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Access channels to the buried active site control substrate specificity in CYP1A P450 enzymes

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ABSTRACT

Background: A cytochrome P450 active site is buried within the protein molecule and several channels connect the catalytic cavity to the protein surface. Their role in P450 catalysis is still matter of debate. The aim of this study was to understand the possible relations existing between channels and substrate specificity.
Methods: Time course studies were carried out with a collection of polycyclic substrates of increasing sizes assayed with a library of wild-type and chimeric CYP1A enzymes. This resulted in a matrix of activities sufficiently large to allow statistical analysis. Multivariate statistical tools were used to decipher the correlation between observed activity shifts and sequence segment swaps.
Results: The global kinetic behavior of CYP1A enzymes toward polycyclic substrates is significantly different depending on the size of the substrate. Mutations which are close or lining the P450 channels significantly affect this discrimination, whereas mutations distant from the P450 channels do not.
Conclusions: Size discrimination is taking place for polycyclic substrates at the entrance of the different P450 access channels. It is thus hypothesized that channels differentiate small from large substrates in CYP1A enzymes, implying that residues located at the surface of the protein may be implied in this differential recognition.
General significance: Catalysis thus occurs after a two-step recognition process, one at the surface of the protein and the second within the catalytic cavity in enzymes with a buried active site.

1. Introduction

Amino acid residues important for substrate recognition are generally expected to lie close or among those flanking the active site; molecular docking is a particularly vivid application of this [1–4]. However, recent works, particularly in the field of directed *in vitro* evolution, revealed that residues located at the periphery of protein could also play a critical role in controlling substrate specificity shifts as observed with promiscuous activities [5–7]. It has also been shown that combinations of amino acid residues, not located only in the active site, control a network of coupled motions that facilitate catalytic activity [8].

Cytochrome P450s (P450s) are heme *b*-thiolate enzymes, found in all kingdoms of nature, that catalyze mono-oxygenation reactions by the insertion of one oxygen atom from dioxygen into a hydrophobic substrate, the other atom being reduced to water [9,10]. In animals, microsomal P450s play a key role in the oxidative phase of the detoxification metabolism converting the majority of drugs, procarcinogens, environmental pollutants and plant secondary metabolites brought by food [11,12]. This work relies on the mammalian CYP1A subfamily consisting of P450 proteins of particularly high sequence similarity. Human CYP1A1 and CYP1A2 proteins are 73% identical in amino acid sequences [13,14], and because of this CYP1A enzymes have attracted a considerable amount of interest due to their involvement in procarcinogen activation and drug metabolism [15]. Since CYP1A enzymes are highly similar in sequences, the number of potential structural elements that explain any difference in functional properties should be limited.

The study of enzymes with a buried active site such as haloalkane dehalogenases and cytochrome P450s [16,17], has shown that no visible path can be seen in the crystal structure for the substrate to reach the catalytic cavity from the surface of the protein, yet substrates indeed reach the active site. This raised the problem of looking for access channel(s) in the protein structure [18]. Moreover, some residues forming the entrance or the wall of the channel could therefore be implied in a primary selection of the substrate before it reaches the active site. To investigate this possibility, we developed an approach mixing a

Abbreviations: 9MAN, 9-methyl-anthracene; ANT, anthracene; BaP, benzo[*a*]pyrene; BCA, bicinchoninic acid; DMF, dimethylformamide; DPHE, 9,10-dihydrophenanthrene; EFEE, 7-ethoxy-fluorescein ethyl ester; EOR, 7-ethoxy-resorufin; LC, liquid chromatography; MDS, multidimensional scaling; MOR, 7-methoxy-resorufin; MPHE, 4,5-methylene-phenanthrene; NAP, naphthalene; P450, cytochrome P450; PAH, polycyclic aromatic hydrocarbon; PCA, principal component analysis; PHE, phenanthrene; PYR, pyrene; RAMD, random acceleration molecular dynamics; VdW, Van der Waals; wt, wild-type

