

Clues from Three-Dimensional Structure Analysis and Molecular Modelling: New Insights into Cytochrome P450 Mechanisms and Functions

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Abstract

Cytochrome P450 is a focus of attention as it comprises one of the largest superfamilies of enzyme proteins. Metabolization of many drugs is affected by cytochrome P450. It is an attractive drug target, e.g., cytochrome P450s of *Mycobacterium tuberculosis* are promising targets in the fight against tuberculosis. The structure provides new insights for investigation of structure/mechanism of cytochrome P450, and for rational design of inhibitor molecules. We will illustrate how biocomputing and bioinformatical techniques reveal details, functions and further secrets of this exciting molecule. Molecular modelling along with site-directed mutagenesis of P450 2B1 elucidated the molecular determinants of substrate specificity. Regioselectivity of progesterone hydroxylation by cytochrome P450 2B1 was reengineered based on the X-ray structure of cytochrome 2C5. Docking approaches rationalized the regioselectivity of the reengineered cytochrome P450 2B1. Furthermore, by methods of molecular dynamic simulations, routes were identified by which substrates may enter into and products exit from the active site of cytochrome P450.

Introduction

Cytochrome P450 enzymes¹⁻⁵ form an ubiquitous heme protein monooxygenase family (EC: 1.14.14.1). They play an important role in the synthesis and degradation of many physiologically important compounds such as steroid hormones, cholesterol, bile acids and in the detoxification of xenobiotics in many species of microorganisms, plants and animals.

P450 are of great medical relevance: Mutations in P450 genes are triggers of human diseases such as primary congenital glaucoma and there are evidences for associations between cytochrome P450 enzyme-polymorphism and cancer. Some P450 enzymes are able to activate procarcinogens to genotoxic intermediates. They play a major role in drug-metabolism, for example the P450 3A family of enzymes are able to metabolize the majority of commercially available drugs such as Codeine (narcotic), Diazepam (Valium), Erythromycin (antibiotic). Drug metabolism polymorphism or interactions with other drugs can cause severe sideeffects in patients.

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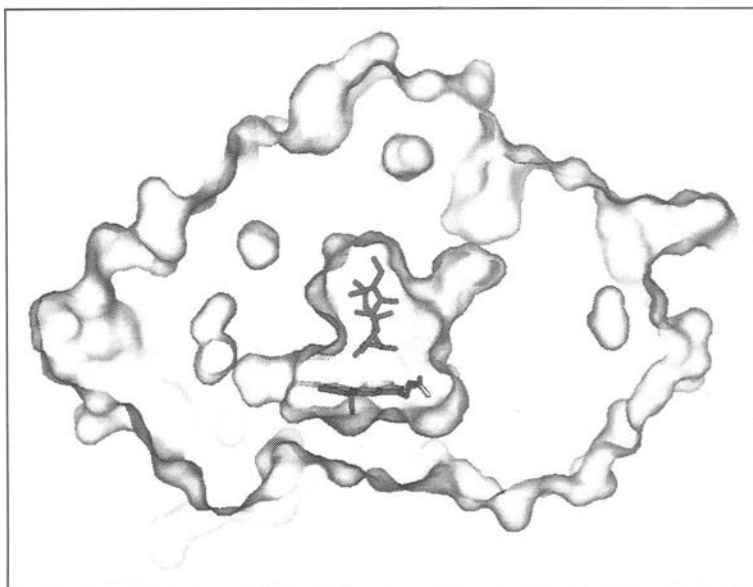


Figure 1. The active site molecular surface and the outer molecular surface of cytochrome P450eryF (CYP107A1) are not connected with each other. A substrate molecule, 6-deoxyerythronolide, is shown in the active site above the heme. The molecular surface was computed with the PyMOL program.⁵³

P450 are heme-thiolate containing proteins where the ligand of the heme iron is delivered by a cysteine residue in a highly conserved region of the enzyme. The active site is buried at the center of the enzyme (Fig. 1). They are named P450 for the absorption band at 450 nm of their carbon-monoxide-bound form. The reactions carried out by cytochrome P450 molecules are very diverse and include hydroxylation, N-, O- and S-dealkylation and oxidation of heteroatoms.

According to their sequence similarity P450 enzymes are subdivided into families (sequence identity greater than 40%) and subfamilies (sequence identity greater than 55%). In humans 57 CYP genes are sequenced (and 58 pseudogenes) which are subdivided into 18 families and 43 subfamilies.

