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Rational Engineering of Cytochromes P450 2B6 and 2B11 for Enhanced Stability: Insights Into Structural Importance of Residue 334

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Abstract

Rational mutagenesis was used to improve the thermal stability of human cytochrome P450 2B6 and canine P450 2B11. Comparison of the amino acid sequences revealed seven sites that are conserved between the stable 2B1 and 2B4 but different from those found in the less stable 2B6 and 2B11. P334S was the only mutant that showed increased heterologous expression levels and thermal stability in both 2B6 and 2B11. The mechanism of this effect was explored with pressure-perturbation spectroscopy. Compressibility of the heme pocket in variants of all four CYP2B enzymes containing proline at position 334 are characterized by lower compressibility than their more stable serine 334 counterpart. Therefore, the stabilizing effect of P334S is associated with increased conformational flexibility in the region of the heme pocket. Improved stability of P334S 2B6 and 2B11 may facilitate the studies of these enzymes by X-ray crystallography and biophysical techniques.

Keywords

Cytochrome P450 2B6; cytochrome P450 2B11; thermal stability; pressure-perturbation spectroscopy; heme pocket compressibility; proline 334

1-Introduction

The cytochrome P450 (P450)¹ heme-containing monooxygenases are involved in the oxidation of a broad range of endogenous and xenobiotic compounds [1]. Multiple species of cytochrome P450 found in the endoplasmic reticulum of various tissues of vertebrates catalyze insertion of a single oxygen atom into a wide variety of xenobiotic substrates of differing shapes and sizes. Besides the central role in drug clearance, the ability of mammalian cytochromes P450

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to convert various inactive precursors to the respective bioactive compounds makes these enzymes of paramount importance for the healthcare and pharmaceutical industries [2–4].

The P450 2B subfamily exhibits a relatively low degree of catalytic conservation across mammalian species, making these enzymes an outstanding model system for investigating structure–function relationships of P450s [5]. Investigations utilizing members of the cytochrome P450 2B subfamily have yielded a wealth of biochemical and biophysical information about substrate binding, protein–protein interactions, and the catalytic mechanisms of the microsomal monooxygenase. These enzymes have been studied at length using chimeragenesis, site-directed and random mutagenesis, molecular modeling, X-ray crystallography and solution biophysics [5,6]. X-ray structures of an engineered rabbit P450 2B4 (N-terminal modified and C-terminal His-tag) in ligand-free (pdb code 1PO5), 4-(4-chlorophenyl)imidazole (4-CPI)-bound (pdb code 1SUO), bifonazole (BIF)-bound (pdb code 2BDM), and 1-biphenyl-4-methyl-1*H*-imidazole (1-PBI)-bound (pdb codes 3G5N and 3G93) forms show a remarkable amount of structural plasticity with retention of function [5,7]. Further studies utilizing isothermal titration calorimetry (ITC) have reinforced the ability of P450 2B4 to accommodate a wide variety of ligands of a wide range of sizes [5]. These studies provide insight into factors that must be considered in understanding and predicting the binding and metabolism of drugs by P450 enzymes.

Despite their importance for human and experimental pharmacology, human P450 2B6 and canine P450 2B11 have not been as extensively studied from a structural or biophysical standpoint as rat P450 2B1 or rabbit 2B4. A major contributing factor is the lower stability of the human and canine enzymes [7]. To surmount these difficulties, a variety of approaches have been used including removal of the membrane associated N-terminal domain, directed evolution, and site-directed mutagenesis [7–10]. Furthermore, rational engineering and directed evolution have been used to locate important non-active site amino acids and alter function of P450s in the 2B subfamily [7,10–13]. Measures of protein stability used to examine 2B enzymes include thermal and pressure tolerance [7,14–16].

Recently, sequence comparisons of P450 2B1, 2B4, 2B6, and 2B11 led to the identification of Leu-264 as a major determinant of the lower thermal stability of P450 2B6 [7]. The objective of the present study was to improve stability of P450s 2B6 and 2B11 in order to allow further investigation of their structure-function relationships by X-ray crystallography and solution biophysics approaches. Based on sequence comparison with the relatively more stable 2B1 and 2B4, seven residues in 2B6 and 2B11 were subjected to site-directed mutagenesis. The mutants were then characterized using catalytic tolerance to temperature, thermal stability, and pressure-perturbation spectroscopy. In particular, residue 334 (Ser in 2B1 and 2B4 and Pro in 2B6 and 2B11) was found to play a key role in thermal stability and compressibility of the heme pocket.

