Modeling cytochrome P450 14α demethylase (*Candida albicans*) from P450cam

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The tertiary structure of cytochrome P450 14α demethylase—Candida albicans *(P450 CA) is modeled on the basis of sequence alignment with two closely related proteins and the crystallographic structure of* Pseudomonas putida *P450cam. The secondary structure prediction system used combines the information from several algorithms and trains the data to offer an optimized prediction of the known P450cam. The trained algorithm was then used to predict the secondary structure of the other P450 sequences. The prediction of the surface coil regions was aided by an alignment between P450 CA and the homologous sequences P450 14α demethylase*—Saccharomyes cerevisiae *(66 SD) and P450 14α demethylase*—Candida tropicalis *(72 SD). The prediction and alignment information was combined to establish an alignment between P450 CA and P450cam, and to assign full secondary structure to the target protein. This secondary structure was folded from the template of P450cam and the predicted structure was relaxed by molecular dynamics. Model checking highlighted minor adjustments in the alignment, correctly orienting hydrophobic and hydrophilic side chains. The model offers explanations for several known experimental results and suggests further investigations that may prove fruitful in understanding the structure and mechanisms of the P450 family (Porter, T.D. and Coon, M.J. Minireview cytochrome P450.* J. Biol. Chem. *1991,* **266,** *13469–13472. Waterman, M.R. Cytochrome P450 cellular distribution and structural considerations.* Current Opinion in Structural Biology *1992,* **2,** *384–387. Aoyama, Y., Yoshida, Y., Sonondo, Y. and Sato, Y. Structural analysis of the interaction between the side-chain of substrates and the active site of lanosterol 14α demethylase (P450 14DM) of yeast.* Biochim. Biophys. Acta *1992,* **1122,** *251–255.).*

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INTRODUCTION

The P450 superfamily presently comprises more than 60 eukaryotic sequences and one prokaryotic sequence. These are divided into 10 families and 18 subfamilies based on sequence alignment, with families sharing greater than 30% sequence identity and subfamilies greater than 46%.[1–3] Each enzyme binds, reduces, activates and then cleaves molecular oxygen to oxidize a range of substrates.[4] Oxidation as part of the detoxification system requires that the enzymes recognize a vast number of both natural and synthetic small molecules for removal from the body. In response to this, the P450 superfamily has developed into one of the most versatile biological catalysts known, exhibiting great sequence diversity with few totally conserved residues. Unfortunately, the only structure from the superfamily to be solved crystallographically is that of the prokaryotic P450cam camphor hydroxylase from *Pseudomonas putida*[5–8] (P450cam or cam). This is the most distant relation in the family and of little pharmaceutical or agrochemical importance. However, despite the diversity in sequence, many of the important eukaryotic target enzymes appear to share a similar fold to that of P450cam,[9–14] making their structural elucidation by homology modeling a possibility.

This is the case for P450 14α demethylase from *Candida albicans* (P450 CA or *Candida*)[15] for which both secondary structure prediction and sequence alignment point to a core structure similar to that of cam. *Candida albicans* is a yeast responsible for the opportunistic disease candidiasis (or *Candida*'s disease).[16,17] Candidiasis has a colloquial name of *thrush* (or *yeast infection*, in the USA) when it affects the genital region and it is a common complaint suffered by up to 75% of women. It may also affect the throat, appearing as a white fungal growth spreading over the roof of the mouth. In these instances, the disease is curable by administering azole compounds, which inhibit the action of P450 CA.[18] It is when the immune system is compromised to a greater extent by physical intervention (such as major surgery) or biological intervention (such as AIDS) that the cures are no longer effective.[19–21] The problem arises because the human P450 14α demethylase enzyme, required for the production of cholesterol, is also inhibited. This limits the dosage of the azole compounds and in severe cases the yeast will spread

throughout the body, eventually causing death. The ultimate aim of this research is to improve the selectivity of the azole compounds for the yeast enzyme over that of the human, for which protein structure is a prerequisite.

P450 CA is a 528 residue, 51 kD protein,[18] which is membrane bound in the endoplasmic reticulum. It forms part of the ergosterol synthetic pathway by oxidatively cleaving the 14 methyl group (C32) of its substrate, 24 methylene 24, 25 dihydrolanosterol[22,23] (Figure 1). Inhibition of the enzyme by fungicides causes a build up of 14α methylated sterols that, through further reaction, destabilize the cell wall and destroy the yeast.

