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Biochimica et Biophysica Acta 1770 (2007) 446–457
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbagen

A combinatorial approach to substrate discrimination in the P450 CYP1A subfamily

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Received 28 April 2006; received in revised form 22 July 2006; accepted 8 August 2006
Available online 10 August 2006

Abstract

A comparison of all known mammalian CYP1A sequences identifies nineteen sequence regions that are conserved within all 1A1s or within all 1A2s but at the same time systematically differ between any 1A1 and any 1A2. The purpose of this study was to explore links between these specific CYP1A sequence signatures and substrate specificity shift through the kinetic analysis of combinatorial variants of increasing complexity. The less complex variants correspond to multiple mutations within a short segment of their sequence. The more complex variants correspond to mosaic P450s recombining 1A1 and 1A2 sequences (up to 5 crossovers per sequence). Fifty-eight such functional CYP1A variants and parental wild-type enzymes were expressed in yeast and assayed with 7-alkoxyresorufins and ethoxyflurorescein ethyl ester as substrates. Observed kinetic data were analyzed by multivariate statistical analyses and hierarchical clustering in order to highlight correlations and identify potential sequence–activity relationships within the three-dimensional function space investigated. Several variants are outliers in these representations and show a redistribution of their substrate specificity compared to wild-type CYP1As. Some combinations of sequence elements were identified that significantly discriminate between 1A1 and 1A2 for these three substrates. The comparison of this combinatorial approach with previous results of site-directed mutagenesis is discussed.
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Keywords: Multivariate statistic; CYP1A1; CYP1A2; Mutant; DNA shuffling; Random mutagenesis; Resorufin; Fluorescein; ProSAR

1. Introduction

Combinatorial mutagenesis approaches have paved the way to the directed evolution of proteins with great success [1–5]. Recently, a mutant of mammalian glutathione S-transferase was identified by applying multivariate statistical tools to screening a library of mutants through the multidimensional structure–activity they exhibit [6]. The purpose of this study is to extend such an approach to the exploration of the multidimensional structure–activity space characterizing the cytochrome P450 (P450) superfamily on a library of CYP1A variants of increasing complexity. In animals, P450s play a key role in the oxidative phase of detoxification metabolism, converting the majority of drugs, procarcinogens, environmental pollutants and plant secondary metabolites brought by food [7,8]. Some animal P450s also metabolize endogenous compounds [9]. One peculiar aspect of microsomal P450s is their potential to accommodate a large number of different substrates in the catalytic cavity, leading to non-classical enzymatic behaviour in some cases [10]. Rendering the situation still more complex to analyze is the fact that for each substrate, multiple metabolites are frequently produced. P450 substrate specificity is a major issue for drug development and chemical toxicity, and is controlled by a complex combination of substrate recognition events, induced haem iron spin-state changes, oxido-reduction potential shifts, that together can give rise to a delicate balance between abortive and productive catalytic cycles [11]. Such a complex set of events generally makes most studies of mutation–activity relationships delicate to rationalize and difficult to predict. However, a promising approach is to explore the P450 function space by using a library of multiply mutated variants, together with a collection of substrates.

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doi:10.1016/j.bbagen.2006.08.005

The current study presents the first functional characterization of a library of combinatorial mammalian CYP1A variants. The CYP1A subfamily was chosen as a model because of its well-known catalytic versatility and important substrate diversity exhibited by 1A1 and 1A2 [12]. These enzymes have attracted a considerable amount of interest due to their involvement in procarcinogen activation and drug metabolisms [13,14] to the point that it has been questioned whether 1A1 was friend or foe [15]. The first subset of variants examined contains amino acid residues shuffled in a short segment of the sequence, namely segment 202–214 of human 1A1. The second subset contains bi- or tri-partite chimeras between mouse 1a1 and rabbit 1A2. The third subset contains mosaic structure obtained by DNA shuffling between human 1A1 and 1A2. Each variant was expressed in yeast and assayed against three fluorogenic substrates, all undergoing a similar *O*-dealkylation reaction producing the fluorophore; and the specific activities at a saturating substrate concentration were determined. The purpose of this work was to harvest large amounts of kinetic data on a combinatorial library of shuffled CYP1A variants and to subject them to multivariate statistical analyses in the hope that interesting correlations between amino acid sequence signatures and variations in substrate specificities could be unveiled.

2. Materials and methods

2.1. Materials

The *Saccharomyces cerevisiae* W(R) strain is a derivative of the W303-1B strain (*MAT*a; *ade2*-1; *his3*-11,15; *leu2*-3,112; *trp1*-1; *ura3*-1; *can*R; *cyr*+) engineered to overexpress the yeast microsomal NADPH-P450 reductase. The multicopy expression vectors pYeDP60 contains *URA3* and *ADE2* as selection markers, whereas the pYeDP8 vector only contains *URA3*. The inserted coding sequence is placed in both vectors under the transcriptional control of a *GAL10-CYC1* hybrid artificial promoter and *PGK* terminator [16]. The following chemicals were used: 7-methoxyresorufin (MOR), 7-ethoxyresorufin (EOR), and NADPH from Sigma-Aldrich (St. Louis, MO); ethoxy fluorescein ethyl ester (EFEE) from Molecular Probes (Eugen, OR). Other reagents were of the highest grade available.

