Cytochrome P450 active site plasticity: attenuation of imidazole binding in cytochrome P450_{cam} by an L244A mutation

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We have identified a P450_{cam} mutation, L244A, that mitigates the affinity for imidazole and substituted imidazoles while maintaining a high affinity for the natural substrate camphor. The P450_{cam} L244A crystal structure solved in the absence of any ligand reveals that the I-helix is displaced inwardly by over 1 Å in response to the cavity created by the change from leucine to alanine. Furthermore, the crystal structures of imidazole-bound P450_{cam} and the 1-methylimidazolide-bound P450_{cam} L244A mutant reveal that the ligands have distinct binding modes in the two proteins. Whereas in wild-type P450_{cam} the imidazolide coordinates to the iron in an orientation roughly perpendicular to the plane of the heme, in the L244A mutant the rearranged I-helix, and specifically residue Val247, forces the imidazole into an orientation almost parallel to the heme that impairs its ability to coordinate to the heme iron. As a result, the imidazolide is much more weakly bound to the mutant than it is to the wild-type enzyme. Despite the constriction of the active site by the mutation, previous work with the L244A mutant has shown that it oxidizes larger substrates than the wild-type enzyme. This paradoxical situation, in which a mutation that nominally increases the active site cavity appears to decrease it, suggests that the mutation actually increases the active site maleability, allowing it to better expand to oxidize larger substrates.

**Keywords:** active site conformation/azole drug resistance/cytochrome P450 inhibition/imidazole binding to P450/ protein maleability

Introduction

Azole-based compounds such as fluconazole, ketoconazole and itraconazole are potent inhibitors of cytochrome P450 enzymes (Little and Ryan, 1982; Loose \textit{et al}., 1983; Murray and Ryan, 1983; Benedetti and Bani, 1999) and are employed in the treatment of topical and systemic fungal infections (Keating, 2005; Krcmery, 2005; Lacroix \textit{et al}., 2005). The potential utility of azoles in other clinical contexts, for example in therapeutic approaches to tuberculosis (Guardiola-Diaz \textit{et al}., 2001; McLean \textit{et al}., 2002), has also received attention. However, azole-resistant fungal strains are now well known (Asai \textit{et al}., 1999; Kelly \textit{et al}., 1999; Cuencaa-Estreilla \textit{et al}., 2005; Meneau and Sanglard, 2005). An understanding of the parameters that control the binding of azoles to hemoproteins is thus relevant to the development ofazole agents that circumvent resistance, possess greater specificity for particular hemoproteins or address new targets. Because azole compounds are still the primary antifungal therapeutics in current use, an understanding of azole binding and resistance is particularly critical in this area.

Until recently, structural insights into the origins of mitigatedazole affinity were derived primarily from homology models and experiments with model P450 enzymes, because no crystal structure existed of a therapeutically relevant target. The crystalization of CYP51 from Mycobacterium tuberculosis in 2001 provided the first structurally characterized potential drug target in the P450 family, as well as a crystallographic model for the CYP51 enzymes that are the actual targets in fungi (Podust \textit{et al}., 2001). Azole-based compounds have been shown to bind and inhibit CYP51 (Podust \textit{et al}., 2001; Matsuura \textit{et al}., 2005). The insights derived from homology models of resistant strains of CYP51 revealed that mutations near the heme cofactor are likely to be responsible for the altered affinity of azole-based agents (Sheng \textit{et al}., 2004).

In previous work, we constructed the L244A mutant of P450cam based on the hypothesis that the active site would be modestly expanded relative to that of the wild-type enzyme, because the alanine side chain is three carbons smaller than that of the normal leucine (De Voss \textit{et al}., 1997). In accord with this hypothesis, and with the predictions of an associated \textit{in silico} docking study, the L244A mutant was found to oxidize larger molecules than the wild-type enzyme. More recently, we reported a computational docking effort to predict the differential binding of substituted imidazoles to the active sites of cytochrome P450_{cam} and its L244A mutant (Verras \textit{et al}., 2004). The goal of the study was to determine whether computational approaches could identify ligands that selectively bind to the mutant but not to the wild-type even though the two proteins differed by only three carbons and six hydrogens owing to replacement of an isopropyl moiety by a hydrogen. As in the earlier studies, the model of the L244A mutant was constructed for this effort by simply deleting the three atoms corresponding to the mutation from the crystal structure of the wild-type enzyme. While P450_{cam} is not itself a target for antifungal azole compounds, it is the most highly characterized P450 enzyme in both biochemical and structural terms (Poulos and Johnson, 2005). Crystal structures are available of P450_{cam} complexed with phenylimidazole, metyrapone and an azole drug (Poulos and Howard, 1987; Raag \textit{et al}., 1993), but no structures are available of this enzyme complexed with smaller imidazoles. The computational studies successfully provided qualitative predictions concerning the binding of mono- and di-substituted imidazoles to P450_{cam} and its L244A mutant, but quantitative predictions were stymied, in part, by an unexplained 40-fold decrease in the affinity of the L244A mutant for imidazole itself, despite an essentially unchanged affinity for the natural substrate camphor.

