Cytochrome P450 monooxygenases form an enzyme superfamily that are ubiquitous in nature, found in organisms from protists to plants to man. Whereas the human genome contains around 40 cytochromes P450 (P450s), over 400 are present in the rice genome and over 250 have been located in the *Arabidopsis* genome. This enzyme superfamily serves to metabolize many physiologically important compounds such as catabolizing compounds for use as a carbon source and detoxifying xenobiotics, as well as aid in the synthesis of biologically active compounds such as horomones, steroids, defense compounds, signaling molecules, and fatty acids. A common feature of the substrates for cytochrome P450 is the hydrophobic nature, where the enzyme acts as a monooxygenase to form products of increased solubility. The majority of the cytochrome P450 enzymes are membrane-bound, such that the hydrophobic substrates are able to access the binding pocket.

The majority of the newly identified cytochromes P450 have undefined function. The nomenclature for cytochrome P450 monooxygenases classifies the enzymes into families and subfamilies based on sequence homology. Sequence identity, however, is frequently less than 20 percent and only share greater than 40% identity within a specified family, yet secondary and tertiary structure is relatively conserved as has been made clear through the study of x-ray crystal structures. A few primary sequence conservations exist among the P450s, most notably the signature motif found in the heme-binding pocket that involves a cysteine ligand to the iron and gives cytochromes P450 their unique spectral properties. An N-termnial hydrophobic anchor and a proline-rich region that may modulate folding and substrate access are moderately conserved among P450s, but vary in sequence and spacing such that an overall unambiguous alignment based on these structures is uncommon.

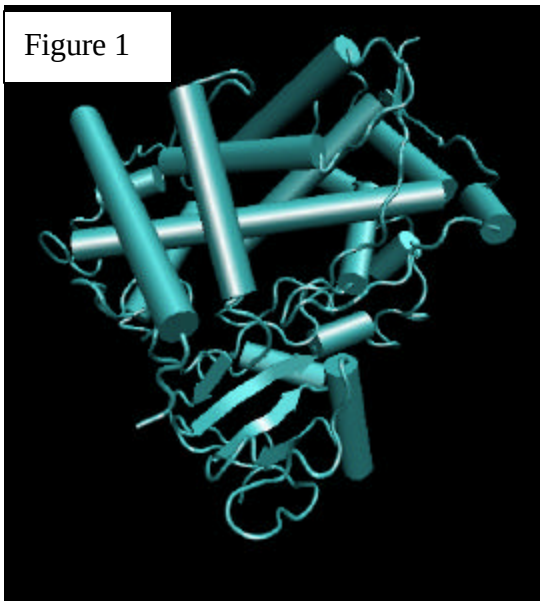
Cytochromes P450 are specific for a wide range of structurally divergent substrates, yet all perform similar chemistries. The general mechanism of P450s has been elucidated through the study of a soluble cytochrome P450, P450cam. The basic cycle consists of ligand binding, followed by binding of a dioxygen molecule. P450s are then capable of accepting another electron, which systematically allows for the formation of a highly reactive compound I complex, which can proceed to perform all sorts of chemistries including hydroxylations, epoxidations, dealkylations, isomerizations,

dehydrations, and deaminations. A single P450 is not able to perform all these oxygenations, however it is common for multiple P450s to act on a single substrate at alternate positions. P450s usually require a redox partner that enables the transfer of electrons and formation of compound I, yet the interactions between cytochromes P450s and its redox partner are still unclear. Analysis of the electron density distribution of crystal structures has helped to elucidate sites of interactions between the enzyme and redox partner. Low sequence identity among the cytochrome P450 superfamily and lack of crystal structures for any but one of these membrane-bound enzymes have created great difficulties in ascertaining P450s to biological pathways.