MATERIALS AND METHODS

A strategy for modeling P450 proteins has evolved with the concomitant development of in-house software. The manual alignment program CAMELEON[24] and secondary structure prediction system Weighted Average Structure Prediction (WASP)[25] were both designed while modeling P450 target proteins. Automatic alignment between P450cam and P450 CA was optimized to a significant score of 3.62 SD using the mutation data matrix[26] with a constant of zero and a fixed gap penalty of four in the software package AMPS.[27,28] From the SD score, the likely accuracy for the aligned secondary structure was estimated to be between 35% and 65% correct[28] and, with this in mind, the alignment was used as a starting position for manual adjustment in CAMELEON.

In a first pass, the alignment was altered using the broader options of the program. Aligning windows of residues from each protein sequence, the mutation data matrix[26] score for each pair of aligned residues are summed to give a value for the window comparison. In this way, a score is calculated between every window on one protein and every window on the other; window alignments above a given cutoff score are highlighted graphically. By interactive variation of the cutoff score, the alignment can begin with the highest scoring window comparisons and progress to lower similarity. The display is capable of showing multiple matches for certain windows, which is information that is lost when an automatic alignment is generated. The broad alignment was assisted by visually comparing amino acid property profiles, calculated as a moving average over each sequence. The hydropathy profile with a window length of 12 residues proved to be the most informative for adjusting this alignment.

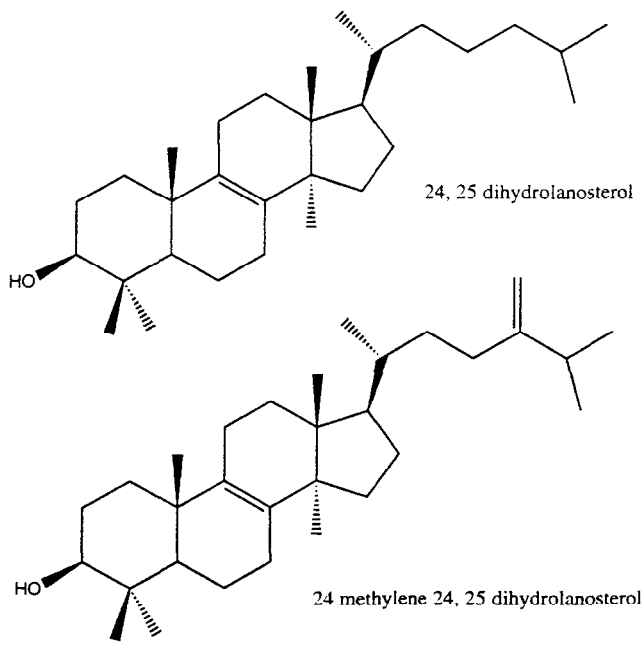

Figure 1. The known substrate of P450 14α demethylase (Candida albicans) *and of P450 14α demethylase* (Saccharomyces cerevisiae).

Having roughly aligned the two sequences, the next stage was to examine the position of each secondary structural unit in greater detail. The detailed analysis in CAMELEON is provided by an interactive vertical scan mode. The short section under review is expanded on the graphics monitor and an amino acid property is chosen to compare the alignment. The three properties most useful in this case related chemical properties, hydrophobicity and identity matches. Having chosen a property, the amino acids are grouped and residues in the same group are displayed graphically in the same color. Vertical scan mode simply draws a connector when aligned residues are of the same color. Each secondary structural unit in P450cam was moved over the local sequence in P450 CA and the number of property matches monitored. As the cam units were moved, the probability that the aligned sequence in *Candida* would adopt the given secondary structure was analyzed separately in the WASP program.

The WASP method[25] enables secondary structure prediction data from four algorithms to be summed as a weighted average. The data from Garnier, Osguthorpe and Robson,[29] Chou and Fasman,[30] Gascuel and Golmard,[31] and Hopp and Woods[32] were combined with different weights and optimized by predicting the known secondary structure of P450cam. The system can offer unambiguous prediction in terms of helix, sheet and coil. However, like CAMELEON, it can be used to a far greater advantage by graphically displaying all relevant information and allowing interactive manipulation of the parameters. When displayed in WASP, the sequence of P450 CA is given together with the secondary structure predictions from the composite methods and the 100 best algorithm combinations from training on P450cam. Helix, sheet and coil probability profiles are displayed together with the cutoff values used to generate the predictions.

