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THE ROLE OF CYTOCHROME P450 2B6 AND 2B4 SUBSTRATE ACCESS CHANNEL RESIDUES PREDICTED BASED ON CRYSTAL STRUCTURES OF THE AMLODIPINE COMPLEXES[†]

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Abstract

Recent X-ray crystal structures of human cytochrome P450 2B6 and rabbit cytochrome P450 2B4 in complex with amlodipine showed two bound ligand molecules, one in the active site and one in the substrate access channel. Based on the X-ray crystal structures, we investigated the interactions of P450 2B4 and 2B6 with amlodipine using absorbance spectroscopy, and determined the steady-state kinetics of 7-ethoxy-4-(trifluoromethyl)coumarin and 7-benzoyloxyresorufin oxidation by some access channel mutants to evaluate the functional role of these residues in substrate turnover. The results of absorbance titrations are consistent with a simple mechanism with two parallel binding events that result in the formation of the enzyme complex with two molecules of amlodipine. Using this model we were able to resolve two separate ligand-binding events, which are characterized by two distinct K_D values in each enzyme. The access channel mutants R73K in P450 2B6 and R73K, V216W, L219W, and F220W in P450 2B4 showed a significant decrease in k_{cat}/K_M with the both substrates. Overall, the results suggest that P450 2B4 and 2B6 form an enzyme complex with two molecules of amlodipine in solution, and R73, V216, L219 and F220 in P450 2B4 may play an important role in substrate metabolism.

Keywords

Cytochrome P450 2B4 and 2B6; substrate access channel 2f and 2a; spectral titrations; amlodipine

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Conflict of Interest Statement

The authors declare no conflicts of interest.

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Introduction

Cytochromes P450 comprise a large superfamily of heme-containing monooxygenases that metabolize a wide variety of structurally different endogenous and exogenous substrates including fatty acids, steroids, drugs, and environmental pollutants [1]. Different P450 enzymes typically show distinct yet overlapping substrate specificity and regio- and stereoselectivity of metabolism of the same substrate [2, 3]. Understanding the structural determinants of substrate selectivity of individual P450 enzymes is of key importance for rational prediction of metabolism of various xenobiotics of pharmacological and toxicological relevance [4, 5]. X-ray crystal structures of mammalian P450 enzymes reveal that the P450 fold is highly conserved, and the heme group is generally buried deep at the center of the enzyme molecule, indicating that the protein must undergo dynamic conformational motions to allow a diverse range of substrates to reach the active site and product to exit the enzyme [6–8].

Possible substrate access or egress channels that connect the protein surface to the active site have been investigated in a number of structural and molecular dynamic studies of several bacterial and mammalian P450s [9, 10]. Substrate entry and binding are mainly controlled by the residues within the B, B', F, G, and I helices and the B/C and F/G loops [11, 12]. In P450cam the access channels located near the F–G region are capable of accommodating hydrophobic substrates, while the channel between the B' helix and the F/G loop is thought to serve as a channel for more hydrophilic products [13, 14]. Similarly, in the crystal structure of P450BM3 with palmitoleic acid, the largest difference from the structure of the substrate-free enzyme is observed in the regions of the F- and G-helices and the F/G loop [15]. P450 2C8 exhibits two access channels to the buried active site, which are located on either side of the helix B' [16], whereas P450 2E1 shows an access channel between the B–B' loop, the β_1 sheet system, and the F' and G' helices [17]. In addition, the channel in P450 51 is near the F–G loop, helix A', and the β_4 loop [18]. Furthermore, molecular dynamics simulations showed that substrates and products could egress from the active site via channels that differ between membrane-bound mammalian P450s and soluble bacterial P450s, and that different gating mechanisms are operative for the corresponding channels in different P450 isoforms [19, 20].

Our laboratory has used a variety of techniques such as site-directed mutagenesis, molecular modeling, isothermal titration calorimetry, and X-ray crystallography to identify key residues responsible for substrate specificity of P450 2B enzymes. Most of the mutagenesis studies were focused on P450 2B1 and involved the helix B' region [21], the F–G region [11], and the N-terminal portion of helix I [22]. In addition, numerous structures of P450 2B4 and 2B6 have been determined by X-ray crystallography. Closed conformations were found in the complexes with small imidazole inhibitors [23], antiplatelet drugs [24], and covalently bound mechanism-based inactivators [25], whereas more open conformations were observed with ligand-free enzyme and some larger inhibitors [26, 27].

