Protein dynamics and imidazole binding in cytochrome P450 enzymes

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Abstract

P450 (cytochrome P450) enzymes have major roles in the biosynthesis of endogenous factors such as steroids and eicosanoids, in the termination of the action of endogenous factors such as retinoic acid, in the metabolism of most drugs and xenobiotics and in the generation of toxic and carcinogenic products. Understanding the determinants of the substrate and inhibitor specificities of these enzymes is important for drug design. The crystallographic analysis of the deformability of two bacterial P450 active sites associated with the binding of azole (a class of inhibitors with an imidazole or triazole ring that co-ordinates to the haem iron) inhibitors described in the present study illustrates the importance of protein conformational malleability in the binding of imidazole derivatives.

Introduction

The three prevalent models of substrate binding to a protein are: (i) the original lock and key hypothesis in which a preformed active site conformation binds a preformed substrate conformer with little or no conformational adjustment [1], (ii) the induced fit paradigm, in which ligand binding triggers conformational changes in the receptor site that lead to appropriate binding [2], and (iii) the conformational ensemble view, in which the protein exists in an equilibrium of multiple conformers and ligand binding shifts the equilibrium towards the one appropriate for ligand binding [3]. The latter formalism can be extrapolated to envision a dynamic conformational continuum that allows considerable diversity in ligand binding.

P450 (cytochrome P450) enzymes fall into two broad classes, one of relatively substrate-specific enzymes that have defined physiological substrates, and a second of enzymes with broad specificities and relatively undefined substrates [4,5]. Examples of the first class are P450cam (CYP101) and lanosterol 14α-demethylase (CYP51), which specifically oxidize camphor and lanosterol respectively. Nevertheless, P450cam does have some breadth of substrate tolerance. Examples of the second class are CYP3A4 and CYP2C9, which are responsible for the bulk of human xenobiotic metabolism. An enzyme such as CYP3A4 that oxidizes substrates as structurally diverse as acetaminophen and cyclosporine A [3], and in some instances binds two substrates simultaneously [5], and does so without becoming completely uncoupled must be able to undergo significant structural accommodation.

The P450 enzymes are unusual in that their primary catalytic role is the activation of molecular oxygen to produce a highly reactive ferryl-oxidizing species {ferryl = the P450-oxidizing species, probably a [Porphyrin+ · FeIV=O]} [6,7]. The role of the protein once this is achieved, apart from binding a substrate near the ferryl species, appears to be negligible. The substrate oxidation regiospecificity is therefore largely determined by the properties of the ferryl species, the relative intrinsic reactivities of the different sites on the substrate and the relative accessibilities of these sites to the ferryl species. A close tailoring of the active site to the substrate, particularly in P450 enzymes with broad substrate specificities, is therefore not required for catalysis, although a dynamic ability to conform to the substrate can enhance substrate affinity and help to prevent the unproductive uncoupling of the enzyme to give hydrogen peroxide and/or water rather than substrate oxidation [8].

It is becoming evident that conformational mobility is a critical determinant of P450 substrate- and regio-specificity, emphatically so for enzymes that are primarily involved in xenobiotic metabolism but to some degree even for enzymes with well-defined natural substrates. Here, we illustrate the role of conformational mobility in the binding of azole (a class of inhibitors with an imidazole or triazole ring that co-ordinates to the haem iron) inhibitors to two bacterial P450 enzymes, CYP119, a thermophilic enzyme whose native substrate is unknown, and P450cam, a substrate-‘specific’ enzyme for which camphor is the natural substrate.

Two CYP119 conformations

CYP119 is a P450 from the thermophile Sulfolobus solfataricus [9]. Unlike most P450 enzymes, which ultimately derive electrons for the catalytic activation of molecular oxygen from NAD(P)H, the electrons for CYP119 derive from pyruvic acid [10,11]. The protein that transfers electrons to CYP119 is a ferredoxin, and thus is similar to the immediate electron donors of many bacterial P450 enzymes, but

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Abbreviations used: OFOR, 2-oxoacid:ferredoxin oxidoreductase; P450, cytochrome P450.
reduction of the ferredoxin is mediated by an OFOR (2-oxoacid:ferredoxin oxidoreductase) protein rather than by a flavin-containing ferredoxin reductase. Reconstitution of CYP119 with the OFOR/ferredoxin system from S. solfataricus provides a P450 catalytic system active at elevated temperatures [11].

The native substrate of CYP119 is not known, but in the presence of its native electron donor partners, the enzyme oxidizes lauric and other fatty acids primarily to the ω-1 hydroxylation products [12]. The crystal structures of CYP119 complexed with imidazole and phenylimidazole reveal two very different conformations of the enzyme [13]. In the imidazole complex, four residues that are part of the F-helix in the phenylimidazole complex unravel to provide closer contacts with the imidazole ring. The loss of favourable interactions upon unravelling of the terminus of the F-helix is compensated for by new interactions between the F/G loop and the I-helix. The conformation involved in catalytic turnover of fatty acids is not known: conceivably, both of the conformations seen in the imidazole and phenylimidazole complexes are catalytically active, or only one, or possibly a different conformation altogether.