2-Material and methods

2.1-Materials

7-Hydroxy-4-trifluoromethylcoumarin (7-HFC), 7-methoxy-4-(trifluoromethyl)coumarin (7-MFC), and 7-ethoxy-4-(trifluoromethyl) coumarin (7-EFC) were purchased from Invitrogen (Carlsbad, CA). Sodium hydrosulfite, β -mercaptoethanol, phenylmethylsulphonyl fluoride and NADPH were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant NADPH-cytochrome P450 reductase (CPR) and cytochrome *b*₅ (*b*₅) from rat liver were prepared as described previously [17]. Oligonucleotide primers for PCR were obtained from Sigma Genosys (Woodlands, TX). 5-Cyclohexylpentyl- β -D-maltoside (CYMAL-5) was purchased from Anatrace (Maumee, OH). The molecular chaperone plasmid pGro7, which expresses GroES/EL [18], was obtained from TAKARA BIO (Shiba, Japan). The QuikChange XL site

directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). Phusion High-Fidelity DNA Polymerase was purchased from New England Biolabs (Ipswich, MA). Nickel-nitrilotriacetic acid (Ni^{2+} -NTA) affinity resin was purchased from Qiagen (Valencia, CA). All other chemicals were of the highest grade available and were used without further purification.

2.2-Site directed mutagenesis

Sequence alignments and identity calculations were performed with the AlignX program in the Vector NTI software package (Invitrogen), using standard parameters. 2B4 was the reference sequence in all cases. Single mutants in 2B6 and 2B11 were created using 2B6 and 2B11 plasmids as the respective templates and appropriate forward and reverse primers; the S334P mutant was created in the 2B1 and 2B4 background using the appropriate forward and reverse primers (Table 1S). Constructs were sequenced at Retrogen, Inc. (San Diego, CA). Mutants were generated by polymerase chain reaction (PCR) using the QuikChange site directed mutagenesis kit for 2B6 and using Phusion High-Fidelity DNA Polymerase and a standard site directed mutagenesis protocol for 2B11.

2.3-Expression and purification

P450 2B6 and mutants were co-expressed with GroES/EL in *Escherichia coli* (*E. coli*) *JM109* cells (Stratagene) as His-tagged proteins [7]. 2B1, 2B4/H226Y, and 2B11 enzymes and corresponding mutants were expressed in *E. coli* TOPP3 cells (Stratagene, La Jolla, CA) as His-tagged proteins. These proteins were purified using a Ni-affinity column as described previously [9]. Eluted protein was dialyzed against 10 mM KPi buffer containing 10% glycerol and 1 mM EDTA (pH 7.4) with three changes. The P450 content was measured by reduced CO-difference spectra. P450 2B6, 2B11 and most of the mutants had an expression level of 200–450 nmol P450/L, except P334S which had higher expression of 600 nmol /l and 400 nmol/l in 2B6 and 2B11, respectively.

2.4-Enzyme assay

The standard NADPH-dependent assay for 7-MFC or 7-EFC *O*-deethylation by 2B6 or 2B11, respectively, was carried out as described previously [11]. Steady-state kinetic analysis of P450 2B enzymes and mutants were performed at varying 7-MFC or 7-EFC concentrations (0–150 μM). The reconstituted system contained P450, NADPH-cytochrome P450 reductase, and cytochrome *b*₅ at molar ratios of 1:4:2. Steady state kinetic parameters were determined by regression analysis using Sigma Plot (Systat Software, Inc., Point Richmond, CA). The k_{cat} and K_{m} values were determined using the Michaelis-Menten equation. Kinetic experiments included wild type and mutant enzymes for more accurate comparison of the data.

2.5-Thermal stability studies

Inactivation of P450 was monitored as described earlier [10]. The reaction mixture contained 1 μM protein in 100 mM NaOH-HEPES buffer (pH 7.4). Thermal inactivation was carried out by measuring a series of absorbance spectra in the 340- to 700-nm range as a function of temperature between 25 and 70 °C with 2.5–5 °C intervals and a 2-min equilibration at each temperature. For inactivation kinetics, the samples were treated at 45 °C, and the spectra (340–700 nm) were recorded at different time intervals. Determination of the changes in the total concentration of the P450 heme protein was done as described below (see *Data processing*). Fitting of the temperature profile and time-dependent inactivation curves was performed by non-linear least-squares regression using Sigma Plot. The inactivation profiles were fit to a two-state model to obtain the mid-point of the thermal transition temperature (T_{m}); a simple pseudo-first-order equation was used to determine the k_{inact} values [10].

2.6-Catalytic tolerance to temperature

The catalytic tolerance to temperature was studied by incubating enzyme (20 pmol in 20 μ l dialysis buffer for two assays) at different temperatures (30–70 °C) with an interval of 2.5–5 °C for 10 min. The samples were chilled in ice for 15 min and then brought to room temperature prior to measuring enzyme activity using a 7-MFC or 7-EFC *O*-deethylation assay as described earlier [11]. The temperature at which the enzyme retains 50% of the activity (T_{50}) was calculated by fitting the data to a sigmoidal curve using a two state function by regression analysis using Sigma Plot.

