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CYP2E1 active site residues in substrate recognition sequence 5 identified by photoaffinity labeling and homology modeling ☆

Samuel L. Collom, Arvind P. Jamakhandi, Alan J. Tackett, Anna Radominska-Pandya, Grover P. Miller *

Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, 4301 W. Markham St. Slot 516, Little Rock, AR 72205, USA

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Abstract

Despite its biological importance, our knowledge of active site structure and relevance of critical amino acids in CYP2E1 catalytic processes remain limited. In this study, we identified CYP2E1 active site residues using photoaffinity labeling with 7-azido-4-methylcoumarin (AzMC) coupled with a CYP2E1 homology model. In the absence of light, AzMC was an effective competitor against substrate *p*-nitrophenol oxidation by CYP2E1. Photoactivation of AzMC led to a concentration-dependent loss in CYP2E1 activity and structural integrity resulting from the modification of both heme and protein. The photo-labeling reaction degraded heme and produced a possible heme adduct. Probe incorporation into the protein occurred at multiple sites within substrate recognition sequence 5 (SRS-5). Based on a CYP2E1 homology model, we hypothesize AzMC labels SRS-5 residues, Leu363, Val364, and Leu368, in the active site. In addition, we propose a series of phenylalanines, especially Phe106, mediate contacts with the coumarin.
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Microsomal cytochrome P450 (CYP)[1] enzymes (EC 1.14.14.1) are major catalysts in the oxidative transformation of a structurally diverse class of compounds including steroids, fatty acids, hormones, antibiotics, and a wide variety of artificially produced chemicals in Phase I metabolism of xenobiotic compounds, e.g., drugs, food additives, and environmental contaminants [1]. CYPs convert lipid-soluble molecules to more water-soluble forms and in effect, modulate transport and other chemical properties. To accommodate a wide array of compounds, typical CYP catalysts have evolved low specificity and activity toward substrates, making interpreting and predicting catalytic properties difficult. An important step forward in the field of xenobiotic metabolism would be a deeper understanding of the structure of the active site mediating substrate recognition and product profile for theses compounds.

In common with other members of the CYP1-3 families, CYP2E1 has broad substrate specificity. More than 70 different chemicals with diverse structures have been identified as substrates for CYP2E1 [2,3]; nevertheless, these molecules are typical small (molecular weight <100) and hydrophobic in character. Among the substrates are alcohols/ketones/aldehydes (e.g., ethanol), nitrosamines, alkanes, halogenated alkanes, and anaesthetics. CYP2E1 also recognizes some molecules possessing one or two rings including benzene, caffeine, acetaminophen, isoniazid, and

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* Corresponding author. Fax: +1 501 686 8169.
E-mail address: millergroverp@uams.edu (G.P. Miller).

[1] *Abbreviations used:* CYP2E1, cytochrome P450 2E1; CPR, cytochrome P450 reductase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pNP, *p*-nitrophenol; pNC, *p*-nitrocatechol; 7HC, 7-hydroxy-4-methylcoumarin; AzMC, 7-azido-4-methylcoumarin; nMC, 7-nitrene-4-methylcoumarin; 7EFC, 7-ethoxy-4-trifluoromethylcoumarin; CHZ, chlorzoxazone; $NADP^+$, nicotinamide adenosine dinucleotide phosphate (oxidized); SRS, substrate recognition sequence.

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resveratrol with *p*-nitrophenol (pNP) [4] and chlorzoxazone [5] being considered typical marker substrates. In addition to xenobiotic metabolism, growing evidence supports an important physiological role for CYP2E1 in gluconeogenesis [3]. CYP2E1 is regulated similarly to enzymes contributing to gluconeogenesis in relation to starvation and diabetes and in fact, recognizes precursors to gluconeogenesis, acetone, acetol (1-hydroxyacetone), and fatty acids [6] as substrates. Taken together, these findings implicate a degree of selectivity for the CYP2E1 activity through a restrictive active site.

Although great strides have been made to determine the X-ray crystallographic structure of several microsomal CYPs [7–9], not all enzymes are amenable to this approach resulting in a continued challenge to define the structure of CYP active sites. Through the alignment of sequences from known members of the CYP2 family, Gotoh [10] assigned possible substrate recognition sites (SRS), which have formed the basis of studies by other groups. Based on those types of analyses, several groups have used site-directed mutagenesis to explore the role of functional residues [11–13]. An alternative technique utilizes the catalytic ability of the enzyme to convert a bound substrate to a reactive product capable of modifying the active site protein or heme [14,15]. Analysis of the site of labeling implicates residues in contact with the substrate or the accessibility of the heme to the substrate. Similarly, the use of photoactivatible substrate analogs provides a methodology to modify the CYP active site following exposure of the bound complex to light. Photoaffinity labeling of CYP1A2 [16] and CYP2B4 [17] have provided valuable information toward their respective substrate binding sites. In contrast, our knowledge concerning active site structure and the relevance of critical amino acids in CYP2E1 catalytic processes remain limited.