2.2. Construction of the combinatorial libraries of increasing complexity

For mutagenesis by PCR amplification, the direct 5′-primers were a population of fifty-four nucleotide-long degenerated primers encompassing codon 202 to codon 214 of the human CYP1A1 coding sequence. Multiple mutations were introduced in a single PCR reaction using semi-random primers with multiple bases inserted at exact positions during primer synthesis. The sequence of the mutated oligonucleotide is: 5′-T T T G C T T T G G C C **R** G C **R** C T **W** T **S** **M** T **S** A **S** A **S** A **R** C **M** **R** C **S** A **M** G A A **W** T G C T T A G C C T A G T C-3′. R stands for A or G, W for A or T, S for C or G, and M for A or C. Fourteen positions out of 54 were degenerate resulting in nine modified codons targeting the sequence substitutions between human 1A1 and 1A2 coding sequences. The reverse 3′-primer was 25 nucleotides long and permitted amplification of an approximately 400 bp fragment of the human 1A1 coding sequence. The amplified DNA fragments were used after purification in a second PCR amplification as 3′-megaprimers. The resulting amplicons were introduced in pYeDP8 vector yielding a library of CYP1A mutants designated as 1AMu (for 1A Mutant) for the name root followed by a number indicating the individual variant.

The plasmid pLM4V8 was described previously [17] and contains the 1551-bp open reading frame of rabbit *CYP1A2*. The plasmid pP1V8 contains the 1575-bp mouse *Cyp1a1* coding sequence. For recombination assays, the plasmid pLM4V8 was linearized at the unique *Bst*EII site situated in the rabbit 1A2 sequence. The resulting ends were Klenow-filled, and the resulting linearized DNA fragment was submitted to BAL31 exonuclease digestion. Different digestion times were pooled so that the fragment extremities were heterogeneous. This pool of DNA fragments was used for cotransformation with the 1785-bp homologous sequence resulting from the *Bam*HI–*Eco*RI digestion of pP1V8. The resulting mutants were designated as 1ACh (for 1A Chimera) for the name root followed by a number indicating the individual variant.

Mosaic human CYP1A structures were obtained by DNA shuffling using the CLERY procedure, as previously described [18], which combines an in vitro PCR-based step of DNA shuffling after DNase I-catalyzed random fragmentation followed by an in vivo recombination step in yeast. The resulting mutants were designated as 1AMo (for 1A Mosaics) for the name root followed by a number indicating the individual variant.

2.3. Preparation of yeast microsomes

Yeast transformants growth and galactose-induction in YPGE medium and microsomal fractions preparation were carried out as previously described [16]. The protein concentration of the microsomes was determined with the bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard.

2.4. Enzyme activities

The alkoxyresorufin-*O*-deethylase activities were measured fluorimetrically using an excitation wavelength and an emission wavelength set at 530 nm and 586 nm, respectively. Incubations were in 1 ml of 50 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, NADPH 150 μM, 7-alkoxyresorufin 5 μM, and 40–90 μg of yeast microsomal proteins. Ethoxyfluorescein ethyl ester *O*-deethylase activities were measured fluorimetrically using an excitation wavelength and an emission wavelength set at 479 nm and 560 nm, respectively. Incubations were in 1 ml of 50 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, NADPH 120 μM, EFEE 1 μM, and 70–150 μg of yeast microsomal proteins.

2.5. Spectroscopy

UV-visible spectra were recorded with a Perkin-Elmer Lambda-2 spectrophotometer (Perkin-Elmer France, Les Ulis). Fluorescence was measured with a double-beam Perkin-Elmer L50 fluorimeter.

2.6. Statistical analyses

The ternary plots were obtained by using SigmaPlot™ software on data normalized by calculating the ratio of the activity for a particular substrate to the sum of the three measured activities. Dendogram constructions and principal component analyses of the kinetic data were performed by using XLSTAT-2006™ software. For similarity hierarchical clustering, the Pearson's correlation coefficient with complete linkage was used. Principal component analysis was carried out on ratios of each activity to the sum of the activities for the three substrates calculated for each enzyme. The Pearson's method of decomposition was used. The first vector keeps 63.4% of the variance of the source data. The dataset and correlation matrices used throughout this work are available upon e-mail request from urban@cgm.cnrs-gif.fr.

3. Results

3.1. Rationale for choosing a sequence shuffling of increasing complexity

The sequences of the four mammalian P450s (human and mouse 1A1s and human and rabbit 1A2s) have been aligned, and the 41 positions systematically differentiating 1A1 from 1A2 in all 19 available mammalian P450 1A sequences have

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