2.7-Pressure-perturbation studies

High-pressure spectroscopic studies were performed using a rapid scanning multi channel MC2000-2 spectrophotometer (Ocean Optics, Inc., Dunedin, FL) equipped with a custom-made light source using an OSRAM 64614 UV-enhanced tungsten halogen lamp (OSRAM, Germany). The instrument was connected by a flexible optic cable to the high-pressure cell connected to a manual pressure generator capable of generating a pressure of 600 bar. All experiments were carried out at 4 °C in 100 mM Na-HEPES buffer, (pH 7.4). This buffer is known to be appropriate for pressure-perturbation experiments, as it exhibits a pressure-induced pH change of only $-6 \cdot 10^{-4}$ pH unit/MPa [14]. All samples were prepared with CO bubbled Na-HEPES buffer, cooled to 4 °C and reduced by the addition of 0.25 M sodium dithionite to a final concentration of 12.5 mM. Formation of the CO complex of the reduced protein was followed by the appearance of an absorbance band at ~ 450 nm until the process was completed. A series of absorbance spectra were recorded at 4 °C, at pressure increasing in 10–20 MPa increments from 0.1 to 520 MPa.

2.8-Data processing

To interpret the changes in the absorbance spectra observed in our temperature inactivation and pressure perturbation experiments in terms of the changes in the concentration of P450 and P420 species, we used principal component analysis (PCA) combined with least-squares approximation of the spectra of principal components with a linear combination of appropriate spectral standards (prototype spectra), as described previously [19,20]. The set of spectral standards used in temperature inactivation experiments consisted of the spectra of ferric high-spin, ferric low-spin and ferric P420 states obtained for full-length 2B4 enzyme [20]. The basis set of the spectral standards used in pressure-perturbation experiments was made of the spectra of ferrous carbonyl complexes of P450 and P420 states also obtained with the full-length 2B4 heme protein [16]. Due to a large pressure-induced displacement (red shift) of the P420 Soret band, the P420 species was represented by two separate spectral standards, namely the prototypic spectra the P420 state at 1 bar and 6 kbar, respectively. The total concentration of the P420 state was calculated as a sum of these two apparent “substates” [14,16]. The spectra obtained in pressure perturbation experiments were corrected for the solvent compression prior to the analysis, as described [14].

To find the exact position of the maximum of the Soret band in the analysis of the heme pocket compressibility we applied the approximation of the spectra digitized in the region 410–470 nm with the step of 1 nm by a combination of two mixed (Gaussian + Lorentzian) peaks (each for P420 and for P450 states) with the second order polynomial added to compensate for the turbidity component. Fitting was performed using GRAMS32/AI software (Thermo Fisher Scientific, Waltham, MA). The fitting was usually very precise, being characterized by the square correlation coefficient >0.998 . The confidence interval for the position of the band found hereby was in the range of 0.05–0.1 nm ($P < 0.05$).

2.9-Analysis of pressure-induced transitions

Our interpretation of the pressure-induced changes is based on the equation for the pressure dependence of the equilibrium constant:

$$K_{eq}(P) = K_{eq}^{\circ} \cdot e^{-P\Delta V^{\circ}/RT} = e^{(P_{1/2}-P)\Delta V^{\circ}/RT}, \text{ where } K_{eq}^{\circ} = e^{P_{1/2}\Delta V^{\circ}/RT} \quad (1)$$

Here $K_{eq}(P)$ and K_{eq}° are the equilibrium constants of the reaction at pressure P and at zero pressure respectively, $P_{1/2}$ is the pressure at which $K_{eq} = 1$ ("half pressure" of the conversion), ΔV° is the molar reaction volume and K_{eq}° is the equilibrium constant extrapolated to zero pressure, $K_{eq}^{\circ} = e^{P_{1/2}\Delta V^{\circ}/RT}$.

This equation was transformed to yield the following relationship:

$$[P420]_p/[P450]_0 = A_0 + F_c \cdot (e^{(P_{1/2}-P)\Delta V^{\circ}/RT} + 1)^{-1} \quad (2)$$

$[P420]_p$ - concentration of pressure-induced P420 state of the hemoprotein, $[P450]_0$ - total concentration of cytochromes P450 and P420 in the sample, F_c - the fraction of cytochrome P450 exposed to the conversion, A_0 - a parameter, reflecting the position of apparent equilibrium at room pressure. Fitting of concentration curves to find F_c , A_0 , $P_{1/2}$ and ΔV° was made using SPECTRALAB software [19].