Recently, the structures of P450 2B6 and 2B4 in complex with the calcium channel blocker amlodipine have been determined by X-ray crystallography [28]. The presence of two ligand molecules with different orientations of the amlodipine more distal to the heme suggested

clear substrate access channels in each P450. According to the accepted nomenclature [10] in both enzymes the second amlodipine molecule is poised to access the active site through the 2*f* entrance pathway, which lies among helices F', G', A', and A. Residues L43, M46, R48, K/R49, F/V212, V/L216, and L219 are located in this channel. In addition, there is a second channel termed 2*a* in P450 2B4 between helices B' and G' near the β_1 sheet. Residues lining this channel are R73, K100, A102, and E387 in the β_1 sheet and S221 in helix G'.

Importantly, multi-step binding mechanisms and multiple substrate occupancy have been reported in X-ray structures of dual ligand complexes of P450 3A4 [29], P450 21A2 [30], and P450 2A13 [31]. However, there were no direct experimental indications of the involvement of multiple substrate binding sites in the catalytic mechanisms of 2B enzymes. Therefore, the relevance of the ternary enzyme-substrate complexes observed in [28] to enzyme catalysis remains to be determined. The functional role of the access channel residues that are located in the proximity of the substrate-access channel and interact with amlodipine molecules in the X-ray structures also requires detailed examination.

The present study was therefore undertaken in order to explore the relevance of the multisite mechanism to the interactions of P450 2B enzymes with amlodipine and to evaluate the functional role of some of the putative access channel residues in enzyme-substrate interactions and catalysis. First, we studied the interactions of amlodipine with P450 2B4 and 2B6 in solution by advanced absorbance spectroscopy. Resolution of two separate substrate binding in these experiments allowed us to demonstrate that the formation of the complexes of P450 2B4 and 2B6 with two molecules of amlodipine [28] are relevant to the mechanism of enzyme-ligand interactions in solution. Furthermore, we evaluated the functional role of some of the amino acid residues of the putative substrate-access channel that interact with amlodipine in X-ray structures. To this end we studied the effect of the respective amino acid substitutions on the steady-state kinetics of metabolism of prototypical 2B substrates 7-ethoxy-4-(trifluoromethyl)coumarin (7-EFC) and 7-benzyloxyresorufin (7-BR). Specifically, we probed the substitutions of amino acids 48, 49, and 73 in both P450 2B4 and 2B6 with alanine or lysine, and investigated the effect of the replacement of residues 212, 216, 219, and 220 in P450 2B4 with tryptophan. Taken together our results confirm the biochemical relevance of the substrate-binding mode suggested by the X-ray structures of the complexes of P450 2B4 and 2B6 with amlodipine.

Materials and Methods

Materials

Amlodipine besylate, β -NADPH, ribonuclease A (RNase), deoxyribonuclease I (DNase), resorufin, and 7-BR were purchased from Sigma-Aldrich (St. Louis, MO). 7-Hydroxy-4-(trifluoromethyl) coumarin (7-HFC), and 7-EFC were purchased from Invitrogen (Carlsbad, CA). Nickel-nitrilotriacetic acid affinity resin was from Qiagen (Valencia, CA), and Macroprep CM cation exchange resin was obtained from Bio-Rad Laboratories (Hercules, CA). The QuikChange XL site-directed mutagenesis kit and TOPP3 and JM109 cells were obtained from Stratagene (La Jolla, CA). The molecular chaperone plasmid pGro7, which expresses GroES/EL, was obtained from TAKARA BIO (Shiba, Japan). Recombinant

NADPH cytochrome P450 reductase (CPR) and cytochrome *b*₅ from rat liver were prepared as described previously [32]. All other chemicals and supplies used were from standard sources.