In prokaryotes P450 are soluble proteins whereas in eukaryotes P450 are usually membrane-associated within the inner mitochondrial membrane or endoplasmic reticulum.

Because of their physiological importance and medical relevance the P450 enzymes are an emerging field of research. Major unresolved issues are structurefunction relationships such as the understanding of substrate specificity, the catalytic mechanism of multi-step reactions, the dynamical properties that allow substrates to enter the active site and products to leave the active site or the identification of essential determinants of drug metabolism or tolerance. In the following paragraphs methods of computational biology are presented which aid our understanding of this interesting enzyme. However the presented methods are applicable to a variety of biomolecules.

Modelling

The gap between the high number of known protein sequences and the only limited available 3-dimensional protein-structures is increasing rapidly. Molecular modelling techniques are valuable tools to fill this gap.^{6,7} In the field of cytochrome P450 research this technique is of high interest. Up to now more than 3700 cytochrom P450 (different named) sequences of different species are known, the determination of all these protein structures is a tedious work, because crystallization of some P450 enzymes, especially of the membrane-associated ones is

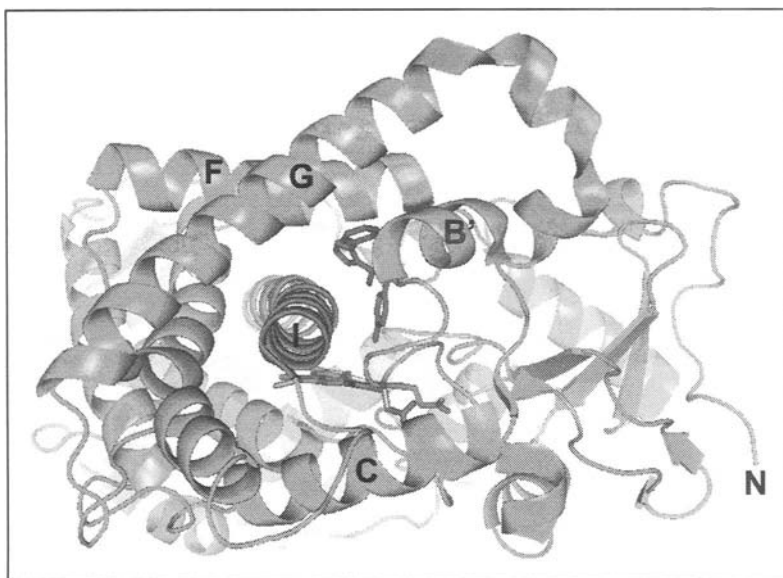


Figure 2. Structure of cytochrome P450 2C5 bound to a substrate. A substrate molecule, 4-methyl-*N*-methyl-*N*-(2-phenyl-2*H*-pyrazol-3-yl)benzenesulfonamide, is shown in the active site above the heme. The principal helices and the NH₂-terminal are labelled. P450 2C5 is membrane-associated by its N-terminal tip.

difficult. So far more than 120 P450s structures are in the brookhaven protein database. Among them two mammalian P450 enzymes (2C5 and 2B4), the first two structures of membrane-associated P450s^{8,9} which were solved recently (Fig. 2). The overall fold of P450s is conserved despite their low sequence identity (as low as 10%).

Homology Modelling

Although the amino acid sequence must finally determine a protein's three dimensional structure and despite of intensive research the development of an algorithm to determine the accurate 3D-structure from amino acid sequence has yet not been achieved.

The most promising approach is the modelling based on structures of homologue proteins. The progress within the field of protein structure prediction by NMR or X-ray crystallography enhances the probability to find a homologue protein of which the 3-dimensional structure is known.

Experimentally determined protein and DNA/RNA structures can be found in the brookhaven protein databank (<http://www.pdb.org>). To compute a good model of the unknown structure the protein homologue should have a sequence identity of more than 30%. The prerequisite of a good model is the generation of an alignment between sequence of the experimentally determined template structure and the protein which has to be modeled. To generate a satisfactory alignment, experimental data should also be taken into account such as site-directed mutagenesis data of residues known to be involved in substrate binding, binding to redox partners; antibody recognition sequences which indicate whether certain residues are located on the surface of a protein.