To date, at least seven soluble bacterial P450 crystal structures have been solved, as well as one mammalian membrane-bound structure. Comparison of the crystal structures has given great insight to the overall conservation of P450 tertiary structure, highlighting contributions of the regions surrounding the catalytic core for substrate specifications. Crystallization of the membrane-bound P450, CYP2C5 was obtained by truncation of the hydrophobic N-terminal membrane-anchor (residues 3-21) and mutation of five additional residues (residues 202, 206, 207, 209, and 210) to enhance solubilization of the protein by decreasing the aggregation tendency of the protein, mutation of five residues (residues 2, 22, 23, 25, and 26) to improve protein expression, and addition of a c-terminal histidine tag to ease protein purification. McRee et al. states that the mutated version of CYP2C5 exhibits a "similar" Km for progesterone hydroxylation and a Vm that is about 1/3 of that found for an N-terminal truncation-only mutant enzyme. Therefore, still unclear is how the crystallized enzyme catalyzes the hydroxylation of progesterone as compared to the native enzyme. With knowledge that the proposed mechanism of substrate entry to the binding site is through the membrane bilayer, it seems unlikely that the N-terminally mutated, aqueous soluble enzyme would perform catalysis on the hydrophobic substrate near as efficiently as the native enzyme. However, analysis of this CYP2C5 crystal structure and comparison to the crystal structures of the soluble P450s has yielded several interpretations of protein structure and correlations for roles of specific residues in enzyme catalysis.

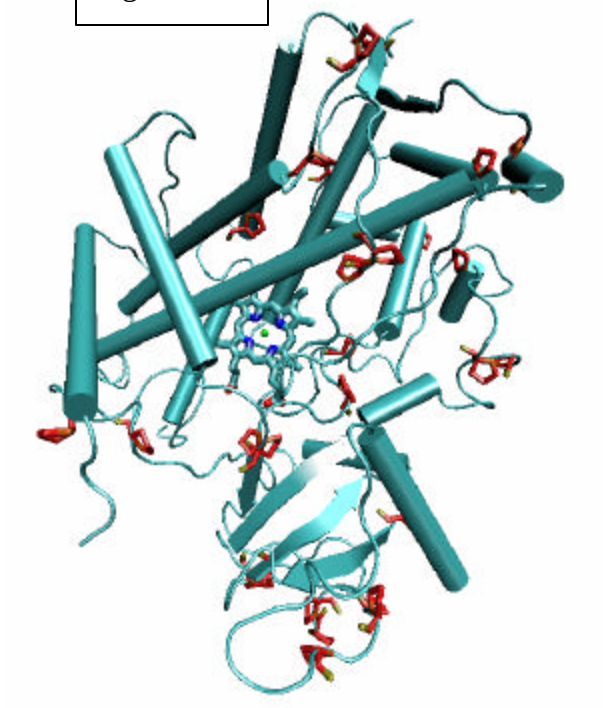
The overall fold of cytochromes P450 is shown:

Figure 1



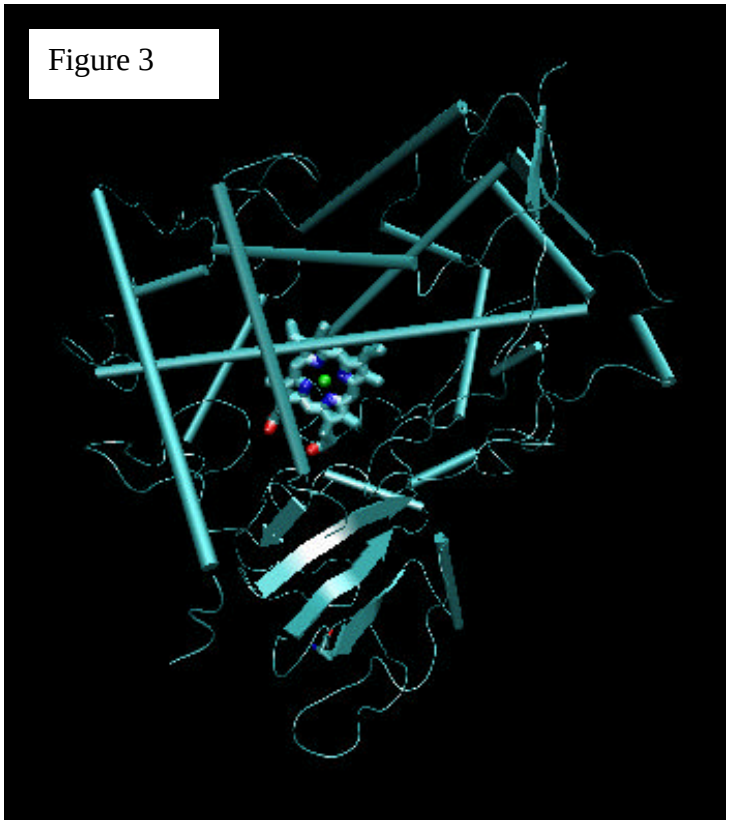
The structure is divided into two domains, the alpha-helical domain that contains the heme binding site as well as the substrate binding site and the beta sheet domain near the N-terminus that interacts with the membrane. As expected, a number of proline residues reside within the more flexible portions of the protein to help in the overall fold of the enzyme:

Figure 2

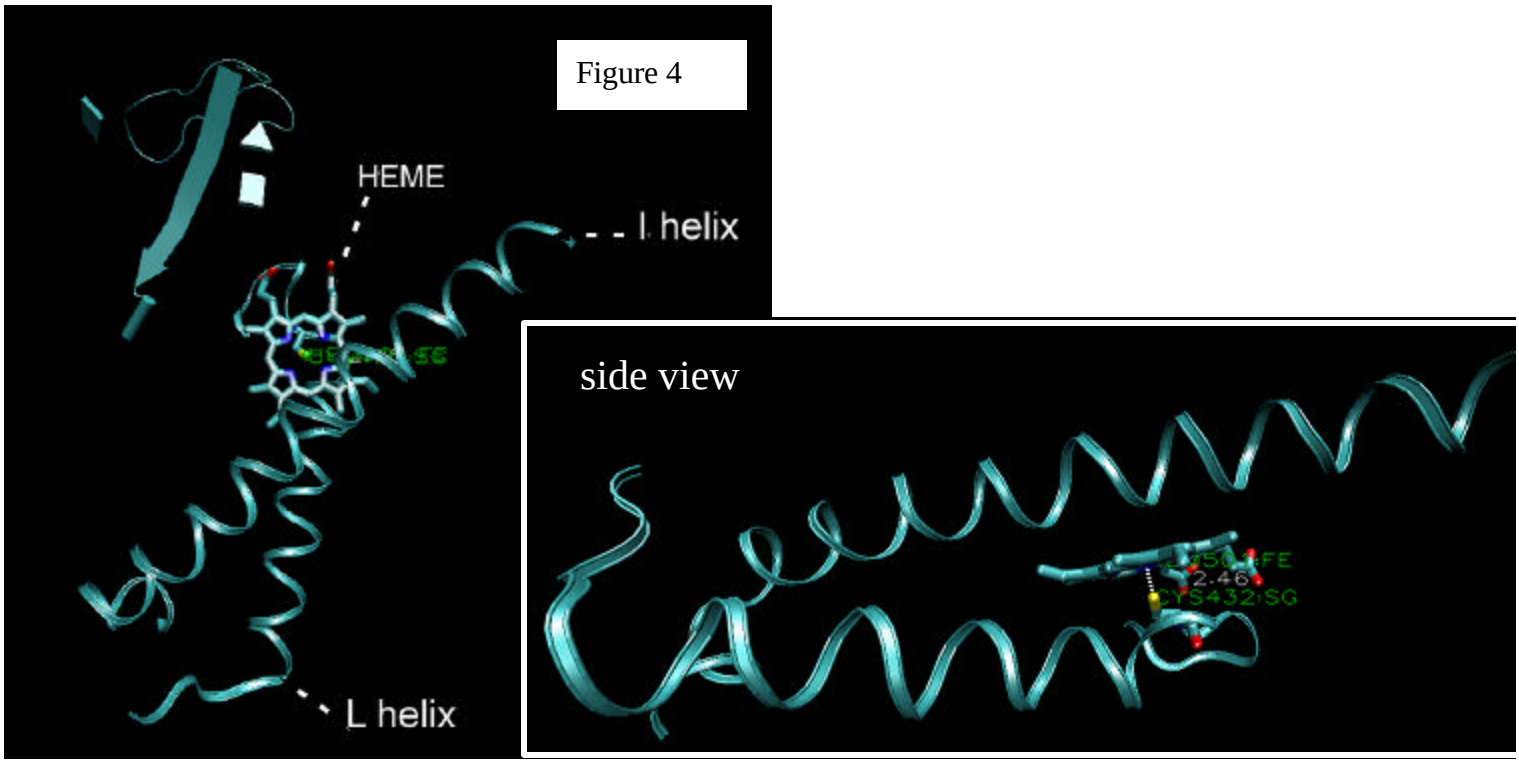


As often seen in proteins, Figure 2 shows that the Pro residues (red) are present either in random coil portions of the protein or in regions that lie between a helix-helix or helix-ß sheet motif.