As the secondary structure units of cam were moved along the sequence of *Candida*, the aligned portion of the *Candida* sequence was monitored in WASP. The optimized prediction of P450cam was compared to the known structure and allowances made in the *Candida* prediction. For example, the B3/B4 region of cam was predicted to be helical by all of the composite algorithms and hence by all combinations of those algorithms. The same "ghost" helix appeared in the *Candida* prediction. By examining the different probability profiles, it was possible to locate the potentially helical regions of *Candida* and ensure that the alignment of cam fitted these data.

Thus, a procedure can be envisaged whereby each secondary structure unit of cam is aligned to a position in *Candida*

by interactively monitoring both the sequence correlation and the propensity of the *Candida* sequence to adopt the given secondary structure. Extra information was correlated to trace the surface coil regions from the alignment of P450 CA to P450 SC[33] and P450 CT.[34] The three homologous sequences show great similarity with automatic pairwise alignment scores of 66 and 72 SD respectively. Conservation and similarity of mutation between the aligned residues was monitored in CAMELEON, but has been displayed in Figure 2 using three connectors (., : and |) in order of increasing similarity.

Having aligned the cam secondary structure to the *Candida* sequence, the final stage before model building was to analyze the *Candida* sequence and fully assign all secondary structures. The alignment predicted ten inserts and one deletion. Bearing in mind the low sequence similarity and distant relationship of the proteins, it was unreasonable to suggest that these inserts were all random coil. Based on the information from CAMELEON and WASP, and the tertiary

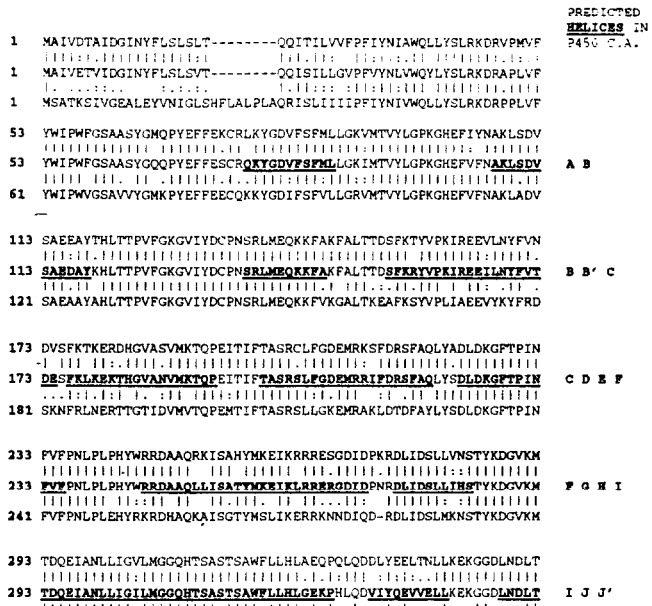

Figure 2. Two pairwise alignments between the middle sequence P450 CA, upper sequence P450 CT and lower sequence P450 SC. The degree of similarity for aligned residues is based on Dayhoff scores and is illustrated by ., : and | in order of increasing similarity. The predicted helices in P450 CA are highlighted in bold/underline and the helix labels are shown at the end of each row.

structure of cam, several helices were extended and two additional helices, J′ and J″, were created. The striking periodicity of hydrophobic and hydrophilic residues in the insert after the J helix was highlighted in CAMELEON, by the color grouping file "tendency to be a core or surface residue," and finally predicted as two surface helices. The predicted secondary structure for P450 CA is shown together with the alignment to cam in Figure 3. Given the low similarity between the sequences, the alignment generated here would never have been created by an automated package owing to the localization of the inserted residues.

With the secondary structure of P450 CA fully assigned, it was then folded around the template structure of P450cam. Mutation of the residues in the cam tertiary structure to those of the aligned *Candida* sequence was completed using the molecular modeling software package QUANTA[35] and coil inserts with less than four residues were built using the BUILD and REGULARIZE features. This uses an algorithm to build the new backbone based on bond lengths and phi/psi angles, places the side chain atoms from structural template files and relaxes the region by minimization. The initial results were viewed on the graphics display and modified manually when necessary. Larger inserts were found by searching a fragment database of fifty nonhomologous proteins. In the case of extending helical structure, the process was split into building the required helix and then fragment searching a turn or coil section to anneal the sequence.