Site-directed Mutagenesis

P450 2B4^a and 2B6^b mutants were generated by polymerase chain reaction (PCR) with Stratagene's QuikChange XL site-directed mutagenesis kit using as a template pKK2B4dH (H226Y) [33] or pKK2B6dH (Y226H/K262R) [28, 34] plasmids that express an N-terminal truncated and modified P450 2B enzymes. The forward mutagenic oligonucleotides for each construct are shown in Table 2. The altered nucleotides are in bold italics. All mutants generated in this study were verified by sequencing at Retrogen Inc (San Diego, CA, USA) to ensure the intended mutations and the absence of extraneous mutations.

Protein Expression and Purification

P450 2B4 and 2B6 enzymes were expressed in *Escherichia coli* TOPP3 cells and JM109 cells respectively. 2B6 and mutants enzymes were coexpressed with the chaperone GroES/EL (pGro7 plasmid) to overcome low expression as described previously [34]. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.5 mM) and δ-aminolevulinic acid (ALA, 1 mM) to Terrific broth medium at A₆₀₀~0.7 at 37°C in the presence of ampicillin (2B4 and mutants) or ampicillin and chloramphenicol (2B6 and mutants). The cells were grown 68–72 h at 30°C and were harvested by centrifugation (4,000 g). Further protein purification was carried out at 4°C according to a protocol described previously [24]. Briefly, the cell pellets were resuspended in 10% of the original culture volume in buffer containing 20 mM potassium phosphate (pH 7.4 at 4°C), 20% (v/v) glycerol, 10 mM 2-mercaptoethanol (BME), and 1 mM phenylmethanesulfonylfluoride (PMSF). After addition of lysozyme (0.3 mg/mL) and stirring for 30 min at 4°C, the spheroplasts were separated by centrifugation for 20 min at 7000 rpm and resuspended in 5% of the original volume in the buffer containing 500 mM potassium phosphate (pH 7.4 at 4°C), 20% (v/v) glycerol, 10 mM BME, 0.5 mM PMSF, 10 μg/mL RNase, and 10 μg/mL DNase. Four rounds of sonication of the resulting suspension for 45 s on ice were followed by incubation in the presence of 0.8% CHAPS for 2 h at 4°C. After ultracentrifugation for 1 h at 45,000 rpm, the supernatant was collected and subjected to purification with the use of Ni²⁺-NTA agarose. The resin was washed with buffer containing 100 mM potassium phosphate (pH 7.4 at 4°C), 100 mM NaCl, 20% (v/v) glycerol, 10 mM BME, 0.5 mM PMSF, 0.5% CHAPS, and 1 mM histidine. The protein was eluted using 40 mM histidine in the same buffer. Pooled P450-containing fractions were diluted 10-fold with buffer containing 5 mM potassium phosphate (pH 7.4 at 4°C), 20% (v/v) glycerol, 1 mM EDTA, 0.2 mM dithiothreitol (DTT), 0.5 mM PMSF, and 0.5% CHAPS and applied to a CM-Macroprep cation exchange column. The column was washed using 5 mM potassium phosphate (pH 7.4 at 4°C), 20 mM NaCl, 20% (v/v) glycerol, 1 mM EDTA, and 0.2 mM DTT, and the protein was eluted with the buffer containing 50 mM

^aP450 2B4 WT will refer to 2B4dH (H226Y) with N-terminal truncations and C-terminal tetra-His tag to facilitate purification.

^bP450 2B6 WT stands for an N-terminally truncated and modified and C-terminally His-tagged form of the cytochrome P450 2B6 genetic variant K262R with an internal Y226H mutation.

potassium phosphate (pH 7.4 at 4°C), 500 mM NaCl, 20% (v/v) glycerol, 1 mM EDTA, and 0.2 mM DTT. The P450 concentration was determined by measuring a difference spectrum of the ferrous carbonyl complex of the heme protein [35].

Spectral Binding Titrations

Interactions of P450 2B4WT and 2B6WT with amlodipine were studied by spectrophotometric titrations of 1 μ M enzyme in 100 mM HEPES (pH 7.4 with 20% glycerol) in 1.0 mL at 25°C. The spectra of absorbance were recorded at 0 – 60 μ M amlodipine. The amlodipine was solved in 20 mM potassium acetate (pH 4.0), which has no effect on the spectral properties of the enzyme. The spectra of absorbance (340 – 700 nm) were measured with an S2000 rapid scanning CCD spectrometer (Ocean Optics, Inc., Dunedin, FL, USA) equipped with an L7893 UV-VIS fiber-optics light source (Hamamatsu Photonics K. K., Hamamatsu City, Shizuoka, Japan) and a custom-made thermostated cell holder with magnetic stirrer using a semi-micro quartz cell with a stirring compartment (10 $\times$ 4 mm light path) from Hellma GmbH (Mülheim, Germany) as described previously [36, 37].