3-Results

3.1 Exploratory analysis of amino acid substitutions affecting the stability of P450 2B enzymes

3.1.1-Identification of amino acids of interest—Among the P450 2B subfamily, which includes the rat 2B1, rabbit 2B4, human 2B6, and dog 2B11 enzymes, 2B1 and 2B4 were found to be more stable than 2B11 and 2B6. The temperature-induced inactivation of the protein is caused by both P450→P420 formation and the heme loss processes [7]. A multiple sequence alignment of the relatively more stable P450s 2B1 and 2B4 with the less stable 2B6 and 2B11 identified seven non-active site sequence positions, where the residues are identical or similar within either (2B6, 2B11) or (2B1, 2B4), but different between the pairs. In addition to these seven residues identified through sequence alignment, we previously identified L295H as a beneficial mutation in 2B1 by directed evolution [10]. We therefore engineered 2B6 and 2B11 by replacing residues V/I81, V234, E254, Y325, P334, I427 and Q473 in 2B6/2B11 with the residues found in P450 2B4 at the corresponding locations (T81, I234, A254, Q325, S334, M427 and K473). In addition, L295H was created in 2B6 and 2B11.

3.1.2-Expression and purification of 2B6 and 2B11 mutants—The P450 2B wild-type and mutant enzymes were first expressed in 100 ml *E. coli* culture and P450 was extracted and measured as described earlier [7]. The low expression of P450 2B6 as a result of rapid inactivation into P420 is overcome by co-expressing P450 2B6 with the molecular chaperones GroEL/ES [7]. Of the eight substitutions made in each enzyme, P334S in 2B6 or 2B11 yielded ~1.5-fold higher expression than the wild-type enzymes; V81T in 2B6 and Y325Q and I427M in 2B11 expressed at similar levels to the respective wild-type enzymes. Interestingly, the mutation L295H that was beneficial with respect to temperature stability in 2B1, proved to be harmful in both 2B6 and 2B11, yielding no active protein when expressed in *E. coli* (Table 1). Furthermore mutant V81T had similar expression as wild type. V234I, L295H and E254A showed very low expression and higher P420 content.

3.1.3-Stability of 2B6 and 2B11 mutants—The temperature stability V81T, V234I, Y325Q, P334S, I427M and Q473K is presented in Table 2. P334S showed ~6 °C higher T_m than 2B6, while the T_m of Q473K was ~5 °C lower than 2B6. Catalytic tolerance to temperature was also determined for 2B6 and the mutant P334S. P334S showed ~4 °C higher T_{50} than 2B6 (46.9 °C vs. 42.3), further confirming its enhanced thermal stability (data not shown). Similarly, 2B11 P334S was found to be the best expressing (Table 1) and most stable (data not shown) mutant. Furthermore, in steady-state kinetic analysis, P334S showed essentially unchanged K_m and k_{cat} with the substrate 7-MFC for 2B6 and 7-EFC for 2B11. Hence, mutation of residue 334 has not affected catalysis of the model substrates of the respective enzymes (Table 2S).

3.2-Role of proline³³⁴ in the stability of P450 2B enzymes

3.2.1-Expression and purification of the mutants—To further investigate the role that residue 334 plays in the stability of P450 2B enzymes, we decided to mutate Ser³³⁴→Pro in 2B1 and 2B4, as found in the less stable 2B6 and 2B11 proteins. The S334P mutants expressed at similar levels to wild-type 2B1 and 2B4. Whereas the T_m values for P334S were higher than 2B6 and 2B11 (Fig. 2A, Table 3), the reverse mutation (S334P) in 2B1 and 2B4 (Fig. 2B, Table 3) yielded a T_m 9.3 and 4.4 °C lower than wild-type proteins 2B1 and 2B4, respectively. As seen from the measurements of k_{inact} (Table 3), the wild-type 2B6 and 2B11 underwent inactivation 2.2- and 7.8-fold faster than their P334S mutants (Fig. 2C), whereas inactivation of 2B1 and 2B4 was 1.72- and 1.6-fold slower than the mutants (Fig. 2D). Thus in all four P450 2B enzymes, the presence of a serine at position 334 provides a more thermally stable enzyme, whereas proline yields a less thermally stable enzyme.

3.2.2-Pressure-perturbation studies of the susceptibility to P450→P420

conversion—Conversion of cytochromes P450 into their inactive “cytochrome P420” state represents an important pathway of inactivation, which is promoted by elevated temperature, increased hydrostatic pressure, high concentrations of KSCN, alkaline pH, and some other factors [21–26]. Formation of the P420 state of the enzyme with the apparent replacement of the axial thiolate ligand of the heme iron with non-ionized thiol group [22,27,28] is known to be associated with an important increase in protein hydration [14,22,29]. Here we study the pressure-induced P450→P420 transition in a series of P450 2B enzymes and their mutants in order to probe possible differences in the dynamics of protein hydration as related to the susceptibility of these enzymes to their inactivation via formation of the P420 state. We also used pressure-perturbation spectroscopy to explore the role of residue 334 in the compressibility of the heme pocket, which was assessed from the pressure-induced displacement of the Soret absorbance band of the carbonyl complex of ferrous heme protein [16,30,31].

