to constitute a portion of the effector binding site, were replaced with the larger Phe and Glu, respectively, to mimic the action of the effector by reducing the size of the active site. The L211F/ D214E double mutant displayed an increased rate of testosterone and progesterone 6β-hydroxylation at low substrate concentrations and a decreased level of heterotropic stimulation elicited by α-NF. Kinetic analyses of the double mutant revealed the absence of homotropic cooperativity with either steroid substrate. At low substrate concen-
trations the steroid 6β-hydroxylase activity of the wild-type enzyme was stimulated by a second steroid, whereas L211F/D214E displayed simple substrate inhibition. Moreover, based on spectral binding studies, testosterone binding by the wild-type enzyme displayed hyperbolic cooperativity, whereas substrate binding by L211F/D214E displayed hyperbolic behavior.

As illustrated above, the 3D enzyme model suggested a plausible explanation of P450 3A4 activation by flavonoids and the choice of residues to be targeted for site-directed mutagenesis. Experimental evidence supported the initial idea and gave additional insight into the mechanism of enzyme activation. However, more questions concerning that mechanism arise. If both substrate and effector are present in the active site and can interact with each other, it may be difficult to distinguish residues that directly affect effector binding from residues that indirectly affect effector action. For example, alteration of a residue in the substrate binding site can change the binding orientation of the substrate. This, in turn, can affect substrate-effector interactions and lead to apparent altered response to the effector. Further analysis of 3A4 mutants using additional substrates and effectors will be required in order to fully map the effector site. Detailed knowledge of the structural requirements of the substrate binding and effector sites should allow prediction of interactions among compounds that bind to P450 3A4.

Conclusions

The combination of homology modeling and site-directed mutagenesis has provided an important insight into P450 structure-function relationships. The simplest application of the homology models involves the determination of the “hot spots,” or key residues. The location of key residues in the active site and their interactions with docked enzyme substrates can be readily ascertained. Moreover, additional residues that may be important for enzymatic activity can be pinpointed. However, we should keep in mind that the identity of residues able to contact the substrate depends upon the structure of the compound and the specific binding orientations it assumes in the active site. P450 models have been successfully utilized to explain changes in regio- and stereospecificity of substrate oxidation, as well as alterations in susceptibility toward inactivation upon site-directed mutagenesis. These changes can be related to the removal or appearance of van der Waals overlaps in the mutant proteins and changes in substrate/inhibitor mobility. With P450 models, we can postulate plausible mechanisms for enzyme catalysis, inhibition, and activation, based on the 3D structure. In the last case, the model of P450 3A4 suggested a plausible hypothesis concerning the location of the effector site, which has been supported experimentally. In summary, homology modeling allows for a mechanistic interpretation of various aspects of P450 function and, in conjunction with experimental methods, is likely to continue as an important tool in studies of mammalian P450s. We can expect further development of homology modeling methods, as well as methods for structural verification of the models. The improvement of forcefields for protein modeling and generation of better parameters for heme will allow for the introduction of molecular dynamics methods to analyze substrate or inhibitor motion in the active site and to calculate binding free energies, as has been done for P450cam (Paulsen and Ornstein, 1992 and 1996, Paulsen et al., 1993). When the structure of a eukaryotic enzyme is solved, the methodology established and verified in studies utilizing homology models can be easily adapted and refined for use with the “real” structures.

References