Enzymatic Assays

The rates of *O*-dealkylation of 7-EFC and 7-BR were measured as described earlier [38, 39]. Specifically, 100 μ L of the reaction mixture contained 10 pmol P450, 40 pmol cytochrome P450 reductase, and 20 pmol cytochrome *b*₅ in 50 mM HEPES, 15 mM MgCl₂, 0.1 mM EDTA (pH 7.6). The concentrations of 7-EFC or 7-BR were in the range of 2 ~ 200 μ M or 0.5 ~ 10 μ M, respectively. The samples were preincubated for 5 min at 37°C before initiation of the reaction by addition of 1 mM NADPH. After 5 min of incubation at 37°C the reaction was stopped by the addition of cold acetonitrile (50 μ L), and 50 μ L of the reaction mixture was diluted into 950 μ L of Tris-HCl buffer (pH 9.0). The content of 7-HFC, the product of the reaction, was determined from the intensity of fluorescence at 500 nm measured with excitation at 410 nm. 7-BR *O*-dealkylation was examined in a reconstituted system as described earlier [39]. The composition of the incubation mixture was similar to that described above for 7-EFC *O*-deethylation. Formation of resorufin was monitored fluorometrically using $\lambda_{\text{ex}} = 550$ nm and $\lambda_{\text{em}} = 585$ nm. The K_{m} and k_{cat} values were calculated using Michaelis-Menten nonlinear regression analysis with GraphPad Prism (GraphPad Software, San Diego, CA).

Data Analysis

Absorbance spectra obtained in titration experiments were analyzed by principal component analysis (PCA) as described previously [36, 40]. To interpret the changes in the spectra of absorbance in term of the concentration of P450 low-spin, high-spin and P420 species, we used a least squares fitting of the spectra of the first and second principal components to the set of spectral standards of pure low-spin, high-spin, and P420 species of P450 2B4. All data treatment procedures and curve fitting were performed with the SpectraLab software package [36].

Curve Fitting

The fitting of the absorbance titration data was performed based on models with either one or two substrate binding sites in the enzyme. In the case of the one binding site model we used the equation for equilibrium of binary association also known as “square root” or “tight binding equation” ([41], page 73, Eq. II-53):

$$[ES] = \frac{[E]_0 + [S]_0 + K_D - \left\{ ([E]_0 + [S]_0 + K_D)^2 - 4 \cdot [E]_0 \cdot [S]_0 \right\}^{1/2}}{2} \quad (1)$$

where $[E]_0$, $[S]_0$, and $[ES]$ designate the concentrations of the enzyme, substrate and enzyme-substrate complex respectively, and K_D is the dissociation constant. The analysis with the model with two binding sites was based on the following scheme:

(2)

Here E stands for the substrate-free enzyme; ES and SE designate the complexes of the substrate bound at each of the two sites; and SES stands for the ternary complex with both binding sites occupied. K_{D1} , K_{D2} , K_{D3} and K_{D4} are the dissociation constants. This general model is, however, too complex to be applicable for reliable fitting of the actual steady-state titrations. In our analysis we simplified it to the case where substrate binding at each site has no effect on the interactions at the second one, so that $K_{D1} = K_{D3}$, $K_{D2} = K_{D4}$. This simplification results in a “random order” or “parallel” binding scheme with two binding sites.