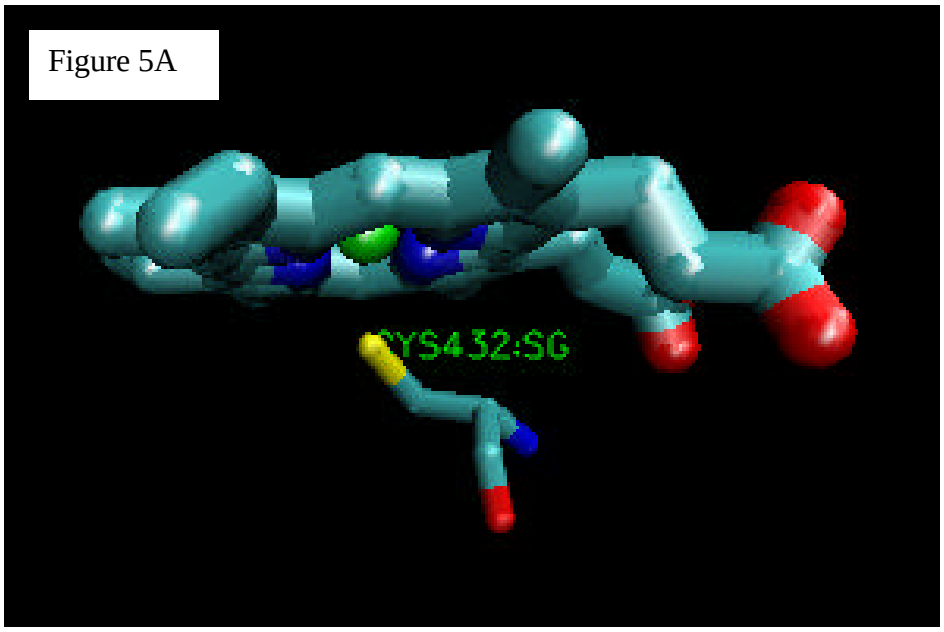
The cytochrome P450s are hemeproteins and the crystal structure, of course, reveals where the heme is located and how it is incorporated into the enzyme structure (Figure 3).

