Identification of Binding Sites of Non-I-Helix Water Molecules in Mammalian Cytochromes P450

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ABSTRACT:

The cytochromes P450 (P450s) enzymes are integral in determining the disposition of many therapeutic compounds. At the molecular level, the details of P450 catalysis are still under investigation, but the importance of water-mediated enzyme intermediates. The study of P450-bound waters has been largely restricted to bacterial enzymes that may or may not reflect the location or function of waters in human drug-metabolizing P450s. However, in recent years, 16 structures of mammalian P450s containing crystallographic waters have been deposited in the Protein Data Bank. Described herein is the identification of seven well defined water clusters in mammalian P450s identified by calculating the density of globally aligned waters as reported by Tanner and coworkers (Bottoms CA, White TA, and Tanner JJ (2006) Proteins 64:404–421 (DOI: 10.1002/prot.21014)). All water binding sites were in or within the immediate vicinity of the active sites of the P450s, but most were not near the conserved I-helix threonine often implicated in P450 catalysis. Therefore, it is possible that some of the water binding sites identified here ultimately determine P450 catalytic efficiency either by working as an extension of the I-helix water network, or by acting in novel proton shuttles that modulate the nonproductive shunting of reactive oxygen species.

The heme-thiolate enzymes known as cytochromes P450 (P450s) are widely recognized for their role in the metabolism of drugs and other xenobiotics. One property that makes the P450s such versatile catalysts is their ability to generate reactive iron-oxygen species (reviewed in Denisov et al., 2005). Water networks have long been proposed to act as proton shuttles that help the P450s generate various iron-oxygen intermediates during the catalytic cycle (Denisov et al., 2005). Much of the evidence for the existence and role of water networks in P450 catalysis has come from the study of the bacterial iron-oxygen enzyme intermediates. The study of P450-bound waters has been largely restricted to bacterial enzymes that may or may not reflect the location or function of waters in human drug-metabolizing P450s. However, in recent years, 16 structures of mammalian P450s containing crystallographic waters have been deposited in the Protein Data Bank. Described herein is the identification of seven well defined water clusters in mammalian P450s.

Seven well defined water clusters in close proximity to the active site correlate with observed isoform differences in water binding sites. Searches for nearby H-bonding polar backbone and side-chain partners for water clusters were also conducted and seemed to appear critical in controlling water positioning. In addition to being central in P450 catalysis, water is almost certain to affect enzyme stability, dynamics, and substrate binding. However, a substantial hurdle to studying water-protein interactions, even beyond understanding their function, is that of identifying where water actually binds. Until recently, the location of waters or water-binding residues in mammalian, microsomal P450s could only be inferred based on information derived from bacterial enzymes. For instance, it has become apparent that the same highly conserved I-helix threonine residue studied in bacterial P450s is involved in several aspects of catalysis (Vaz, 2001) and the water-mediated reversibility of covalent heme adducts (Blobaum, 2006) in the mammalian P450s 2E1 and 2B4. However, over the past few years, crystallization of mammalian P450s by Williams and coworkers, Johnson, Stout and coworkers, and Halpert and coworkers have greatly added to our knowledge of the drug-metabolizing P450 isoforms by reporting some of their structural features (reviewed in Johnson and Stout, 2005). There are currently 16 crystal structures encompassing seven mammalian isoforms, all with ordered waters, but no reports regarding the positions of these water molecules have been presented.

This work describes an attempt to evaluate whether there may be conserved water molecules and/or water networks in the mammalian xenobiotic-metabolizing P450s. After globally aligning the P450 structures, the three-dimensional coordinates of all the crystallographic waters were analyzed by calculating the density of water clusters. Searches for nearby H-bonding polar backbone and side-chain partners for water clusters were also conducted and seemed to correlate with observed isoform differences in water binding sites. Seven well defined water clusters in close proximity to the active site were identified. The possible roles of these water clusters in P450 structure and function are discussed based on these findings.

Materials and Methods

Hardware and Software. All analyses were carried out on a UNIX workstation using the Mauoster (version 7.5) interface of Schrödinger, LLC (New York).