In the general case when the concentration of the enzyme is comparable to the concentrations of the substrate and changes in free substrate concentration cannot be neglected, the steady-state concentrations of the free enzyme $[E]$ and the ternary complex $[SES]$ for this mechanism may be described with the following relationships [42]:

$$\begin{cases} [S]^2 - (2 \cdot [E]_0 - [S]_0 + K_{\Sigma}) \cdot [S] - (K_{\Sigma} \cdot [E]_0 - K_{\Sigma} \cdot [S]_0 + K_{\Pi}) \cdot [S] - K_{\Pi} \cdot [S]_0 = 0 \\ [SES] = \frac{[E]_0 \cdot [S]^2}{[S]^2 + K_{\Sigma} \cdot [S] + K_{\Pi}} \end{cases} \quad (3)$$

where

$$K_{\Pi} = K_{D1} \cdot K_{D2}; \quad K_{\Sigma} = K_{D1} + K_{D2}$$

Although the explicit analytical solution of this system is intricate, the concentrations $[E]$, $[SES]$ and the total of the $[ES]$, and $[SE]$ may be found from a numerical solution of (3), as previously described [42, 43].

Similar to the approach used to analyze the substrate-induced spin shift in P450 3A4 [44, 45], we added to the above model an allowance for a difference in the amplitude of the substrate-induced spectral changes between the ternary and binary enzyme-substrate complexes. For that purpose the observed amplitude was represented as a function of the concentrations of ternary and binary complexes:

$$A_s = A_B \cdot \left(\frac{[ES] + [SE]}{[E]_0} \right) + A_T \cdot \left(\frac{[SES]}{[E]_0} \right) = A_{\Sigma} \cdot \left[F_B \left(\frac{[ES] + [SE]}{[E]_0} \right) + (1 - F_B) \cdot \left(\frac{[SES]}{[E]_0} \right) \right] \quad (4)$$

Here A_s is the amplitude of spectral changes observed at a given concentration of substrate, A_B , and A_T are the maximal relative amplitudes of the spectral changes observed upon the formation of the binary (either ES or SE) and ternary (SES) complexes respectively, and $A_{\Sigma} = A_B + A_T$. The concentrations $[ES] + [SE]$ and $[SES]$ are determined according to (3). The parameter F_B , which characterizes the relative amplitude of the changes observed in the binary complex, is determined as follows:

$$F_B = \frac{A_B}{A_B + A_T} \quad (5)$$

The case when $F_B = 0$ corresponds to the case where substrate-induced spectral changes are observed in the ternary complex only. The situation when the full-amplitude spectral changes takes place upon the formation of the complex ES and the binding of the second molecule has no further effect corresponds to $F_{ES} = 0.5$. The intermediate situation, when the binding of the first substrate molecule results in some partial spectral signal, although the full amplitude requires the formation of the ternary complex, is observed when $0 < F_{ES} < 0.5$. The case when $F_{ES} > 0.5$ represent the situation where the formation of the ternary complex results in a backward changes (decrease in the amplitude) relative to that observed in the binary complex.

The fitting of the experimental data sets to the above equations was done using a combination of Nelder-Mead and Marquardt non-linear regression algorithms as implemented in our SpectraLab software package [36, 45].

Results and Discussion

Interactions of Amlodipine with P450 2B4 Studied by Absorbance Spectroscopy

As seen from the series of spectra obtained upon titration of P450 2B4 with amlodipine (Fig. 1A), ligand additions resulted in the appearance of an extensive absorbance band from

amlodipine centered at 366 nm. Application of PCA to this series yields two significant principal components that cover over 99.9% of the total changes (Fig. 1A, inset). While the second component reveals a Type-II change in the enzyme (Fig. 1A inset, dashed line), the main contribution to the spectrum of the first component (Fig. 1A inset, solid line) is from the absorbance band of amlodipine. Correspondingly, the dependence of the loading factor (the amplitude) of the first principal component on the concentration of amlodipine was nearly linear (data not shown). In order to further analyze the ligand-induced changes in the enzyme we eliminated the absorbance band of amlodipine using the technique applied in our earlier studies of P450 interactions with 1-pyrenebutanol [42] or Fluorol-7GA [46]. Specifically, we approximated the spectrum of the first principal component with the absorbance spectrum of amlodipine combined with a set of the prototypical absorbance spectra of the high-spin, low-spin and the Type-II substrate-bound P450 2B4. This procedure is illustrated in Fig. 1B, where the overall approximation of the first principal component (empty circles) is shown as a thin solid line, and the amlodipine (dashed lines) and P450-specific contributions (thick solid line) are illustrated. The amlodipine-specific part of this approximation (i.e., the spectrum shown in dashed line in Fig. 1B) was then subtracted from the experimental spectra with the coefficients equal to the respective loading factors found from the PCA procedure.