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combinatorial library of chimeric enzymes built from four parental CYP1A enzymes and a combinatorial library of 25 polycyclic substrates, three of the alkoxyaryl class and 22 belonging to the class of polycyclic aromatic hydrocarbons (PAHs). It was hypothesized that shuffling sequence elements could help to identify combinations of amino acid residues critical for substrate specificity through the analysis of substrate specificity changes induced from one variant to the next. The identified sequence elements were compared to the location of putative substrate access channels found by using CAVER [19,20]. A network of channels thus seems to be involved in the control of global substrate specificity in P450 enzymes of the CYP1 family by exhibiting different substrate specificity from one channel to the other depending on the size of the substrate. It is thus proposed that distinct channels would be specific of small *versus* large substrates.

2. Materials & methods

2.1. Substrates

The polycyclic series comprises twenty-two aromatic hydrocarbons and three fluorogenic alkoxyaryl compounds. Naphthalene was from Merck. 2-Methyl- and 9-methyl-anthracenes, 9-vinyl-anthracene, 9-phenyl-anthracene, 9,10-dihydrophenanthrene, 4,5-methylenephenanthrene, benzo[*e*]pyrene, benzanthracene, triphenylene, fluorene, fluoranthene, benzo[*a*]- and benzo[*b*] fluorenes, 7,12-dimethyl-benzanthracene, and pyrene were from Fluka. Anthracene, chrysene, acenaphthene, *trans*-stilbene, benzo[*a*]pyrene, and phenanthrene were from Sigma. The two 7-alkoxyresorufins (MOR and EOR), 7-ethoxyfluorescein-ethylester (EFEE) and NADPH were from Sigma. The different substrates were solubilized either in methanol (since ethanol is a known CYP1A inhibitor) or in dimethylformamide (DMF).

2.2. Plasmids and yeast strains

Vectors p1A1/V60 and p1A2/V60 for human wt CYP1A expression, pP1V8 for mouse wt 1A1 expression and pLM4V8 for rabbit wt 1A2 expression were described before [21,22]. The pYeDP60 vector (V60) contains both *URA3* and *ADE2* as selection markers, whereas pYeDP8 (V8) only bears *URA3*. The inserted coding sequence is placed under the transcriptional control of a *GAL10-CYC1* hybrid artificial promoter and *PGK* terminator. *Saccharomyces cerevisiae* W(R) strain is a derivative of the W303-1B which, when cultivated onto galactose, overexpresses yeast NADPH-P450 reductase which, in turn, optimizes the activities of any recombinant P450 [23]. Transformations, culture media, cell cultures, and galactose induction procedure of individual clones were as described previously [24].

2.3. Chimeric CYP1A enzyme variants

Four parental P450s were used in DNA shuffling experiments: human CYP1A1 and CYP1A2, mouse CYP1A1 and rabbit CYP1A2 coding sequences. The CYP1A variants were obtained by using three types of sequence shuffling of increasing complexity as described previously [25] (see Fig. S1 in Supplementary information). Only variants found functional were kept for further analysis. The two first shuffling methods each yields a library of increasing complexity ranging from bi- or tripartite chimera up to highly mosaic structures (average 5–6 crossovers per sequence). The bi- or tri-partite 1ACh chimeras were obtained by *in vivo* gap-repair technology between mouse CYP1A1 and rabbit CYP1A2 [26]. The mosaic 1AMo variants were obtained using the mixed *in vivo–in vitro* CLERY recombination procedure between human CYP1A1 and CYP1A2 [27]. The third sequence shuffling method consisted in segment-directed saturated mutagenesis targeted to the segment 202–214 of human CYP1A1. This sequence segment was targeted because it is found as hypervariable between CYP1A1s and CYP1A2s [28]. This last procedure resulted in 1AMu variants that exhibit an average of 4–5 amino acid positions mutated per sequence, all the rest of the sequence being that of human CYP1A1.

