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Archives of Biochemistry and Biophysics 454 (2006) 42–54
ABB
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Cysteine 98 in CYP3A4 contributes to conformational integrity required for P450 interaction with CYP reductase ☆

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Received 18 June 2006, and in revised form 31 July 2006
Available online 22 August 2006

Abstract

Previously human cytochrome P450 3A4 was efficiently and specifically photolabeled by the photoaffinity ligand lapachenole. One of the modification sites was identified as cysteine 98 in the B–C loop region of the protein [B. Wen, C.E. Doneanu, C.A. Gartner, A.G. Roberts, W.M. Atkins, S.D. Nelson, Biochemistry 44 (2005) 1833–1845]. Loss of CO binding capacity and subsequent decrease of catalytic activity were observed in the labeled CYP3A4, which suggested that aromatic substitution on residue 98 triggered a critical conformational change and subsequent loss of enzyme activity. To test this hypothesis, C98A, C98S, C98F, and C98W mutants were generated by site-directed mutagenesis and expressed functionally as oligohistidine-tagged proteins. Unlike the mono-adduction observed in the wild-type protein, simultaneous multiple adductions occurred when C98F and C98W were photolabeled under the same conditions as the wild-type enzyme, indicating a substantial conformational change in these two mutants compared with the wild-type protein. Kinetic analysis revealed that the C98W mutant had a drastic 16-fold decrease in catalytic efficiency (V_{max}/K_m) for 1′-OH midazolam formation, and about an 8-fold decrease in catalytic efficiency (V_{max}/K_m) for 4-OH midazolam formation, while the C98A and C98S mutants retained the same enzyme activity as the wild-type enzyme. Photolabeling of C98A and C98S with lapachenole resulted in monoadduction of only Cys-468, in contrast to the labeling of Cys-98 in wild-type CYP3A4, demonstrating the marked selectivity of this photoaffinity ligand for cysteine residues. The slight increases in the midazolam binding constants (K_s) in these mutants suggested negligible perturbation of the heme environment. Further activity studies using different P450:reductase ratios suggested that the affinity of P450 to reductase was significantly decreased in the C98W mutant, but not in the C98A and C98S mutants. In addition, the C98W mutant exhibited a 41% decrease in the maximum electron flow rate between P450 and reductase as measured by reduced nicotinamide adenine dinucleotide phosphate consumption at a saturating reductase concentration. In conclusion, our data strongly suggest that cysteine 98 in the B–C loop region significantly contributes to conformational integrity and catalytic activity of CYP3A4, and that this residue or residues nearby might be involved in an interaction with P450 reductase.
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Keywords: Photoaffinity labeling; Cytochrome P450 3A4; Conformational integrity

Cytochrome P450 (P450) 3A4, the major P450 isoform present in human liver, is involved in the metabolism of more than 50% of clinically used drugs, making it one of the single most important human xenobiotic-metabolizing enzymes [1]. The large binding pocket in CYP3A4 can accommodate a wide variety of structurally diverse substrates while maintaining remarkable regioselectivity and stereoselectivity with many compounds. For example, the enzyme catalyzes the 1′- and 4-hydroxylation of midazolam [2], the 2β-, 6β-, and 15β-hydroxylation of testosterone, the 6β- and 16α-hydroxylation of progesterone [3], and the M1-, M17- and M21-oxidation of cyclosporine A [4]. The size of

☆ This work was supported by NIH Grant GM32165 (to S.D.N.) and the UW NIEHS sponsored Center for Ecogenetics and Environmental Health: NIEHS P30ES07033.
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0003-9861/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2006.08.003

the active site may also be responsible for the phenomenon of cooperativity that could be clinically significant due to the role it can play in enhancing drug–drug interactions [5]. Because of its general importance in drug metabolism and carcinogen bioactivation, elucidation of the key structural elements responsible for substrate recognition leading to oxidation by CYP3A4[1] is of considerable interest.

Despite the wealth of information on the importance, regulation, and substrate specificity of the cytochrome P450 3A subfamily, structure–function analysis of these enzymes is only now being rigorously approached. A major step toward this goal has recently been achieved with the determination of high-resolution structures by X-ray crystallography of modified forms of human CYP3A4 [6,7]. However, X-ray crystallography, as important as it is, primarily provides information on static CYP3A4 structures. Several other techniques also have been employed to characterize P450 active sites and substrate binding sites for enzyme–substrate complexes, including spectroscopic analysis [8,9], site-directed mutagenesis [10–12], photoaffinity labeling [13,14] and mechanism-based inhibition [15,16]. Unlike cytochromes P450 of family 2, CYP3A enzymes within or across species exhibit few dramatic substrate specificity differences that could provide obvious leads for site-directed mutagenesis of particular residues. Most of our present knowledge has come from studies using a combination of site-directed mutagenesis and molecular modeling based upon alignment of sequences with bacterial P450s [17,18]. Additional studies by Halpert and colleagues with the use of alanine-scanning mutagenesis identified some amino acid residues necessary for substrate specificity and flavonoid activation of CYP3A4 [19].