We report here the structures of both the substrate-free and 1-methylimidazolide-bound P450_{cam} L244A mutant. We have
also co-crystallized wild-type P450\textsubscript{cam} with imidazole in order to compare the structures and explore the basis for the decreased imidazole affinity of the L244A mutant. We have found that the single L244A mutation, despite inducing a very different crystal morphology and crystal space group, affords a structure highly similar to that of the wild-type protein. However, a shift in the distal helix caused by the L244A mutation contracts the active site and causes an imidazole to bind in a drastically different orientation relative to the heme iron atom compared with the orientation of imidazole in the wild-type enzyme. The difference readily rationalizes the difference in affinity and offers a model for a mechanism that could result in resistance to azole agents. In view of the earlier substrate oxidation studies, the results furthermore suggest that a primary effect of the mutation is to enhance the dynamic plasticity of the active site.

Materials and methods

Protein expression

The L244A and C334A double mutant of P450\textsubscript{cam} was constructed as described elsewhere (Verras et al., 2004). The C334A mutation exists solely to prevent adventitious dimerization of the protein and thus to facilitate crystallization. It has no other discernible effect on the structure or activity of the protein and is referred to here as the wild-type protein (Nickerson and Wong, 1997; Nickerson et al., 1998).

Cytochrome P450\textsubscript{cam}, and the P450\textsubscript{cam}, L244A mutant were expressed in \textit{Escherichia coli} as previously reported (Verras et al., 2004). The proteins were purified as before with the exception that camphor was not included in any wash, elution or loading buffer. No ammonium salt cut was performed in purifying the proteins, but an additional gel filtration column was run after the Q-Sepharose column. The proteins thus obtained were sufficiently pure for crystallization. The proteins were concentrated to 50 mg/ml in Amicon-2000 centrifugal concentrators prior to storage but, when possible, the proteins were used for crystallization without freezing.

Crystal screening, data collection and refinement

Crystal trays were set up at the temperatures indicated with mother liquor that consisted of 50 mM KCl containing 250 mM KPi and 36–52% ammonium sulfate (Poulos et al., 1982). The protein was combined in a ratio of 1 μl to 1 μl of mother liquor in the hanging drop. The cryo-protectant was 50 mM KPi, 25% ammonium sulfate and 30% glycerol. Imidazole and 1-methylimidazole were added to the protectant was 50 mM KCl, 25% ammonium sulfate and 30% glycerol. Imidazole and 1-methylimidazole were added to the mother liquor to bind in a drastically different orientation relative to the heme iron atom compared with the orientation of imidazole in the wild-type enzyme. The difference readily rationalizes the difference in affinity and offers a model for a mechanism that could result in resistance to azole agents. In view of the earlier substrate oxidation studies, the results furthermore suggest that a primary effect of the mutation is to enhance the dynamic plasticity of the active site.

Results and discussion

Binding of imidazole to P450\textsubscript{cam}, and L244A P450\textsubscript{cam}

The binding affinities of P450\textsubscript{cam} and its L244A mutant for the natural substrate camphor are very similar, $K_s = 1.3 \pm 0.2$ μM and $K_s = 1.6 \pm 0.3$ μM, respectively. Furthermore, the spectroscopic shifts induced by titration of the protein with the substrate are identical for both proteins. Thus, beginning in both instances with a water coordinated heme, one observes a decrease at 416 nm and an increase in the high spin species at 390 nm.

The affinity of the protein for imidazole as judged by spectroscopic methods, however, is drastically different for the wild-type and L244A mutant, with $K_s = 7.53 \pm 1.4$ μM and $K_s = 300 \pm 45$ μM, respectively. Nevertheless, the spectroscopic shifts observed upon titration of the proteins with imidazole are indistinguishable. Beginning with the water-coordinated heme, a decrease is observed at 416 nm and an increase at 424 nm. The spectroscopic shifts indicate that in both cases the binding of the ligand involves coordination of theazole nitrogen to the heme iron, giving rise to a low spin species.

The spectroscopically determined constants for binding of 1-methylimidazole to the two enzymes diverge similarly, the affinities being $K_s = 2.4 \pm 0.4$ μM and $55.9 \pm 9.0$ μM for the wild-type and L244A proteins, respectively. Again, the spectral shifts are the same for both proteins, resulting in a low spin...
424 nm species. The L244A protein was crystallized with 1-methyl imidazole, which is larger and more asymmetric than imidazole, to facilitate the ligand placement within the density.