A series of spectra of ferrous carbonyl complex of 2B4 (2B4) recorded at increasing hydrostatic pressure is shown in Fig. 3. The dependence of the concentration of the P420 2B4 on pressure obeys equation (1) with $\Delta V^\circ = -36 \pm 4$ ml/mol and $P_{1/2} = 250 \pm 30$ MPa (Fig. 3 inset). It is important to note that, in contrast to the behavior observed earlier with the oligomeric full-length 2B4, where no more than 65% of the total enzyme content underwent a P450→P420 conversion [14], the susceptibility of 2B4 to pressure-induced inactivation approaches 90%.

The behavior of wild-type 2B1, 2B6 and 2B11 was qualitatively similar to that observed with 2B4, although the values of the barotropic parameters vary (Table 4). P450 2B11 exhibited the most important difference from the other 2B enzymes. While for the other three 2B enzymes the values of ΔV° and $P_{1/2}$ were in the ranges of -33 to -36 ml/mol and 25 – 31 MPa, respectively, the half-pressure of the inactivation of 2B11 is as low as 18 MPa, and the volume change is as small as -22 ml/mol. Therefore, as the Gibbs free energy of the reaction is defined as the product of ΔV° and $P_{1/2}$ values, 2B11 is characterized by the lowest value of ΔG°_{P420} (around

4 kJ/mol as compared to 9–13 kJ/mol for the other 2B enzymes). Consequently, 2B11 is extremely susceptible to a spontaneous conversion to the P420 state, and the content of the P420 state in this enzyme at the ambient pressure was as high as 30–40%. In contrast, the initial content of P420 heme protein in 2B1, 2B4 and 2B4 enzymes at 1 bar does not exceed 15–20%.

Although the effects of the mutation at residue 334 on the pressure-induced P450→P420 transition are fairly pronounced for all four P450 2B enzymes (Table 4), these changes do not reveal any systematic relationship. Thus, while the P334S mutation had a negligible effect on P420 formation in 2B6, there was a pronounced protective effect in 2B11, as revealed in the increased ΔG°_{P420} from 4.1 to 8.4 kJ/mol. The reverse (S334P) substitution in 2B4 and 2B1 also stabilized both enzymes by a considerable increase in $P_{1/2}$ and, consequently, ΔG°_{P420} values (Table 4).

3.2.3-Effect of S334P and P334S substitutions on the compressibility of the heme pocket of 2B enzymes—An increase in the hydrostatic pressure results in a displacement (“red-shift”) and broadening of the absorbance band, indicating a compression of the chromophore environment that results in tightening interactions of the excited state with adjacent polar groups and the solvent molecules [30,32]. The slope (α) of the dependence of the Soret band wavenumber (ν) on pressure may therefore be used as a measure of the compressibility of the heme pocket [30].

The effect of pressure on the position of the Soret band in a series of P450 2B enzymes and their P334S or S334P mutants is illustrated in Fig. 4 and Table 4. As judged from the values of α , the “wild type” P450 2B enzymes reveal a compressibility of the heme pocket lower than most of the substrate-free P450 enzymes studied to date, where the values of α typically fall in the range of -0.22 to -0.39 $\text{cm}^{-1}/\text{MPa}$ [30,31,33]. This observation is consistent with the results obtained earlier with the full-length P450 2B4, where the value of α was found to be as low as 0.09 $\text{cm}^{-1}/\text{MPa}$ [16].

As seen in Fig 4A, the P334S substitution in 2B6 and 2B11 results in a striking increase in the slope of the pressure dependence of the Soret band wavenumber. The value of 0.46 $\text{cm}^{-1}/\text{MPa}$ observed with 2B11 P334S (Table 4) represents the largest negative value of α observed with P450 heme proteins up to date. Although the effect of S334P substitution on the compressibility of the heme pocket in P450 2B4 and P450 2B1 was much less pronounced (Fig. 4B, Table 4), the direction of the changes caused by this “reverse” mutation was opposite. These results suggest that the nature of the amino acid at the 334 position is an important determinant of the conformational plasticity of the heme pocket of the substrate-free P450 2B enzymes.

4-Discussion

The realization that an increasing number of drugs are metabolized by human P450 2B6 and that canine P450 2B11 has unique ability to metabolize the anti-cancer prodrugs cyclophosphamide and ifosfamide with high efficiency and to detoxify certain polychlorinated biphenyls has prompted a major effort to understand the structural basis of enzyme action [34,35]. The recent discovery of the lower inherent stability exhibited by P450s 2B6 and 2B11 compared with the better characterized 2B1 and 2B4 indicated the need to engineer more stable enzymes amenable to advanced structural and biophysical techniques [7]. Comparative structural and mutagenesis studies of other proteins have revealed some general strategies for increasing protein stability. These include increasing the hydrophobic packing in the interior, extending networks of salt-bridges and hydrogen bonds, increasing the extent of secondary structure formation, shortening or strengthening solvent-exposed loops and termini, and replacing residues responsible for irreversible chemical alterations of the protein structure [36]. Our approach in the present study was to build upon the lessons learned

through site-directed mutagenesis, directed evolution, genetic polymorphism, and conserved sequence motif analysis studies of P450 2B enzymes that show the important role of non-active site residues for P450 expression, stability, ligand binding, and/or catalytic activity [5,7,10, 37].