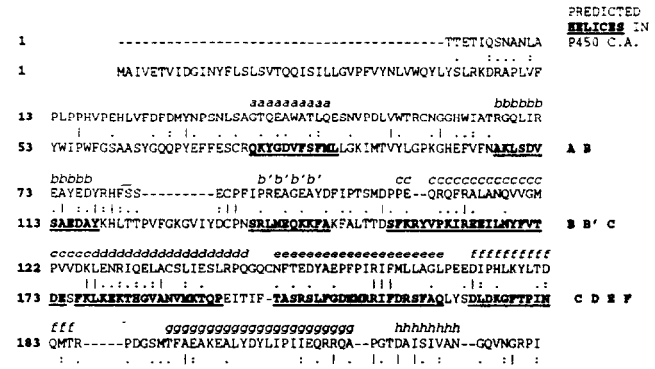

Figure 3. The alignment between the upper sequence P450cam and lower sequence P450 CA. The degree of similarity for aligned residues is based on Dayhoff scores and is illustrated by ., : and | in order of increasing similarity. The known helical secondary structure of P450cam is shown in lower case above the sequence and the predicted helices in P450 CA are highlighted in bold/underline with the helix labels given at the end of each row.

Placing the new J′ and J″ helices involved considerable surface analysis of the *Candida* model around the C-terminal end of the I helix. Areas of the protein surface were found to be sufficiently hydrophobic to enable them to be covered by an additional layer of helices. The completed structure was subjected to constrained minimization and 10 ps of molecular dynamics at 400 K to relax the new backbone structure and remove any side chain clashes. The program CHARMm[36] was used throughout.

The substrate of P450 CA is 24 methylene 24, 25 dihydrolanosterol (Figure 1). This was built using CHEMX,[37] docked manually and the active site again relaxed by constrained minimization.

RESULTS AND DISCUSSION

The predicted structure

The *Candida* sequence is 114 residues longer than P450cam. This comprises a 40-residue N-terminus section and 74 residues inserted into the globular framework. The 74 residues were divided into ten insertions, with eight of the insertions having an average length of just over two residues. The remaining two insertions of 9 and 49 residues respectively, and the 40-residue N-terminus addition, appear to account for the known significant differences between the proteins.

The *Candida* structure retains the basic triangular structure of cam with the addition of a transmembrane anchor at the N-terminus and two large inserts into the globular framework. The two large inserts are shown in blue and are superimposed on the tertiary structure of cam in Color Plate 1. All but one of the necessary insertions were performed on surface areas of the structure, the core insertion being residue MET 508 at the turn of the B5 subunit. The model was analyzed for the conservation of a hydrogen bond network and the appropriate distribution of polar and apolar residues. The results are consistent with a membrane bound, globular protein. *Candida* has a similar distribution of secondary structure to that of P450cam; the percentages are given in Table 1. (The results for *Candida* do not include the 40-residue N-terminus region.)

The active site and access channel

The relative positions of the *Candida* secondary structure units in the region of the active site are similar to those of P450cam, with two exceptions. The position of the B′ helix will be altered by a 9-residue insertion between the B and C helices. However, the exact location of this insertion cannot be pinpointed by sequence similarity. The insertion has been modeled before the B′ helix as shown in Color Plate 1. It could equally have been modeled after the B′ helix or split into two insertions, one on either side. The second alteration is in the coil between the F and G helices where a proline-rich section in *Candida* alters the relative orientations of the two helices.

Table 1. Percentages of secondary structure for P450cam and for the predicted structure of P450 CA.

Protein	Helix %	Sheet %	Coil %
P450cam	51	18	31
P450 CA	51	16	33

The active site remains buried in the core of the protein and the heme group (forming one side of the active site cavity) remains located between the proximal (L) and distal (I) helices. In this conformation, the substrate of *Candida* will fit sterically into the active site. However, there is no access channel large enough through which the substrate may enter or leave. Entry to the active site is enabled by movement of the B′ helix. This movement is facilitated by the first of the large insertions to the original cam structure, which is a 9-residue coil insertion modeled prior to the helix. Having modeled the coil region, the B′ helix was manually manipulated using computer graphics and found to be capable of moving several angstroms to produce an active site entrance with dimensions well in excess of the known substrate and inhibitors. With the insert modeled before the B′ helix, the active site entrance is modeled between the B′ helix and the coil between the F and G helices. If the insert is modeled after the B′ helix, the active site entrance can lie along the I helix. The specific orientation of the B′ helix, and therefore of the active site entrance, must await further experimental evidence for clarification.