To understand how the mutation affects the binding affinity of large ligands, we measured the binding affinity of the wild-type and L244A proteins for ketoconazole. The affinities of the wild-type and L244A mutant for ketoconazole are $K_s = 0.5 \pm 0.1 \mu M$ and $0.6 \pm 0.1 \mu M$, respectively, which indicates that the large discrepancy in imidazole affinity is ablated in the presence of large iron-coordinating ligands. Prior work with 1,5-disubstituted imidazoles reinforces this finding (Verras et al., 2004).

**Crystal structure of substrate-free L244A P450cam**

The substrate-free L244A unit cell contained two molecules in the asymmetric unit. The density of monomer A was slightly clearer than that of monomer B, so when referring to structural characteristics, we refer to monomer A. The Cα rmsd between the monomers was less than 1 Å.

While the crystal morphologies and unit cell dimensions are different between the wild-type (P212121) and the mutant protein (P21) (Table I), the proteins are highly similar to wild-type P450cam [PDB code 1PHC (Poulos et al., 1986, 1987)] with a global rmsd of 1.6 Å for all atoms. The primary differences between P450cam and the P450cam L244A mutant are within the active site. A difference map was constructed by using the structure factor file from the L244A and the wild-type (1PHC) as a model. Viewing the negative difference map (Fo–Fc) at $2.5 \sigma$ revealed a change in cavity size smaller than expected for the deletion of an isopropyl moiety. When

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**Table I. Data collection statistics for the ligand-free P450cam L244A mutant protein, the L244A mutant co-crystallized with 1-methylimidazole and wild-type P450cam co-crystallized with imidazole**

<table>
<thead>
<tr>
<th></th>
<th>L244A with no ligand</th>
<th>L244A with 1-methylimidazole</th>
<th>WT with imidazole</th>
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<td><strong>Crystal data</strong></td>
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<td>P2$_1$</td>
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<td></td>
<td>b 59.853</td>
<td>58.784</td>
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<td></td>
<td>c 114.124</td>
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<td>Wavelength, Å</td>
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<td>18 642</td>
<td>73 041</td>
</tr>
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<td>3.9 (3.0)</td>
<td>6.9 (6.5)</td>
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<td>98.3 (94.3)</td>
<td>98.7 (96.8)</td>
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<td>(last shell), %</td>
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<tr>
<td>R$_{sym}$</td>
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<td>12.8 (2.2)</td>
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<tr>
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<td>146 503</td>
<td>56 915</td>
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<td>Reflections in test set (7.4%)</td>
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<td>1603</td>
<td>6404</td>
</tr>
<tr>
<td>R$<em>{crystal}$/R$</em>{free}$, %</td>
<td>23.54 (24.89)</td>
<td>21 (28.4)</td>
<td>21.34 (24.42)</td>
</tr>
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</table>

*Adjusting the wavelength improved the shape of the low resolution spots for this protein, which otherwise were too large, i.e. had too high a sigma value and occasionally overlapped with each other.

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**Fig. 1.** Stereo view of the P450$_{cam}$ L244A substrate-free active site. The density difference map of the wild-type (red) and mutant (yellow) P450 enzymes indicates points of deletion at Leu244. A comparison of the L244A mutant with the wild-type structure shows only a small patch of negative density (red contour) at the leucine mutation site. Comparison of substrate-free wild-type P450$_{cam}$ (red), camphor-bound wild-type P450$_{cam}$ (blue) and substrate-free P450$_{cam}$ L244A mutant (yellow) shows a shift in the helix about the L244A mutation to fill the gap created by the mutation. Electron density map (Fo–Fc) contoured at $2.5 \sigma$. 

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![Image of crystal structure](image-url)
overlaying the structures of P450<sub>cam</sub> L244A, substrate-free wild-type P450<sub>cam</sub> and substrate-bound wild-type P450<sub>cam</sub> [PDB code 2CPP (Poulos et al., 1987)] we see that the cause of the small negative density at position 244 is a shift of the I helix toward the active site by over 1 Å (Figure 1).