Comparison of wild type and mutant 2B6 or 2B11 enzymes showed no correlation between expression levels and stability. For example, although V81T and V234I showed increased and decreased expression levels, respectively, compared with wild-type 2B6, V81T exhibited a slight decrease and V234I a marked increase in thermal stability. The lack of correlation between expression level and stability is also seen when looking at 2B1, 2B4 and 2B11 in previous reports and in this study [7,9].

Of the mutants that express at similar or higher levels than wild-type enzyme, only P334S proved to impart an increase in thermal stability in both 2B6 and 2B11. This mutation resulted in an increase of the T_m of 7.6 and 2.4 C and a decrease in the k_{inact} 2.17- and 7.8-fold for 2B6 and 2B11, respectively. Furthermore, the S334P mutant in both 2B1 and 2B4 shows significantly decreased thermal stability. At the same time, the alteration of residue 334 does not significantly change enzyme activity with either 7-MFC (2B6) or 7-EFC (2B11), which are model substrates for the respective enzymes. These results allowed us to identify the residue at the sequence position 334 as an important determinant of the structural stability of orthologous P450 2B enzymes studied here.

According to the crystal structure of P450 2B4 complexed with 4-CPI [5] and homology modeling 2B1 based on this structure, Ser³³⁴ in 2B1 and 2B4 is located in a loop between the J- and J'-helices, outside of the active site, and the mechanism by which it affects stability does not seem obvious. This residue does not seem to be directly involved in the P450 catalysis but may be important for the interactions of the protein with the heme group and/or the adaptation of the structure of the heme to temperature-dependent conformational fluctuations in the protein (Fig. 5).

In order to probe the molecular basis for the role of residue 334 as a determinant of the P450 2B stability we employed pressure-perturbation spectroscopy to compare P334S and S334P in P450 2B enzymes in terms of susceptibility to a P450→P420 transition and the compressibility of their heme pocket. Earlier studies with full-length P450 2B4 showed that its conversion to P420 is characterized by a partial volume change (ΔV°) of -50 ± 8 ml/mol and the half-pressure ($P_{1/2}$) of the transition of 300 ± 50 MPa. Similar to earlier observations with the full-length 2B4 [14] and other P450 enzymes [29,31,38], increase in hydrostatic pressure results in a gradual disappearance of the P450 Soret band of truncated P450 2B4 at ~ 451 nm, concomitant with an ample increase in the absorbance bands of the P420 state. The truncated P450 2B4, as well as 2B1, 2B6, and 2B11 enzymes, showed smaller volume change in the P450→P420 transition than the full-length 2B4. The value of $P_{1/2}$ for 2B4 and 2B11 is also lower than that of the full-length 2B4 (Table 4). Due to these differences, the truncated wild type 2B enzymes exhibit lower ΔG°_{P420} than that observed with the full length 2B4 (15 kJ/mol). Therefore, truncation of the enzymes appears to result in some sensitization to P450→P420 inactivation. Another difference from the full-length 2B4 is related to the maximal amplitude of P420 formation. While for the full-length P450 2B4 susceptibility to the P450→P420 transition does not exceed 65%, the maximal extent of the P450→P420 conversion observed with the truncated enzymes approaches 90% (Fig. 3). This result is consistent with lower degree of aggregation of the truncated P450 2B enzymes, which makes their pool more homogenous in terms of sensitivity to pressure-induced hydration and subsequent P450 formation.

Although the effect of mutating residue 334 on P450→P420 transition is fairly pronounced for all four P450 2B enzymes (Table 4), these changes do not reveal any systematic relationship

(Table 4). Therefore, a direct role of this residue in the mechanisms of P420 formation seems unlikely, and the stabilizing effect of P334S mutation in 2B6 in 2B11 does not involve any apparent alteration of their susceptibility to P420 formation. In contrast to the erratic effect of the substitutions at position 334 on P420 formation, the effect on the compressibility of the heme pocket revealed a well-pronounced general trend. Replacement of Pro³³⁴ with Ser in 2B6 and 2B11 resulted in a considerable increase in the compressibility of the heme pocket, while replacing Ser³³⁴ with Pro in 2B4 and 2B1 had the opposite effect. This finding suggests that the residue 334 (Fig. 5) plays an important role in structural plasticity of the heme environment. The presence of the conformationally rigid proline residue must decrease the flexibility of the loop between the J- and J'-helices, which may be important for adaptation of the geometry of the heme environment to the conformational fluctuation of the protein. High conformational flexibility in this region may be therefore important for preventing the heme loss that appears to be the main cause of low stability in P450 2B6 and 2B11.