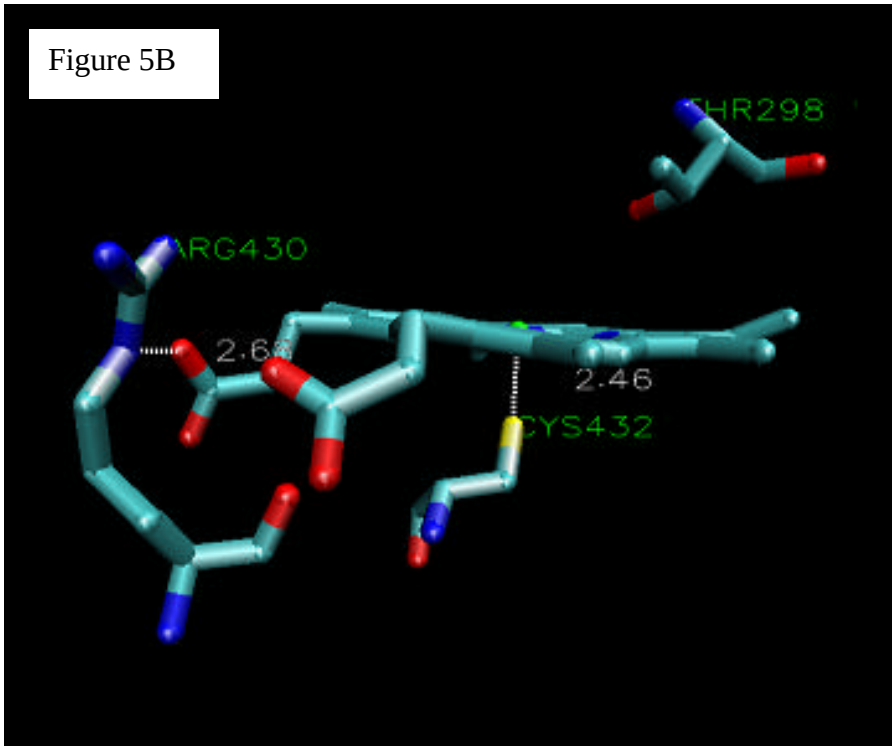


Comparison to that of soluble P450 structures, the spatial arrangement of the heme binding site is conserved. The structure clearly illustrates the orientation of the heme, where helix L and helix I help to hold the heme in place (Figure 4).



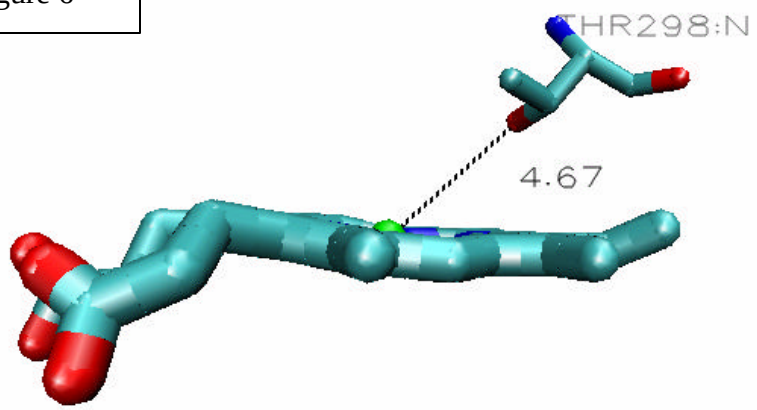
The P450 signature motif is a F—G-R-C-G sequence surrounding the protoporphyrin ring. The cysteine at position 432 serves as the axial ligand to the heme iron and allows the enzyme to perform an unfavorable scission of dioxygen (Figure 5A). The conserved Arg-430 (Figure 5B) interacts with the propionate side chain of the heme by forming a hydrogen bond.



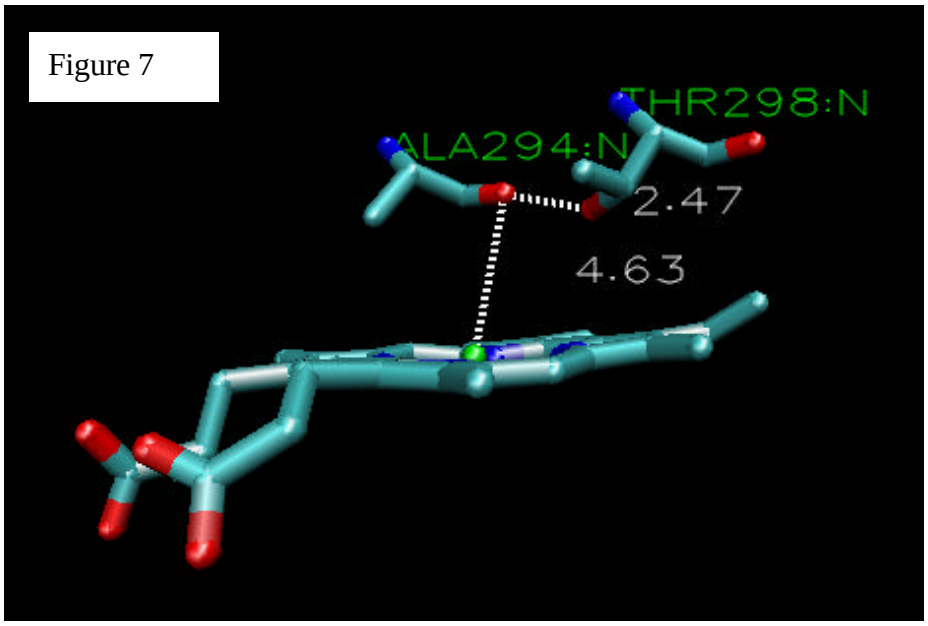


The threonine residue at position 298 (Figure 6) presumably participates in a proton relay which eventually protonates the reduced oxygen-bound intermediate that precedes the formation of the highly reactive compound I intermediate.