Abbreviations: P450, cytochrome P450; rmsd, root mean square deviation; pdb, Protein Data Bank.
CNS version 1.1 (Brunger et al., 1998). Water density maps and contouring were visualized with PyMOL version 0.99 (DeLano Scientific, San Carlos, CA).

Alignment of P450 Crystal Structures. The following pdb files were obtained from the Protein Data Bank (http://www.rcsb.org) (Berman et al., 2000): P450"sub" (1DZ4) (Schlichting et al., 2000), CYP2C9 (1O6G and 1O5G) (Williams et al., 2003), CYP2C5 (1N6B) (Wester et al., 2003a), CYP2C5 (1NR6) (Wester et al., 2003b), CYP2B4 (1P05) (Scott et al., 2003), P450_"bam" (1PV0) (Ost et al., 2003), CYP2C9 (1IP0) (Schoch et al., 2004), CYP2C9 (1R90) (Wester et al., 2004), CYP2B4 (1SUO) (Scott et al., 2004), CYP3A4 (1TQNY (Yano et al., 2004), CYP3A4 (1WOF, 1WOG, and 1WOE) (Williams et al., 2004), CYP2A6 (1Z10 and 1Z11) (Yano et al., 2005), CYP2B4 (2BDM) (Zhao et al., 2006), and CYP2D6 (2FQK) (Rowland et al., 2006), representing all of the currently deposited crystal structures of human and rabbit microsomal P450s. In addition, the model bacterial isoforms P450"cam" (CYP101A1) and P450BM3 (CYP102A1) were added for comparison. After trimming the pdb files of any extra polypeptide chains in the unit cell, the files were imported into Maestro with all of their assigned water oxygens. A global structure alignment, performed using the Protein Alignment Tool, also successfully translated the positions of all water oxygens, allowing all coordinates to be updated and saved. The rmsd of the I-helix Cα atoms between any two P450 structures did not exceed 2.0 Å and averaged 0.99 Å between all structures when the 2B4 structure, 2BDM, was excluded. Furthermore, the rmsd values between the heavy atoms of the heme groups averaged 0.96 Å without the 2BDM structure. At this point, the coordinates of the waters from all structures were added to a single pdb file for further analysis.

Water Density Peak Calculation. The density of overlapping water oxygens was calculated exactly as described by Tanner and coworkers (Bottoms et al., 2006) using the program CNS. By allowing CNS to search for water clusters, record their coordinates, and assign a quantitative number describing their density, the identification of potential water binding sites remains objective since no previous knowledge is needed. Four CNS scripts were used to generate the “pseudo-electron density” map and water cluster density by using the theoretical reflections of the pdb file containing the water oxygens. A P1 lattice with unit cell angles of 90 degrees and unit cell lengths of 30 Å was used in the following analysis. The first three scripts (generate_easy.inp, model_f-calc.inp, and make_cv.inp) generate the necessary coordinate, topology, and cross-validated (10% in the test set) reflection files for input into the fourth script, model_map, as described previously (Bottoms et al., 2006). Model_map.inp was then used with a uniform B-factor of 20 Å2 for all water to output a list of coordinates for each water peak, which can be thought of as the average position of waters belonging to a single cluster. The CNS script also lists the density of the water peaks and outputs a density map (2Fo - Fs). Potential H-bond donors and acceptors near the water peaks were manually identified using the polar group distance tool in PyMOL. The 2-Å cutoff was chosen because distances between water oxygens in the water network ranged from 2.0 to 2.10 Å based on the protein structure alignment. Therefore, the assignment of multiple water molecules to a single binding site was avoided. Furthermore, all but three of the waters from all seven water binding sites were less than 2.0 Å from their respective calculated water peaks. There were a total of 66 water molecules in the alignment (excluding I-helix waters bound to unique sites), which means that any given water was correctly assigned to a particular water binding site 95% of the time. In those rare cases where the water oxygen lies 2.0 Å beyond a water peak calculated from our alignment, an examination of the H-bonding partners confirms whether the water belongs to a conserved site, or whether it is unique.