Photoaffinity labeling, like mechanism-based inhibition, has the advantage of providing direct information concerning active site topology, and photoaffinity ligands (PALs) do not require enzymatic activity to be utilized as active site probes [20]. In our previous work [21,22], CYP3A4 was efficiently and specifically photolabeled by the substrate and PAL, lapachenole. Cysteine 98 in the B–C loop region was subsequently identified as one of the modification sites using a combination of techniques, including tandem mass spectrometry. The labeled protein showed loss of CO binding capacity and a dramatic decrease of catalytic activity, which strongly suggested that large aromatic substitutions on residue 98 triggered critical enzyme conformational changes and subsequent loss of catalytic activity [21].

In the present work, we have tested this hypothesis by generating different substitutions on position 98 of CYP3A4 using site-directed mutagenesis. One goal was to use a mutational substitution to mimic the labeling effects of lapachenole on the wild-type protein. Our results strongly suggest that cysteine 98 in the B–C loop region significantly contributes to conformational integrity and catalytic activity of CYP3A4, and that this residue or residues nearby might be involved in an interaction with P450 reductase.

Experimental procedures

Materials

MDZ, NADPH, 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate (CHAPS), HEPES, EDTA, reduced glutathione (GSH), imidazole, Triton X-100 and CF_3CO_2H (TFA) were purchased from Sigma (St. Louis, MO). Lapachenole was chemically synthesized in our laboratory (22). 1′-OH MDZ and 4-OH MDZ were obtained from GENTEST (Bedford, MA). L-α-Dilaurylphosphatidylcholine (DLPC), L-α-dioleoylphosphatidylcholine (DOPC), and L-α-Dilaurylphosphatidylserine (DLPS) were purchased from Avanti Polar lipids Inc. (Alabaster, AL). The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Slide-A-Lyzer dialysis cassettes were from Pierce (Rockford, IL). A POROS R2 perfusion column was from Applied Biosystems (Framingham, MA). HPLC solvents were of the highest grade commercially available and were used as received. All other reagents were analytically grade.

Mutagenesis and expression of CYP3A4

Recombinant CYP3A4 was produced in *Escherichia coli* XL1-Blue cells using the expression vector pCW 3A4-His6 kindly provided by Dr. Ron Estabrook (University of Texas SW Medical Center, Dallas, TX). The same plasmid pCW 3A4-His6 was used as the template for amplification reactions with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotide primers used in the mutagenesis procedure were as follows (mismatches indicated by the underlined bases): C98A forward, 5′ CTAGTGAAAGAAGCTTATTCTGTCTTCACAAACC 3′; C98A reverse, 5′ GGTTTGTGAAGACAGAATAAGCTTCTTTCACTAG 3′. C98S forward, 5′ AAACAGTGCTAGTGAAAGAAAGTTATTCTGT CTTCACAAACCG 3′; C98S reverse, 5′ CGGTTTGTGAAGACAGAA TAACTTTCTTTCACTAGCACTGTTT 3′. C98F forward, 5′ CTAGT GAAAGAATTCTATTCTGTCTTCACAAACC 3′; C98F reverse, 5′ G GTTTGTGAAGACAGAATAGAATTCTTTCACTAG 3′. C98W forward, 5′ GCTAGTGAAAGAATGGTATTCTGTCTTCACAAACC 3′; C98W reverse, 5′ GGTTTGTGAAGACAGAATACCATTCTTTCACT AGC 3′. *Dpn*I digested DNA was transformed into XL1-Blue cells, and DNA from several of the resulting colonies was isolated. The cDNA sequence was checked for the presence of the desired mutation and the absence of extraneous mutations (University of Washington Sequencing Facility).

Growth and induction of *E. coli* were performed as described by Gillam et al. [23]. Solubilized membranes were prepared and the P450s were purified on ProBond nickel resin columns (Invitrogen) under conditions described previously [21]. The column was washed with 20 column volumes of wash buffer: 100 mM Tris–HCl, pH 7.4, 20% glycerol, 40 mM imidazole, 0.05% cholate, and 50 μM testosterone. The column was eluted with a minimal volume of elution buffer: 100 mM Tris–HCl, pH 7.4, 20% glycerol, 500 mM imidazole and 0.02% cholate. The eluted protein was dialyzed against 100 mM potassium phosphate, pH 7.4, in 20% glycerol, and stored at −80 °C. Total protein concentrations were determined by the bicinchoninic acid method. Bovine serum albumin was used as a standard [24]. SDS-polyacrylamide gel electrophoresis was done according to the procedure of Laemmli [25]. Cytochrome P450 content was determined by

[1] *Abbreviations used:* P450, human liver microsomal cytochrome P450; CYP3A4, histidine-tagged cytochrome P450 3A4; b_5, human cytochrome b_5; P450 reductase, rat NADPH-cytochrome P450 reductase; MDZ, midazolam; APAP, acetaminophen; PAL, photoaffinity ligand; DLPC, L-α-Dilaurylphosphatidylcholine; DLPS, L-α-Dilaurylphosphatidylserine; DOPC, L-α-dioleoylphosphatidylcholine; CHAPS, 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate; GSH, reduced glutathione; HPLC/ESI MS, high-performance liquid chromatography electrospray ionization mass spectrometry; WT, wild type; TFA, CF_3CO_2H; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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