Further evidence for the location and mechanism of the access channel is provided by analyzing the distribution of charge over the *Candida* structure compared to that of cam. The B′ helix, forming the moving side to the access channel, exhibits a net positive character from the presence of arginine and lysine residues 138, 143 and 144. These are seen as important residues for attracting the substrate from its probable location in the membrane and ensuring that it enters the active site correctly oriented.

Binding to the membrane

The protein is membrane bound via a transmembrane section at the N-terminus that extends as far as a four-residue signal motif.[38,39] The transmembrane-spanning region (1–43) and terminating signal (44–47) were immediately apparent when the sequence was colored by a charge in the display program CAMELEON and viewed together with an average hydropathy profile. This indicated that the roughly triangularly shaped protein was anchored to the membrane from its N-terminal point. Comparative analysis of the aligned hydropathy profiles between the two sequences, and the subsequent display of the information on a structural template, showed this point of the *Candida* structure to be considerably more hydrophobic than in cam.

The model indicates the burial of this point of the structure into the membrane, which includes the coil before the A helix, the B1 sheet, the B helix and the B3 sheet (marked in red in Color Plate 1). Should the N-terminal transmembrane region of certain P450 proteins be removed, evidence suggests continued binding to the membrane, although only one transmembrane segment is apparent.[40] This model infers that membrane binding without the transmembrane segment is due to the hydrophobic character of burying the protein in the lipid membrane and favorable interactions between the phosphate surface and charged residues of the protein, nota-

bly Lys 78/90/99/108/119/128/143/144, Arg 76/138/245/246 and His 101/120. Within this sequence length there are eight negative residues, of which two pairs (70/73 and 115/116) are located on a coil or turn protruding above the proposed membrane level. The buried region is thus predominantly hydrophobic and at the phosphate surface the charged residues are apportioned 12 positive and four negative. Placing the protein to bury the hydrophobic residues does require that the plane of the heme be at an angle[39] to the membrane rather than parallel.[41]

Recognizing the P450 reductase protein

The majority of the inserted residues needed to complete the *Candida* structure formed a cluster of three helices at the C-terminal end of the I helix (Color Plate 1). This region of the *Candida* alignment to both P450 CT and P450 SC showed higher than average conservation (Figure 2). Initial speculation is that these helices are involved in recognizing and docking the electron transfer protein. This explains both their high conservation in the closely related endoplasmic reticulum, membrane-bound proteins and their complete absence in P450cam.

Interactions of the substrate

Detailed analysis of the active site is continuing with QSAR work on known inhibitors. To date, there are two possible orientations of the substrate that it is hoped will be discriminated by point mutation studies on the *Candida* sequence. The favored orientation is described here with its supporting evidence.

The active site of *Candida* forms a triangular pocket with the heme at the back, the I helix above and to the right, the B5 and B3 units below and to the right, and the B′ helix and coil to the left. The pocket is covered by the F and G helices and the rigid, proline-rich coil between them. The substrate and oxygen molecule were placed manually (Color Plate 2) to facilitate oxygen interaction with the iron, the 14α methyl group of the substrate and a possible interaction with Thr 311 of the I helix. It is possible that a 120° rotation about the Cα–Cβ bond of Thr 311 stabilizes the Fe–O_2 complex[42] and acts as a proton shuttle from an internal solvent pocket[43] to facilitate O_2-bond cleavage. The hydroxyl group on the substrate was then in position to hydrogen bond to Thr 315, as suggested previously for P450 SC,[42] and accounts for experimental data on lanosterol derivatives.[44]

The sterol is predominantly surrounded by the hydrophobic residues of the active site. The first anomaly in this is in the top of the pocket where an unusual sequence of 14 residues comprises three phenylanalines and four proline residues, and gives an aromatic character. It is possible that one of these Phe residues (228, 233 or 235 on the F helix and coil) forms a π interaction with the eight double bond of the sterol substrate. Both Thr 315 and the Phe residues previously mentioned are conserved in the other related sequences.