Figure 6



A threonine residue analogous to Thr 298 in CYP2C5 that is located above the heme, opposite that of the cysteine ligation, is also found in other P450s. In the well studied soluble P450cam crystal structure, a water molecule is found between this threonine residue and the heme. The mechanism whereby a water molecule is delivered to the reduced oxygen remains under strong debate, however evidence supports the water molecule to play an important role in delivery of the proton to the oxygen molecule. In the crystal structure of CYP2C5, no water molecules are present. The threonine molecule resides 4.67Å from the heme iron. Recognizing the fact that this distance is measured from the heme iron to the oxygen of threonine, the actual distace of proton transfer from the iron-bound oxygen molecule to the hydrogen on the hydroxy group of the protonated threonine may be significantly shorter and may allow sufficient proton transfer. However, based on the facts that a water molecule has been observed in several other P450 structures and that two oxygen, or hydroxy, groups of surrounding amino acids reside within 3 angstroms (Figure 7) of each other that could orient a water molecule in such a way that would allow a water mediated proton transfer to the reduced oxygen intermediate, it is interesting to speculate about the mechanism by which proton transfer occurs in this enzyme.



Several annotations buried within the substrate-binding pocket give insights as to modes for substrate specificity and binding. The volume of the binding site was found to be 360 Å. Coupled to the ability to computationally dock substrates within the protein of interest, McRee et al. were able to identify key contact residues that lie within the six substrate recognition sites (image not shown due to unavailability of substrate-bound structure). Interestingly, earlier mutagenesis studies of the enzyme showed these residues to affect catalytic activity and regiospecificity of hydroxylation. Variability of these contact residues is found among other cytochrome P450s that catalyze similar reactions and, moreover, earlier mutagenesis studies demonstrated that substitutions of these residues could alter the reactions of the enzymes.

The N-terminus of the membrane-bound P450 enzymes acts as a signal sequence that co-translationally targets and anchors the protein to the membrane. In 1998, Shank-Retzlaff et al. measured the insertion area of CYP2B4 to be about 680Å, which is nearly three times greater than would be expected for a single transmembrane anchor. In accordance, it was observed for many P450s that removal of the first 30 N-terminal sequence residues resulted in a protein that still associated with the membrane, but would disassociate in high ionic strength buffers. Anti-peptide antibody binding data also supported additional regions of the protein that were membrane associated, where epitopes in the N-terminal domain and the F-G and B-C loops elicited antibodies that reacted with soluble protein but not significantly with membrane-bound protein. The

crystal structure of 2C5 provides support for a membrane association that involves, not only a single transmembrane helix, but also several surrounding portions of the enzyme:

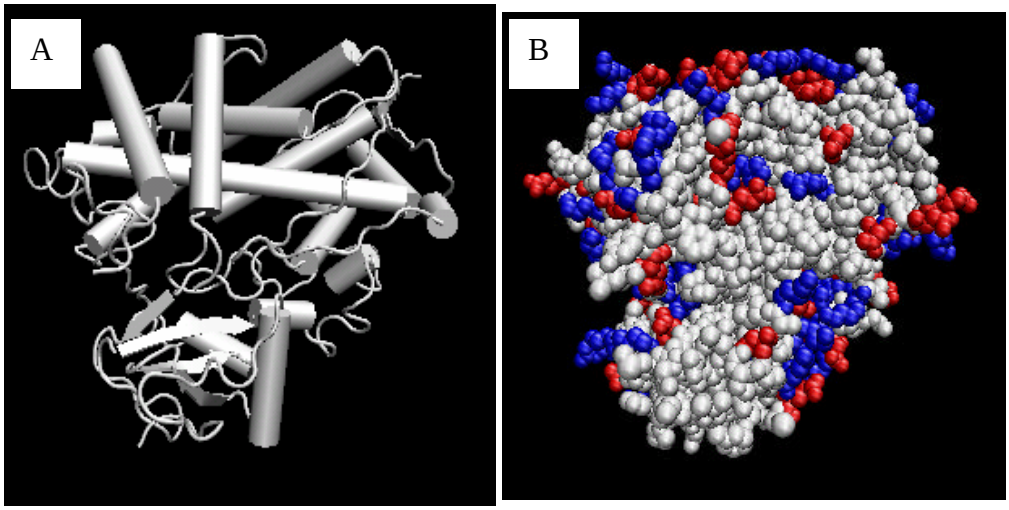


Figure 8: A, cartoon representation of CYP2C5. B, (same view as part A) VDW representation, cluster of hydrophobic residues are seen in the lower portion, near the N-terminus