The prediction of the secondary structure elements is usually reasonable accurate (~75%) for a three state prediction using sequence alignments whereas the prediction within the loop-region and of dynamic sidechains could be problematic. In order to refine the structure the model should be energy minimized using molecular mechanics.

There are comparative modelling servers on the web such as SWISS-MODEL,¹⁰ CPHmodels.¹¹ Software packages for detailed protein structure analysis include Modeller,¹² WHATIF,¹³ Insight¹⁴ and Sybyl.¹⁵

The automated SWISS-MODEL software works in several steps as follows:

- superposition of homologue 3D-structures
- generation of a multiple alignment
- calculation of peptidchain from the averaged coordinates
- reconstruction of loops based on a coordinate library
- addition and correction of sidechains
- validation of the structure (checking of stereochemical quality)

Substrate specificity and type of reactions catalyzed are governed by less conserved regions of P450s and are therefore not well understood. In this context the prediction of the regioselectivity of an enzymatic reaction is of particular interest. In a regioselective reaction one possible product out of two or more is formed preferentially (it is often the case that addition and elimination reactions may, in principle, proceed to more than one product—these are often isomers of each other).

In the following approach based on the 3D model of P450 2B1 it was shown that active site residues are responsible for regioselectivity.¹⁶ P450 2B1 belongs to the 2B subfamily comprising enzymes with a broad range of substrates, including drugs, environmental carcinogens and steroids. A model of 2B1 was built using the X-ray structure of P450 2C5 as a template. The location of the active site residues within the 3D P450 model of 2B1 can be visualized. The active site residues of 2B1 could be deduced from the 2C5 structure and were verified experimentally by site-directed mutagenesis. 2B1 has progesterone 16 α -hydroxylase activity whereas 2C5 has progesterone 21-hydroxylation activity. By replacing seven active site residues of 2B1 by the corresponding active site residues of 2C5 a novel progesterone 21-hydroxylation activity was conferred to 2B1. The mutated 2B1 showed 80% regioselectivity for progesterone 21-hydroxylation.

Threading

In case that there is no homologue protein or the sequence identity is very low (e.g., a novel cytochrome from Archaeobacteria) the threading approach is a good alternative.¹⁷⁻¹⁹ The starting point of a threading approach are protein folds. It is known that proteins having no sequence similarity can have similar 3D-structures. Examples are actin and hexokinase which exhibit the same Ribonuclease H-like folding topology,²⁰ despite having different sequences. In the course of evolution only a limited number of protein folds emerged (~1000).²¹ The sequence for which a prediction is required is threaded onto all known protein folds. As current databases have already covered a large part of protein folds generally used in nature, this approach is successful. Useful Webservers include: genThreader^{22,23} and 3D-PSSM.²⁴

Ab Initio Modelling

Even novel protein folds could be predicted by ab initio modelling. Ab initio structure prediction requires only the sequence of a protein to generate a 3-dimensional model. This approach is computationally demanding, there are several algorithms which for example rely on physicochemical energetics, or on methods that use predicted secondary structure in combination with distance constraints.²⁵⁻²⁷ The catalytic arrangement of the cytochrome P450 center provides useful distance constraints²⁸ and can be confirmed by conserved residues in these positions. This technique could be used to improve ab initio models of cytochromes P450 with completely novel folds.

Assessing Tertiary Structure Prediction

No matter which method was used to establish a structural model, it is essential to assess the validity of the generated model by checking how well the new model conforms to protein

stereochemical quality. There is some agreement about which measurements are good indicators of stereochemical quality; these include planarity; chirality; phi/psi preferences; chi angles; nonbonded contact distances; unsatisfied donors and acceptors. The ProCheck package comprises a number of complementary procedures for evaluating protein structures and identifies regions of the modeled protein which may require further refinement. The following web servers are very useful: Whatcheck,²⁹ ProCheck.³⁰ These tools can be used to check the model quality in the study on cytochrome regioselectivity as residue positions are critical here.

Modelling of Protein-Ligand Complexes

An important and useful area of molecular modelling is the modelling of protein-ligand complexes. Comparative methods and docking approaches can be applied. Protein docking methods help understanding the mechanism of molecular interaction and are also useful in the development of novel pharmaceutical agents because it helps to screen out unfavorable ligands at an early stage.