Highly expressed, stable and homogeneous P450s 2B6 P334S and 2B11 P334S should prove an invaluable template for further study using biochemical and biophysical methods, especially X-ray crystallography and hydrogen/deuterium exchange-mass spectrometry. Furthermore, interesting questions about heme solvation and compressibility arise from P450s 2B1 S334P and 2B4 S334P, which can be examined utilizing our current expertise in solution methods [14,15,19,30].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

P450	cytochrome P450
4-CPI	4-(4-chlorophenyl)imidazole
BIF	bifonazole
1-PBI	1-biphenyl-4-methyl-1 <i>H</i> -imidazole
2B1	2B4, 2B6 and 2B11, an N-terminal truncated and modified and C-terminal 4-His-Tagged form of cytochromes 2B1, 2B4, 2B6 and 2B11, respectively
pdb	protein data bank
7-HFC	7-hydroxy-4-trifluoromethylcoumarin
7-MFC	7-methoxy-4-(trifluoromethyl)coumarin
7-EFC	7-ethoxy-4-(trifluoromethyl)coumarin
CYMAL-5	5-cyclohexylpentyl- β -D-maltoside
Ni ²⁺ -NTA	nickel-nitrilotriacetic acid
ITC	isothermal titration calorimetry
β -ME	β -mercaptoethanol
PMSF	phenylmethylsulphonyl fluoride

NADPH	nicotinamide adenine dinucleotide phosphate
CPR	recombinant NADPH-cytochrome P450 reductase
<i>b</i> ₅	cytochrome <i>b</i> ₅
PCR	polymerase chain reaction
EDTA	ethylenediaminetetraacetic acid
CO	carbon monoxide
PCA	principal component analysis
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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	76	*	86	229	*	239	249	*	259	290	#	300
CYP2B1dH	...	VMLCG T DTIKE.....	GAHRQ I SKNLQ.....	VEKHRA T LDPS.....	ISLLS L FFAGT...							
CYP2B4dH	...	VVLCG T DAIRE.....	GTHRQ I YRNLQ.....	VEKHRA T LDPS.....	LTVLS L FFAGT...							
CYP2B6dH	...	VMLCG V EAIRe.....	GAHRQ V YKNLQ.....	VEKHRE T LDPS.....	LNTLS L FFAGT...							
CYP2B11dH	...	VMLCG I DAIRE.....	GTHRQ V YNNLQ.....	VEKHRE T LDPS.....	DTALS L FFAGT...							

	320	*		*	339	422	*	432	468	*	478
CYP2B1dH	...	VAEKV Q KEIDQVIG S HRLPT.....	KSEAF M PFSTG.....	IDLTP K ESGIG...							
CYP2B4dH	...	VTERV Q KEIEQVIG S HRPPA.....	RNEG F MPFSLG.....	IDLTP R ESGVG...							
CYP2B6dH	...	VAERV Y REIEQVIG P HRPPE.....	KTEAF I PFSLG.....	IDLTP Q ECGVG...							
CYP2B11dH	...	IAERI Y KEIDQVIG P HRLPS.....	KNEAF I PFSIG.....	IDLTP Q EIGVG...							

Figure 1.

Comparison of CYP2B amino acid sequences from rat, rabbit, human, and dog. Locations of mutations described herein are in bold font. The beneficial mutant L295H from CYP2B1 is marked with #; the differences between CYP2B1/2B4 and CYP2B6/2B11 are marked with *. The residues numbers correspond to the full-length rabbit CYP2B4 sequence.

Figure 2.

Thermal stability of CYP2B mutants.

A. Thermal inactivation of CYP2B6 and 2B11 along with P334S

B. Thermal inactivation of CYP2B1 and 2B4 along with S334P (1 μ M) monitored by a decrease in the amount of heme protein as a function of temperature, as described under *Materials and Methods*. Determination of the total concentration of the heme protein was done by nonlinear least square approximation of the spectra by a linear combination of spectral standards of 2B4 low-spin, high-spin, and P420-states. The data were fit to a sigmoidal curve to obtain T_m .

C. Time courses of inactivation of CYP2B6, 2B11 and P334S

D. Time courses of inactivation of CYP2B1, 2B4 and S334P (1 μ M) at 45°C monitored as described above. The data were fit to a pseudo-first order equation to obtain k_{inac} . (●) 2B1; (○) 2B1 S334P; (▲) 2B4; (△) 2B4 S334P; (▼) 2B6; (▽) 2B6 P334S; (■) 2B11; (□) 2B11 P334S

Figure 3.

Pressure-induced transitions of P450→P420 in cytochrome P450 2B4. The main panel shows the series of spectra obtained at 0.1, 20, 60, 100, 140, 180, 260, 300, 380, and 420 MPa. The spectra were recorded with 8 μM protein in CO saturated Na-HEPES buffer containing 12.5 mM dithionite at 4 C. The inset illustrates the corresponding changes in the concentration of the carbonyl complexes.