Results and Discussion

The unbiased water cluster analysis method used here with the 13 microsomal P450 and 2 bacterial P450 structures listed in Table 1 identified seven distinct water binding sites in or within a few angstroms of the enzyme active sites. Numbering of the sites follows the N to C terminus direction as closely as possible. Interestingly, the locations of the highest density water binding sites are not associated with the conserved I-helix threonine. In fact, waters near the con-
K-helix and loop between the K-helix and the defining a water network (sites 3, 4, 5, and 6, Fig. 1A) were found largely reflects the mammalian 2 family of P450s. Four clusters were discovered in two other regions of the data set, which likely adds to the more dispersive water density found at water binding site 2 (Schlichting et al., 2000). In contrast, well defined water at sites 3 and 4. In addition, the water at site 5 of P450s 3A4, BM3, and cam explain the absence of water binding site 7 near the loop N-terminal to helix K in P450 2C5 (gray) versus the lack of water in P450s 3A4 (blue) and cam (magenta). The position of this loop in P450s 3A4 and cam explain the absence of water binding site 7. D, comparison of water binding site 1 in P450 2C5 (gray) with P450 3A4 (blue) explains how a lack of polar side chains and a different B-helix structure does not allow P450 3A4 to bind water at this site. Waters aligned from all of the clusters listed in Table 1 are shown as red spheres and the corresponding density map contoured at 5.0σ denotes distinct water binding sites. The heme group is shown in black. H-bonding partners of conserved waters are shown with the numbering of the corresponding enzyme; a complete list is available in Table 1.

Five of the enzymes included in the analysis, P450s 2C8, 2D6, 3A4, BM3, and cam, contained only a few of the waters clustered at sites 1 to 7 (Table 1). For P450s 3A4, BM3, and cam, the absence of polar groups can account for the lack of crystallographic waters in most cases. Threonine and glutamine side chains involved in water binding to site 3 are replaced with valine, leucine, and phenylalanine in these enzymes and, hence, would be predicted to disrupt the water network at sites 3 and 4. In addition, the water at site 5 of P450s 3A4, BM3, and cam forms an H-bond with the enzyme directly, rather than networking with another water, thereby eliminating site 5. P450s 3A4 and cam were the only two enzymes that had significantly different loop structures in the region where site 7 is proposed to exist in the P450 2 family enzymes, and no corresponding water was found at this site in these well hydrated structures (Fig. 1C). No water at site 1 in P450 3A4 is found, presumably because it contains an isoleucine instead of an aspartate in the I-helix and because the position of the helix in the B-C loop allows it to form H-bonds with itself, decreasing the available space for a water to bind (Fig. 1D). Hence, isoforms lack particular water(s) when there is an unfavorable geometry of polar backbone groups or substitution of polar side chains for hydrophobic side chains. When water was absent from any of the water binding sites, there did not appear to be any compensation by polar protein side chains.

Conversely, very few waters were assigned throughout the P450 2C8 and 2D6 structures, and it is speculated that this may be due to their lower resolution (>2.8 Å) rather than a lack of water binding sites, since the positions and residues at most of the water binding sites are the same as the 2C and 2B isoforms. Water was also sparse in the lower resolution P450 3A4 structures reported by Williams et al. (2004), so that these enzymes were excluded from the analysis.

The locations of the non-I-helix waters may prove to be significant for several reasons. First, site 5 appears to be conserved across species, although this might not be detected by sequence alignments since the H-bonding partners are either other waters or backbone groups of different residues (e.g., Pro or Thr). This region is also suspiciously close to two positions of naturally occurring P450 2C9 variants, 2C9.3 (I359L) and 2C9.5 (D360E), and might explain, in part, the altered kinetic parameters of these enzymes (Fig. 1A) (Dickmann et al., 2001). For example, the D360 side chain forms part of the K-helix, the seemingly conservative D360E substitution may produce changes in both the water network and tertiary structure of P450 2C9. Second, the location of a water binding site in the B-C loop (site 1) may enable a water to bind directly with substrate or help control the egress of metabolites of the P450 because this glycine-rich region has been postulated to be flexible and to adopt multiple conformations (Wester et al., 2003b; Honma et al., 2005; Li et al., 2005). Third, like site 1, water binding site 7 is in a position where it could regulate the structure or stability of a loop (region between the two β3 strands) that
WATER BINDING SITES IN MAMMALIAN P450s

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Abstract

This is the abstract of the paper.

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2. Results

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References

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