A docking procedure³¹⁻³⁴ can be subdivided into three processes: identification of the binding site, sampling of possible ligand orientations and positions in the binding site and scoring of the possible sampled solutions. The rigid-body model is historically the first docking approach where the flexibility of the interaction partners is not considered. However most of the current rigid-body methods could address ligand flexibility by accounting an ensemble of ligand conformations or allowing some intermolecular interactions.

Software programs carrying out docking calculations are for example DOCK,³⁵ GRID,^{36,37} AutoDock,³⁸ FTDock.³⁹ The DOCK program suite is one of the oldest and best known ligand-protein docking programs. In newer versions of DOCK the ligand flexibility is incorporated.

In a first step a 'negative image' of the binding site is constructed by overlapping spheres of varying radii. In a second step the ligand atoms are matched to these sphere centres to position the molecule within the binding site. GRID allows the identification of ligand binding sites of a protein. The protein is put into a 3-dimensional grid, at each point of the grid the molecular mechanics interaction energy between the protein and a series of probe molecules is computed. Probe molecules are a series of chemical functional groups such as phosphate, methyl, hydroxyl, carboxyl. AutoDock identifies the binding site by a genetic algorithm search. Estimated binding free energies can also be computed.

Many computational methods used for modelling of protein-ligand complexes can also be applied to model protein-protein-complexes. For example the DOCK program has been used to model protein-protein complexes. To accelerate the search for the best geometric fit between two proteins, Fourier transform methods are applied, a shape recognition algorithm in which molecules are discretised onto grid. All possible translations of the molecules are scanned by superposition of the grid points. The program FTDock is based on Fourier transform procedure and a method to determine electrostatic complementarity.

Docking of inhibitors or substrate molecules into the active site of P450 aid understanding the key enzyme-substrate-interactions as well as the role of particular residues in catalysis. Steric considerations as well as orientating the site of metabolism toward heme and ferryl oxygen has to be taken into account.

In the following we describe one particular drug target and how docking methods can help in the development of new therapeutics. Cytochrom P450 (CYP121) of *Mycobacterium tuberculosis* (Mtb) is a promising drug target to combat the multidrug-resistant strains of *Mycobacterium tuberculosis*.⁴⁰ The genome of *Mycobacterium tuberculosis* reveals an exceptional high number of 20 different encoded P450—the highest number in any bacterium. Several azole drugs which are known inhibitors of cytochrome P450 have been shown to have potent antimycobacterial activity, especially high affinity for CYP121. But many azol anti-fungal drugs show cross-reactivity with human P450 isoforms. Docking studies should help to rationalize the key determinant that dictate tight CYP121-drug interactions in order to design

novel azole-based drugs that have high selectivity for CYP121. First docking studies could rationalize binding of certain azole drugs, e.g., miconazole whereas bulkier azole drugs failed to dock into the active site.

Molecular Dynamic Simulation

Molecular dynamic simulations^{41,42} have become a standard tool for the investigation of biomolecules. Simulations give insights into the natural dynamics of biomolecules and thereby aid our understanding of biochemical processes. Especially in protein-ligand interaction the flexibility of the interaction partners is of great importance. In order to fit in the active site of the protein the ligand has to adopt a certain 3-dimensional conformation. However, a protein is despite being a rigid entity not completely stiff. Especially the positions of the amino acid side chains can be very flexible and change their positions while the protein is „at work“. For lots of enzymatic processes and binding of ligands this flexibility is essential for a protein's specific function. These subtle movements are the basis for all metabolic processes and thus are a key process of what life, a living protein „breathes“. Even protein domains can change their position relatively to each other. The experimental approaches to study biomolecular dynamics are still limited. With continuing advances in the speed of computers and the methodology of molecular dynamic simulations the time scales that are becoming available are making it possible to study phenomena of biological interest in real time.