(○) P450 (Fe²⁺); (▽) P420 (Fe²⁺); (□) total protein concentration.

Figure 4.

Pressure-induced displacement of the Soret band of ferrous carboxy-complexes of CYP2B mutants.

Panel A shows the results obtained with 2B4 (▲), 2B4 S334P (△), 2B1 (●), and 2B1 S334P (○). Panel B represents the data sets obtained with 2B6 (▼), 2B6 P334S (▽), 2B11 (■), 2B11 P334S (□). Solid lines show linear approximations of the respective data sets with the parameters given in Table 5.

Figure 5. Schematic representation of a three-dimensional structure of CYP 2B4. The heme is shown in red, whereas helices J and J' are in teal and Ser-334 is in orange.

Table 1

Expression of 2B6 and 2B11 mutants

	2B6 (JM109 + pGro7) mol P450/L	2B11 (TOPP3) nmol P450/L
Wt	468 ± 40	256 ± 9
V81T	438 ± 30	102
V234I	30, P420	86
E254A	N.D	135
L295H	N.D	N.D
Y325Q	270 ± 25	262
P334S	678 ± 50	349 ± 100
I427M	270 ± 30	286 ± 62
Q473K	190 ± 20	182

Results are the mean ± standard deviation ($n = 3$)

N.D., Not detectible

Table 2

Thermal Stability of 2B6 mutants

P450	T_m (°C) P450 (HS, LS, and P420)
2B6	47.3 ± 0.6
V81T	45.9 ± 0.6
V234I	49.4 ± 0.4
Y325Q	46.3 ± 0.8
P334S	53.1 ± 0.7
I427M	49.7 ± 0.3
Q473K	42.7 ± 0.6

HS, high spin; LS, low spin

Results are the mean ± standard error for fit to the appropriate equation. The data are representative of at least two independent determinations.

Table 3

Thermal Stability of CYP2B and mutants

		P450 (HS, LS, and P420)	
	P450	T_m (°C)	k_{inact} (min ⁻¹)
2B6	WT	47.3 ± 0.4	0.19 ± 0.02
	P334S	54.5 ± 1.1	0.09 ± 0.01
2B11	WT	45.2 ± 0.1	0.28 ± 0.03
	P334S	47.6 ± 0.4	0.04 ± 0.01
2B4	WT	58.9 ± 1.8	0.08 ± 0.01
	S334P	54.5 ± 1.9	0.13 ± 0.01
2B1	WT	58.4 ± 1.8	0.06 ± 0.01
	S334P	49.1 ± 1.2	0.09 ± 0.01

HS, high spin; LS, low spin

Standard errors for fit to the respective equations are shown. Results are representative of at least two independent determinations. The variation between the experiments is ≤10%.

Results of pressure-perturbation studies with CYP2B wild type and the mutants: Barotropic parameters of P450→P420 transition and pressure-induced displacement of the Soret band.

Table 4

	P450	$P_{1/2}$ MPa	$-\Delta V^\circ$ ml/mol	ΔG° P420 ^a , kJ/mol	ΔP° cm ⁻¹ /MPa	ν° b cm ⁻¹	λ° c nm
2B6	WT	374 ± 5	33.9 ± 0.2	12.7 ± 1.8	-0.157 ± 0.007	22187 ± 1	450.7
	P334S	413 ± 23	25.0 ± 2.9	10.3 ± 1.8	-0.369 ± 0.013	22223 ± 2	450.0
2B11	WT	182 ± 93	22.4 ± 1.7	4.1 ± 3.9	-0.103 ± 0.02	22221 ± 2	450.0
	P334S	275 ± 32	30.5 ± 10.7	8.4 ± 3.9	-0.457 ± 0.03	22134 ± 2	451.8
2B4	WT	247 ± 26	35.8 ± 3.9	8.9 ± 1.9	-0.143 ± 0.03	22173 ± 3	451.0
	S334P	392 ± 43	36.5 ± 3.3	14.3 ± 2.9	-0.129 ± 0.012	22296 ± 1	448.5
2B1	WT	309 ± 64	32.9 ± 1.5	10.2 ± 2.6	-0.241 ± 0.03	22259 ± 3	449.3
	S334P	469 ± 43	37.0 ± 1.5	17.4 ± 2.3	-0.149 ± 0.01	22189 ± 1	450.7

Standard errors for fit to the respective equations are shown. Results are representative of at least three independent determinations.

^aRepresents the Gibbs free energy of the reaction calculated as a product of $-\Delta V^\circ$ and $P_{1/2}$ values

^bPosition of the Soret band calculated from the wavenumber ν°

^cThe slope α , and the offset ν° characterize the linear approximation of the pressure dependence of the Soret band in 0.1–170 MPa region.