The substrate tail lies between the I helix and the B′ helix in a hydrophobic pocket and agrees that introduction of hydrophilic character into the tail would destabilize the hydrophobic interaction.[45] A good steric fit for the tail is not necessary, for binding has been shown using lanosterol derivatives.[46] But similar experiments[45] show the region around the tail to be sterically crowded. This region of the sequence on the B′ helix is highly conserved with P450 SC, despite the latter having the slightly differing substrate lanosterol (Figure 1). However, among the striking similarity is a glaring mutation where Gly 156 in *Saccharomyes* becomes Phe 148 in *Candida*. A possible complementary mutation of Val 154 in *Saccharomyes* to Ala 146 in *Candida* may further modify the shape of the pocket occupied by the substrate tail. It seems reasonable to speculate that these mutations provide some, if not all, of the selectivity between the two possible substrates in a similar manner to the results of Lindberg and Negishi.[47] They may also account for other subtle differences shown to exist by experimental alteration of the natural substrates.[23,45,46]

It is known that some residues of the protein can move to increase or decrease the size of the active site and/or its entrance channel.[48] The substrate binding described next requires that pyrrole ring D of the heme be sterically unhindered, in contradiction to the recent results of Yoshida et al.[49] It therefore implies that the active site is able to open and close to dock differing sizes of substrate and inhibitor—the movement being made possible by alterations in the region of the B′ helix. Indeed, the schematic active site shown in Figure 4 is very similar to that proposed by Yoshida et al.,[49] with the B3 sheet having been moved outward to accommodate a superposition of several structures and thereby uncovering pyrrole ring D.

The model is in agreement with the remaining conclusions from the paper, which speculate that the A and B rings of the porphrin are sterically covered by protein residues, whereas the C ring is accessible. The B ring is covered to the top of the active site by residues on the I helix, however the A ring is only covered by a single methyl group from residue Thr 315. This methyl would prevent binding to the nitrogen of the A ring, but the active site area above the methyl is utilized by the substrate and inhibitors. It is interesting to see that in the orientation given, the substrate is only able to clear the methyl group, and avoid steric contact, by the presence of the eight double bond. This unsaturation is necessary as the *Candida* will not oxidize the 8–9-saturated derivative of its substrate.[50]

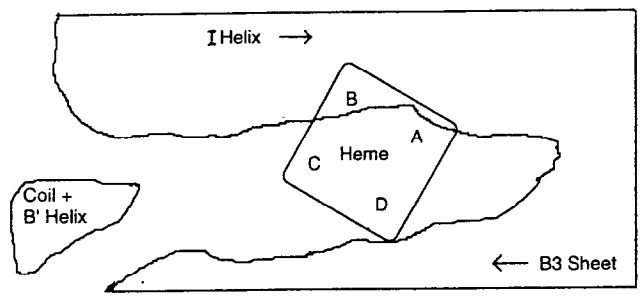

Figure 4. A schematic diagram of the active site, in the plane of the heme, shows the entrance partly covered by the B′ helix. Pyrrole ring B is covered by the I helix, ring A is partly covered, ring C is in space and the B3 sheet can move to expand or contract the active site, exposing or covering the D ring.

CONCLUSION

Modeling P450 CA has led to the following conclusions:

(1) Sufficient sequence and profile similarity exist between P450cam and P450 CA for the latter to modeled from the former. *Candida* retains the roughly triangular shape of cam, with the majority of inserted residues forming one cluster of helices.

(2) The large insert, modeled as three helices, shows good conservation between the closely related proteins and may be involved in recognizing the electron transfer protein, explaining its complete absence in cam.

(3) The protein is membrane bound with a single transmembrane-spanning section at the N terminus. Further stabilization is provided by burying the hydrophobic N-terminal corner of the protein in the membrane to a depth where the phosphate membrane surface meets a number of Lys and Arg residues.

(4) The active site entrance is opened and closed by movement of the B′ helix, assisted by an inserted piece of coil. The active site itself may be able to increase and decrease in size to accommodate differing sizes of substrate and inhibitor.

(5) The substrate fits a predominately hydrophobic active site pocket. In its present orientation, the hydroxyl group forms a hydrogen bond to Thr 315 and the ring double bond of the sterol may interact with Phe residues around the F helix.

(6) The substitution of Gly 156 in P450 SC to Phe 148 in *Candida* may explain their differing substrate selectivity.

The modeled structure of P450 CA, together with the authors' conclusions about membrane binding, attracting and docking the substrate, and recognition of the electron transport protein provide a useful platform from which to undertake experimental analysis. Such work has already begun to try and establish the validity of the structure in the region of the active site and additional modeling is currently rationalizing the activity of the known fungal inhibitors. The model allows a small degree of flexibility in the position of certain residues and only positive experimental evidence can be used to locate these. Should the model be proved correct, within the scope of experimental techniques, it will provide an invaluable framework from which to design more selective inhibitors to *Candida*'s disease. Coordinates are available from the authors on request.

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