The series of spectra obtained after subtraction of the amlodipine absorbance is shown in Fig. 1C. As seen from the inset to this figure, the differential changes in P450 absorbance reveal a typical Type-II transition in the enzyme. PCA of this series shows that the first principal component covers 99.9% of the observed changes and corresponds to the transition of a mixture of the low spin (83%) and the high-spin (17%) states of the substrate-free enzyme to the Type-II complex (Fig. 1C inset, circles). The corresponding changes in the content of the three states of the enzyme are shown in Fig. 1D.

Approximation of the titration curves with an equation for the equilibrium of binary association (1) gave a relatively low square correlation coefficient ($r^2 = 0.975$) and revealed important systematic deviations of the fitting curves (Fig. 1D, dashed lines) from the experimental data set. Poor quality of fitting suggests that the mechanism of the enzyme interactions with amlodipine may be more complex than a simple binary association reaction that was reported in previous study [28].

Based on our recent X-ray structure of a dual-ligand complex of P450 2B4 and amlodipine [28], we tested the possibility that the amlodipine-induced Type-II spectral changes reflect the formation of the enzyme complex with two ligand molecules. Therefore we approximated the titration curves with a model with two independent (parallel) substrate-binding events (see scheme (2) in Materials and Methods). Fitting of the titration curves with the corresponding equations (3) resulted in very accurate approximations ($r^2 > 0.995$) that showed no systematic deviation from the experimental data points (Fig. 1D, solid lines). Importantly, fitting of the titration curves reflecting the concentrations of the low-spin, high-spin enzyme, and the Type-II complex yielded very similar parameters of the binding (K_{D1} , K_{D2} and F_B). This similarity indicates that the formation of all three types of complexes of P450 2B4 with amlodipine (*ES*, *SE* and *SES*) results in the Type-II spectral changes only and is not associated with any displacement of the spin equilibrium. The parameters of the P450

2B4 interactions with amlodipine are summarized in Table 1. As seen from this table, the dissociation constants of the two amlodipine binding steps differ by over two orders of magnitude, and the value of F_B is equal to 0.30 ± 0.04 . According to our definition of the parameter F_B (5), the ratio of the amplitudes of the spectral signal observed upon the formation of the binary and ternary complexes may be determined as $A_B/A_T = F_B/(1-F_B)$. According to this relationship the averaged amplitude of the Type-II changes observed upon the formation of the binary complexes (either *ES* or *SE*) constitutes only about 43% of that characteristic of the ternary complex (*SES*). This fact indicates that the full amplitude of the amlodipine-induced Type-II spectral transition is attained only upon the formation of the ternary complex. The amplitude of the spectral changes resulting from the formation of the binary complexes *ES* and *SE* are only partial. Our results are consistent with a mechanism where the Type-II spectral changes reflecting the formation of the complexes *ES* and *SE* (the individual parameters of which cannot be mathematically resolved) are due to only one of the two types of binary interactions, while the association at the second site (presumably the distal one) may be spectrally silent by itself.

The fact that the full amplitude of the Type-II changes is attained only upon the formation of the ternary complex suggests that the binding of the second (distal) amlodipine molecule affects the orientation and/or the mobility of the proximally-bound one, thereby increasing the probability of ligation of its aromatic nitrogen to the heme iron and enhancing the amplitude of the Type-II spectral changes. Strictly speaking, this mechanism suggests that the distal binding event would enhance the binding affinity for the proximally bound molecule (i.e., $K_{D2} < K_{D4}$ in terms of the scheme (2)). It should be noted, however, that the numeric solution of the system of equations depicting the two-binding sites model (2) is possible only for the extreme cases of sequential ($K_{D1} \ll K_{D4}$) or parallel independent ($K_{D1} = K_{D3}$, $K_{D2} = K_{D4}$) binding mechanisms. The intermediate situation, where $K_{D2} < K_{D4}$, presumably represents better the actual mechanism but the respective system of equations is too complex and contains too many parameters to be used for non-linear regression of the experimental data. Therefore, the mutual effects of the two binding events cannot be quantitatively assessed from our results.