In the absence of a known structure for CYP2E1, we employed 7-azido-4-methylcoumarin (AzMC) as a probe for identifying active site residues that participate in the recognition of the relatively large two-ringed CYP2E1 substrates, e.g., chlorzoxazone [5], quinoline [18], and coumarin [19]. This photoaffinity probe has been used successfully to characterize the active sites of the Phase II xenobiotic-metabolizing enzymes, SULT1A1 [20] and UGT1A6 [21]. To demonstrate the specificity of AzMC for rabbit CYP2E1, we assayed the ability of the reagent to compete with *p*-nitrophenol (pNP) during the oxidation of pNP to *p*-nitrocatechol (pNC). Photoactivation of AzMC led to the breakdown of the azido group of AzMC to form N_2 and the reactive 7-nitrene-4-methylcoumarin (nMC), which can insert into organic molecules, e.g., CYP2E1 protein and heme. The effect of incorporation on CYP2E1 structural integrity was determined by measuring the reduced CO spectra for CYP2E1, a hallmark for competent CYP enzymes, and the presence of intact heme by HPLC. To identify site of incorporation in the CYP2E1 protein, we digested the modified protein and subjected the fragments to MALDI-MS analysis. As a complement to this approach, we generated a CYP2E1 homology model to confirm the location of active site residues identified by photoaffinity labeling and provide insights to other potential active site residues.

Materials and methods

Reagents

C41 (DE3) cells used in CYP2E1 expression were purchased from Avidis (France). Topp3 cells, which are no longer commercially available, were propagated in the laboratory. Terrific broth modified for genomics was bought from US Biological (Swampscott, MA). Protein purification resins, 2′, 5′-ADP–agarose and Reactive Red 120 Type 3000-CL, were obtained from Sigma–Aldrich (St. Louis, MO). SP–Sepharose was purchased from GE Healthcare Life Sciences (Piscataway, NJ). Components of the NADPH regenerating system ($NADP^+$, glucose 6-phosphate, and torula yeast glucose 6-phosphate dehydrogenase) were purchased from Sigma–Aldrich. In addition, dilauroyl-L-α-phosphatidylcholine, *p*-nitrophenol, *p*-nitrocatechol, 2-nitroresorcinol, bovine erythrocyte superoxide dismutase, catalase, and sodium dithionite (hydrosulfite) were obtained from Sigma–Aldrich. In addition to HPLC grade acetonitrile (CH_3CN) and trifluoroacetic acid, ampicillin, isopropyl-β-D-thiogalactopyranoside, lysozyme, diethylaminoethyl cellulose (DEAE), dithiothreitol (DTT), protease inhibitors, and other basic chemicals were purchased from Fisher Scientific (Houston, TX). The photoaffinity probe, 7-azido-4-methylcoumarin (AzMC) was synthesized as described previously [21]. Purified rabbit liver cytochrome b_5 was provided as a generous gift from Wayne L. Backes (LSU Health Science Center, New Orleans).

Construction and expression of rabbit CPR-K56Q

A proteolytically-resistant mutant form of rabbit CPR-K56Q was prepared by site-directed mutagenesis and then expressed in Topp3 *Escherichia coli* cells (Stratagene). The pSC-WT (gift from Lucy Waskell, Univ. of Michigan Medical School) expression vector was used for site-directed mutagenesis using QuickChange (Stratagene, La Jolla, CA) protocol. Oligonucleotide primers used in the generation of CPR-K56Q were as follows (mismatch indicated by the underlined base): rabCPR-K56Q-For 5′-CCCGAGTTCACCCAGATCCAGGCCCCGACG-3′; rabCPR-K56Q-Rev 5′-CGGGGCCTGGATCTGGGTGAACTCGGG CAC-3′. Expression of pSC-rabCPR-K56Q vector was carried out using a slight modification of the procedure described by Hanna et al. [22,23]. In brief, CPR eluted from 2′,5′-ADP–agarose column was loaded on to a pre-equilibrated DEAE column and washed extensively (40 column volumes) with buffer containing 20 mM potassium phosphate pH 7.6, 1 mM EDTA and 20% glycerol to remove residual amounts of detergents from the protein sample. Bound CPR was eluted with buffer containing 50 mM potassium phosphate, pH 7.6, 300 mM KCl, 1 mM EDTA, and 20% glycerol. CPR elutes in a small volume which was then applied on Zeba desalting spin column (Pierce Biotechnology, Rockford, IL) pre-equilibrated with 50 mM potassium phosphate, pH 7.4, 1 mM EDTA, and 20% glycerol. The flow through from the desalting column was quantitated spectrophotometrically using the extinction coefficient for difference in absorbance between 456 and 690 nm (baseline) ($\varepsilon = 21\ mM^{-1}\ cm^{-1}$) [24]. Protein was then aliquoted into 2 mL screw-capped tubes and stored at −80 °C. Apart from using protease inhibitor cocktail, 0.4 KIU/mL aprotinin was included in all buffers used throughout the purification protocol to prevent N-terminal degradation of CPR.

Preparation of CYP2E1 protein

Rabbit CYP2E1 was expressed in *E. coli* C41 (DE3) (Avidis, France) strain transformed with the pLW01/2E1 expression plasmid (gift from W.L. Backes, LSU, NO) using a modification of the procedure described by Cheng et al. [25]. Transformed cells were grown in special terrific broth

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