Structural information on the membrane associated P450 provides a map of charge residues within the enzyme. With knowledge that a surface of cytochrome P450 reductase (CPR) has a largely negative electrostatic potential, the largely positive surface of the 2C5 molecule proximal to the heme provides a good site for interaction of the P450 with its redox partner. In fact, mutagenesis studies that have altered these positive residues have disrupted P450-CPR association. Additionally, the distal surface is negatively charged and the dipole existing between the proximal and distal surface of P450s is thought to guide the CPR to the proximal surface, nearer to the heme, of the P450. Because all family 2 cytochrome P450s (CYP2) interact with CPR, Williams et al. identified residues that were absolutely conserved among the 93 members of the CYP2. When the conserved residues were mapped onto the CYP2C5 crystal structure, the majority of these residues were basic and resided on the proximal face of the enzyme:

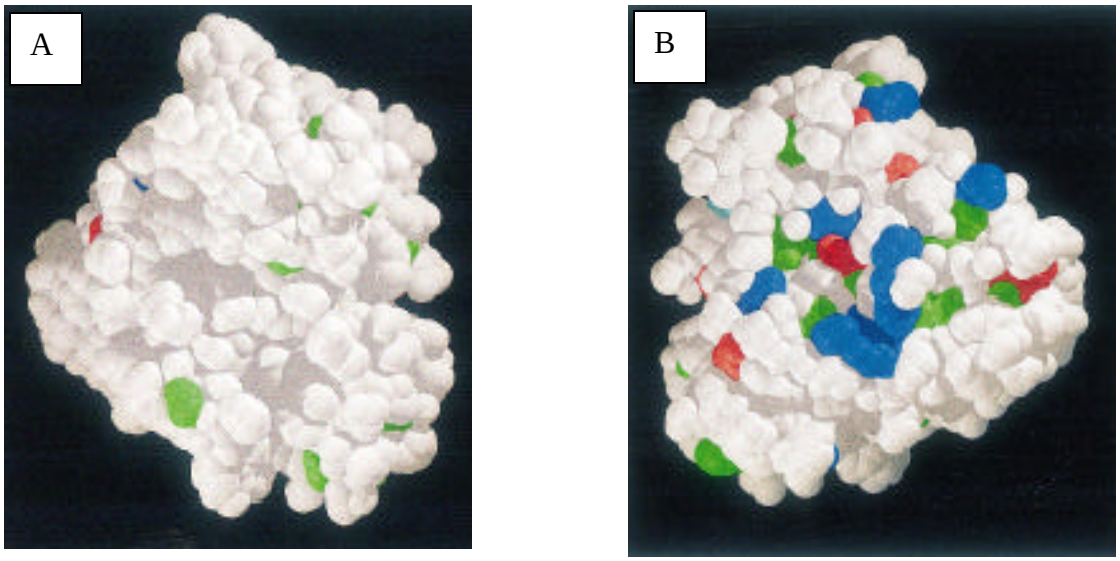


Figure 9: VDW representation of CYP2C5. A, distal face B, proximal face
Note: This figure was taken from Williams et al., Journal of Inorganic Biochemistry 81 (2002) because the residues that are conserved among the CYP2C family are not listed anywhere.

The structure of CYP2C5 serves as an important advancement towards our understanding and characterization of membrane-bound cytochrome P450 monooxygenases to an extent that was previously limited to soluble enzymes. Analysis of the 2C5 structure indicates conservation in topology and in spatial orientation of the heme among P450s. Together with experimental data, the 2C5 structure may serve as a template for other membrane-bound P450s to help identify important residues that mediate substrate specificity and enzyme catalysis. The comparison of the membrane-bound structure to those of soluble P450s is interesting and may help to clarify the evolutionary divergence of this enzyme superfamily.

References:

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* Figures were made with the program VMD, downloaded from www.ks.uiuc.edu/Research/vmd/