Ligand DOCKing into P450cam and its L244A mutant

We have explored the utility of DOCK, a computer-based algorithm for the in silico docking of compound libraries into crystallographic active sites [14], for the prediction of P450 ligand and substrate specificities [15–19]. We have focused in these studies on P450cam, a protein for which multiple crystal structures are available and which is thought to have a relatively rigid active site. The initial docking studies were carried out according to a lock and key model in which preset substrate conformations were docked into a rigid crystallographic model of the P450cam active site. To test the ability of the approach to discriminate between active sites that only have small differences, we constructed a model of the P450cam L244A mutant by deleting the three terminal atoms of the leucine residue from the wild-type crystallographic active site. In our first study, a 20,000-compound subset of the ACD (Available Chemicals Directory) was docked into both the wild-type and L244A mutant models, and 11 compounds predicted to fit within both active sites and five predicted to fit only in the L244A site were selected and tested experimentally. The definition of a substrate was taken as an elevation of NADH consumption, a definition that incorporates both coupled and uncoupled turnovers. The results demonstrated that the algorithm could correctly identify substrates but did so more effectively if the parameter that defined the allowed contact distance between ligand and active site atoms was relaxed to allow a closer approach than is physically acceptable. This ‘fix’, in essence, allowed the active site to become slightly larger than in the crystal structure [15–17].

More recently, we examined the utility of a modified version of DOCK in the design of imidazole-based inhibitors of P450cam [19]. The DOCK algorithm was modified by the introduction of an energy penalty for a long iron–nitrogen bond distance, as the original program did not recognize the nitrogen–iron bond in the imidazole complex. Again, we compared the predicted binding of mono- and di-substituted imidazoles with the rigid wild-type active site and with the L244A model used in the earlier studies. Modified DOCK did well qualitatively, in that it correctly predicted that monosubstituted imidazoles would bind to both enzymes but dissubstituted imidazoles would only bind to the nominally larger L244A active site [19]. Quantitatively, however, the binding affinities of the dissubstituted ligands to the L244A mutant site were weaker than expected.

To address this puzzle, we have crystallized and determined the structure of the P450cam L244A mutant with and without an imidazole ligand, and have also determined the structure of the wild-type enzyme with imidazole bound in the active site as a reference point (A. Verras, A. Alian and P.R. Ortiz de Montellano, unpublished work). The crystal structures of the P450cam–2-phenylimidazole complex were determined some years ago [20]. Comparison of these structures immediately reveals that the L244A active site is smaller, not larger, due to an inward shift of the distal I-helix by over 1 Å (1 Å = 0.1 nm). Furthermore, the structure of the L244A complex with 1-methylimidazole demonstrated that the imidazoles bind very differently in the wild-type and mutant proteins. In the wild-type protein, as expected, the imidazole is co-ordinated to the haem iron and is essentially perpendicular to the haem plane (Figure 1). In the L244A–1-methylimidazole complex, however, the imidazole appears to remain co-ordinated to the haem iron, as judged from the expected spectroscopic shift, but the structure shows that the imidazole ring is tilted into a position almost parallel to the haem plane by interaction with Val247 of the inwardly shifted 1-helix (A. Verras, A. Alian and P.R. Ortiz de Montellano, unpublished work). This unusual co-ordination geometry readily explains the decreased affinity for imidazole observed in the L244A mutant relative to the wild-type enzyme.

The paradox, however, is that the L244A mutation allows the oxidation of larger substrates than is possible with the wild-type enzyme, and allows the binding of dissubstituted imidazoles despite the smaller size of the crystallographic active site! This paradox suggests that the L244A mutation, in effect, increases the malleability of the P450cam active site, so that the active site, although smaller in the crystal structures,

![Figure 1](image_url)
can more readily expand than the wild-type active site to accommodate large ligands. As in the case of CYP119, here again we have a clear example of an induced fit of ligands into a P450 active site.

Conclusions
Azole inhibitors such as ketoconazole and fluconazole are currently employed as antifungal agents in the clinic, and agents such as flutriafol are similarly employed in the field. They also find current use, in the form of anastrozole and letrozole, in the treatment of hormone-dependent breast cancer. Azoles have considerable potential in other areas, for example, as antimycobacterial agents [21]. Clarification of the mechanisms that control ligand binding, and of the active site modifications that can be used to develop resistance to azoles, is important not only for the development of novel azole agents but for the more general prediction of P450 ligand affinity and substrate specificity. As illustrated here for two bacterial enzymes, the conformational mobility of P450 active sites poses a challenge to the predictive design of selective or specific agents [22].

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