**Crystal structure of imidazole-bound wild-type P450<sub>cam</sub>**

To understand the structural differences between the L244A and wild-type proteins we co-crystallized the wild-type protein with imidazole. Imidazole is a small ligand, with few contacts in the active site. To date, only one crystal structure, that of CYP119 [PDB code 1F4U (Yano et al., 2000)], has been resolved with an unsubstituted imidazole bound in the active site. The wild-type protein structure was determined to a resolution of 1.5 Å in the presence of imidazole (Table I). The overall structure is very similar to that of the substrate-free wild-type enzyme and, even in the active site, no backbone or side chain rearrangements are evident. The imidazole, as expected, is bound perpendicular to the heme (Figure 2a). The temperature factor (7.7) of the imidazole N3 is very

![Figure 2](http://example.com/figure2.png)

**Fig. 2.** The binding orientations of imidazoles relative to the heme in P450<sub>cam</sub> and its L244A mutant. (a) Imidazole bound perpendicular to the heme in P450<sub>cam</sub> and (b) the 1-methylimidazole bound structure of the L244A mutant showing the density of 1-methylimidazole relative to the heme and the proximity of the Val244 residue to the ligand. Electron density map (2F<sub>c</sub>−F<sub>c</sub>) contoured at 1 sigma.
low at occupancy of 1.0, whereas the other atom occupancies lie between 0.5 and 0.85 with temperature factors ranging between 18 and 22. This is in agreement with our knowledge of imidazole binding, suggesting that the position of the nitrogen in imidazole that coordinates to the heme is more tightly restricted than those of the other atoms of the ligand.

**Crystal structure of the 1-methyl imidazole-bound L244A mutant**

The L244A protein was crystallized in the presence of 1-methylimidazole, a larger and more asymmetric ligand than imidazole, to improve the fitting of the ligand to the active site density and the structure was determined to a resolution of 2.1 Å (Table I). While the overall structure is again very similar to that of wild-type P450cam (Cα rmsd of <1 Å), the active site density is significantly distinct between the substrate-free L244A and substrate-free wild-type protein. The density indicates that the ligand lies almost parallel to the plane of the heme (Figure 2b). As in the substrate-free L244A structure, the I helix is shifted toward the active site. Most notably Val247 shifts 1.8 Å toward the heme. The density of this residue is well defined. Because of the position of Val247, the 1-methylimidazole is unable to assume the orientation perpendicular to the heme that is observed in the wild-type protein. Because coordination of imidazole to the heme iron involves sigma bonding between the N3 imidazole nitrogen and heme iron, as well as π backbonding from the electron deficient imidazole to the dxy and dxz iron orbitals, a perpendicular binding mode is necessary to optimize affinity. The steric restraints imposed by the shifted backbone, specifically Val247, prohibit optimization of the imidazole coordination and, thus, result in the observed mitigated affinity of 1-methylimidazole and imidazole for the L244A protein. We also crystallized and attempted to determine the imidazole-bound L244A structure (data not shown) and again found a large patch of ligand density almost parallel to the heme. However, the density was not sufficiently defined to permit conclusive placement of the ligand, so we report here the structure of the L244A protein crystallized with 1-methylimidazole.

In earlier work, we prepared the L244A mutant as part of a study of DOCKing methods for the prediction of substrate specificity in cytochrome P450 enzymes (De Voss et al., 1997). This mutant was prepared based on a computer graphics analysis of the crystallographic active site, which suggested that the mutation would delete three carbon atoms from the active site and, thus, expand the substrate-binding cavity by the volume of these three carbons and their associated hydrogen atoms. DOCKing was then carried out with the wild-type crystal structure and with the L244A model, without further refinement of the model, and substrates that docked within the mutant but not the wild-type active site were tested experimentally as ligands and substrates. In fact, the L244A mutant was found to bind and oxidize larger substrates than the wild-type enzyme, as would be expected for a larger active site cavity (De Voss et al., 1997). However, the present crystal structure of the L244A mutant indicates that the I-helix shifts inward, compensating for the volume vacated by the deleted atoms. The binding and oxidation of larger substrates by the L244A mutant, thus, implies that the mutant cavity is more maleable, and can expand to allow the binding of larger substrates, than that of the wild-type enzyme.

**Conclusions**

We present here the first crystallographic analysis of the basis for mitigated imidazole affinity stemming from a point mutation in a P450 enzyme. P450cam is not an antifungal target, but because it is so well characterized it is an excellent model in which to study the phenomenon of drug resistance. As shown here, a single point mutant in the active site results in a 40-fold difference in binding affinity for imidazole and a 25-fold difference in affinity for 1-methyl imidazole.

The structural studies implicate a movement of the I helix as the source of the mitigated imidazole affinity. In the absence of a ligand the helix moves in to fill the cavity created by the leucine to alanine mutation, and in the presence of 1-methylimidazole the helix also shifts into the active site and prevents imidazole from assuming an orientation perpendicular to the heme. Our finding that the ketoconazole binding affinity is not decreased by the lessened affinity of the coordinating imidazole moiety is consistent with the inference that mutations have larger effects on small ligands that have few interactions with other parts of the protein. The observation that the helix shifts inward to diminish the active site cavity, yet the enzyme binds and oxidizes larger substrates than the wild-type enzyme, implies that the mutation increases the deformability of the active site, enhancing its ability to conformationally adjust to accommodate large substrates.

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**References**


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