For the optimization of crystal structures and NMR structures molecular dynamic simulations are applied as a standard tool. The principle of the molecular dynamic simulation is to record the movement of atoms under the influence of a selected force field (OPLS,⁴³ CHARMM,⁴⁴ GROMOS96⁴⁵ or AMBER⁴⁶). It is assumed that the movement of the atoms follows classical mechanics. The theoretical basis of a molecular dynamic simulation is Newton's equation of motion ($F_i(t) = m_i \cdot a_i(t)$, where F is the force acting on atom i at time t , m is the mass of atom i , a the acceleration acting on atom i at time t), which is solved numerically for each atom. Forces between the atoms are neglected during the molecular dynamic simulation. The initial point of a simulation is a starting structure, which is in most cases an experimentally determined structure. To simulate the dynamics of a whole protein it is solvated with water molecules. At the start of a simulation a velocity is assigned to each atom, which corresponds to the selected temperature of the simulation. Newton's equation of motion is solved for each atom taking these initial values into account and the computed coordinates are saved periodically.

In the P450 research molecular dynamic simulations were applied to address the question how substrates enter or exit the isolated cavity of the enzyme. To allow substrate access and product exit the enzyme must undergo structural motions. The understanding of these motions would also help to explain why P450s have such a broad diversity of substrates and such a wide variation in degree of specificity. Furthermore the enzyme kinetic of P450 was shown to be influenced by protein dynamics. In the following we describe two molecular dynamics simulation methods elucidating potential ligand exit pathways in P450s. Steered molecular dynamic simulations (SMD)⁴⁷⁻⁴⁹ of testosterone exit suggested a functional role for the residues in the N-terminal portion of the cytochrome P450 2B1 I helix.⁵⁰ These data are confirmed by site-directed mutagenesis data within the I-helix as these alter the enzymatic activity of the enzyme. SMD is an extended MD simulation method mimicking the principle of the atomic force microscopy (AFM). In SMD simulations, time-dependent external forces are applied to the ligand to facilitate its dissociation from the protein by movement along a trajectory. Because position restraints are removed from the entire protein-ligand complex, the SMD simulation allows the protein to be repositioned in response to the accelerated dissociation process of the ligand.

By random expulsion molecular dynamic simulations (REMD)⁵¹ the substrate exit of different P450 enzymes could be identified. An investigation by REMD of mammalian cytochrome P450 showed that the substrate egress is different from that of soluble, bacterial P450.⁵² In REMD the probability of spontaneous substrate exit in the time range amenable

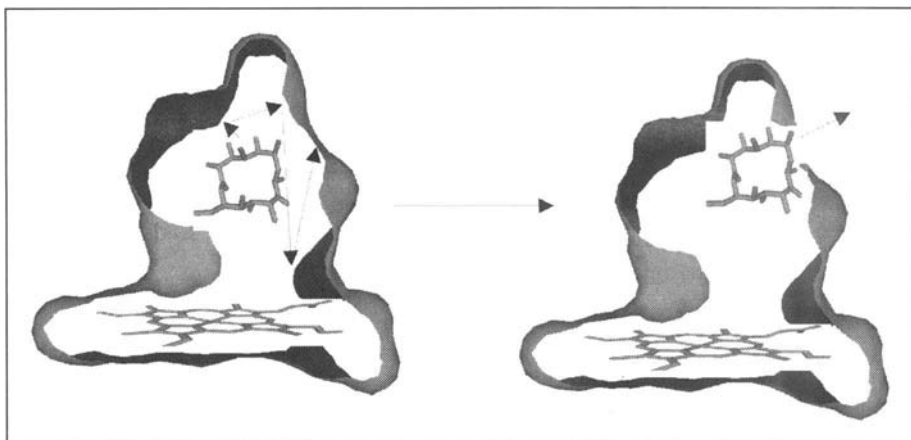


Figure 3. In REMD, routes by which a ligand can exit from an interior cavity in a protein are identified (here the active site of a cytochrome P450 with a substrate molecule above the heme is shown). An artificial randomly orientated force is applied to the center of mass of the ligand.

to molecular dynamic simulation is enhanced by an artificial force with random direction imposed upon the substrate in addition to the standard force field (Fig. 3). The direction of the additional force acting on the center of mass of the ligand is chosen randomly. The direction of the force is kept for a chosen number of time steps, N . During this time period, a specified distance r_{\min} should be covered by the substrate molecule: the substrate is required to travel at an average threshold velocity v during the time period $N\Delta t$, where Δt is the time step of the molecular dynamic simulation. If the substrate encounters relatively rigid parts of the cavity its average velocity will fall below the preset threshold. In this case a new direction is chosen randomly and maintained, as long as the substrate moves in the new direction with an average velocity larger than the preset value. In this way, the substrate probes different regions of the protein during the simulation until it exits.

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