2.4. Microsomal fraction preparation

Briefly, yeast cells were harvested by centrifugation, suspended and washed in a 50 mM Tris–HCl, 1 mM EDTA and 0.6 M Sorbitol buffer Q8 pH 7.3. Cells were disrupted by manual shaking with 0.4-mm diameter glass beads. Cellular debris were removed by centrifugation (10 min at 10,000 rpm). The supernatant was transferred to another centrifuge tube, and NaCl and PEG4000 were added at final concentrations of 0.1 M and 10% respectively, and kept on ice for 30 min. The precipitated microsomes were then pelleted by centrifugation 10 min at 10,000 rpm, washed, and resuspended in a 50 mM Tris–HCl, 1 mM EDTA, and 20% Glycerol, pH 7.4 buffer and stored in −80 °C [24]. Microsomal protein Q9 concentration was determined with the bicinchoninic acid (BCA) assay using bovine serum albumin as a standard.

2.5. Enzyme activities

Alkoxyresorufin-*O*-dealkylase (λexc = 530 nm, λem = 586 nm) and ethoxyfluorescein ethyl ester *O*-deethylase (λexc = 479 nm, λem = 560 nm) activities were measured fluorimetrically as described previously [24]. Incubations with PAH substrates were initiated by NADPH addition and quenched with trifluoroacetic acid (1:60 by vol.). Microsomal fractions of recombinant yeast clones each expressing a particular CYP1A enzyme were assayed with the 22 PAH substrates in a 0.30-mL reaction mixture containing 0.2–0.6 mg/mL protein from yeast microsomal fractions each expressing a unique wt or variant CYP1A proteins, 0.3 mg/mL protein from yeast microsomal fractions expressing recombinant human microsomal epoxide hydrolase, 0.2 mM NADPH, and a saturating concentration of substrate delivered as a methanolic or a DMF solution (final concentration MeOH 0.7% or DMF 0.3%) in a Tris–HCl 50 mM, EDTA 1 mM buffer (pH 7.4) at 28 °C. Q10 The concentration of incubation mixtures in recombinant P450 was ranging from about 5 up to 30 nM as assessed by CO-reduced differential spectrum on some preparations. The concentration of incubation mixtures in yeast microsomal protein was ranging from 0.2 to 0.6 mg/mL for yeast microsomal fractions containing a unique recombinant wt or variant CYP1A enzyme and, for assays with epoxide hydrolase added, 0.3 mg microsomal protein/mL from yeast microsomal fractions expressing recombinant human microsomal epoxide hydrolase. With naphthalene, a 0.5 mM initial substrate concentration was used. With anthracene and its 9-methyl derivative, phenanthrene and its methylene- and dihydro-derivatives, and pyrene, the substrate initial concentration was 130 μM. When incubation was carried out with benzo[*a*]pyrene, the initial concentration of the substrate was 25 μM (delivered as a DMF solution) due to the solubility limit. The acidified mixtures were centrifuged at 10,000 rpm for 10 min and an aliquot of the supernatant (10–30 μL) was analyzed by HPLC separation. Microsomal hydroxylation reactions were shown to be strictly NADPH-dependent. Assays were duplicated using at least two independent microsomal preparations and hidden replicates were included.

2.6. Analytical LC methods

The metabolites were separated at 40 °C and analyzed in an Alliance HT2795 HPLC Waters module onto a Spheri-5 RP18 5 μm Brownlee column (4.6 × 100 mm). The solvent system consisted of H_2O + 0.01% formic acid (by vol.) in acetonitrile + 0.01% formic acid (by vol.) at a flow rate of 1.0 mL/min. Three different separation procedures differing by the total runtime were used depending on the PAH molecule studied. Procedure 1 (total run time of 29 min) corresponds to a gradient from water containing 0.02% trifluoracetic acid to 50% acetonitrile (by volume) in 20 min, followed by a 4 min elution in 100% acetonitrile.

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