Interactions of Amlodipine with P450 2B6

At first glance, the spectral changes observed upon amlodipine binding to P450 2B6 (Fig. 2) are similar to those discussed above for P450 2B4. Here again, the addition of amlodipine results in a profound Type-II spectral transition in the heme protein. However, detailed analysis of the spectra reveals an important difference between the two proteins. In contrast to P450 2B4, where PCA yields only one principal component that adequately depicts over 99.9% of the observed changes, the analysis of the spectral series obtained with P450 2B6 allows resolution of two significant principal components (Fig. 2A, inset). The first principal component is similar to that observed with 2B4 and corresponds to the Type-II changes, whereas the second principal component reveals a typical type-I (low-to-high spin) transition. Accordingly, the titration curves obtained for the changes in the fractions of the low-spin and high spin heme protein and its Type-II complex have clearly distinct shapes (Fig. 2B). As with P450 2B4, attempts to approximate these titration curves with a one binding site model (equation (1)) results in poor fitting and large systematic deviations (Fig.

2B, dashed lines). Moreover the apparent value of K_D obtained from the fitting of the changes in the high spin fraction of the enzyme (1.4 μM) is considerably higher than the values deduced from the fitting of the changes in the concentration of the low-spin state (0.5 μM) or Type-II complex (0.4 μM). In contrast, the approximations of these curves with the equations deduced for the two binding site model (3) are very accurate ($r^2 = 0.995$) and show no systematic deviation from the experimental points (Fig. 2B, solid lines). Importantly, the values of both K_{D1} and K_{D2} for the interactions of 2B6 with amlodipine are considerably higher than those obtained with 2B4 (Table 1).

The validity of the model applied in our analysis is confirmed by mutual consistency of the estimates of the dissociation constants obtained from the fitting of the changes in the concentration of the Type-II complex with those deduced from the changes in the content of the high-spin heme protein. As seen from the data presented in Table 1, the sets of the parameters deduced from the fitting of these two types of titration curves differ only in the value of F_B , while the difference in the values of dissociation constants is insignificant.

In terms of the model (2) the value of 0.37 found for F_B characteristic of the changes in the content of the Type-II state of the enzyme (Table 1) suggests that, while the formation of the ternary complex is associated with a complete transition of the heme protein to the Type-II complex, the Type-II changes that accompany the formation of the binary complexes of P450 2B6 are only partial. The averaged amplitude of these changes constitutes only about 50% of that observed in the complex *SES*. Here again we may speculate that formation of one of the two binary complexes (either *ES* or *SE*) does not cause any Type-II transition in the enzyme.

At the same time, the value of F_B of 0.25 deduced from the dependencies on amlodipine concentration of the content of the high-spin state of P450 indicates that the decrease in its content caused by the formation of the complex *SES* is approximately three times higher than that observed in the complexes *ES* and *SE*. This finding suggests that at least one of the two binary association processes results in some low-to-high spin transition that partially compensates for the disappearance of the ligand-free high spin state of the enzyme upon its interactions with amlodipine. When compared with the amlodipine complex of P450 2B4, the P450 2B6-amlodipine structure showed largest differences in the C–D loop, H–I loop, C-terminal loop, and β_{1-1} and β_{1-2} sheets. In the P450 2B4-amlodipine complex, the side chain of T302 makes a hydrogen bond with the pyridine nitrogen of amlodipine, whereas in P450 2B6 the polar side chain of T302 interacts with the ethoxy oxygen of amlodipine [28]. Moreover, the calculated volume of the P450 2B6 active site cavity (755 Å³) is larger than that of the P450 2B4-amlodipine complex (605 Å³). These specific features may influence the effect of amlodipine on the water network in the heme pocket and thus result in observed difference in the position of spin equilibrium (degree of water ligation to the heme iron) in the binary complexes of the two enzymes with amlodipine.

Construction and Expression of Substrate Access Channel Mutants in P450 2B4 and 2B6

Residues located at the channel 2*a* and 2*f* entrance in P450 2B4 and 2B6 were chosen based on the X-ray crystal structures of the amlodipine complexes. Residues 48, 49, and 73 in both P450 2B4 and 2B6 were substituted with alanine or lysine, and positions 212, 216, 219, and

220 in P450 2B4 were substituted with tryptophan to investigate the effect of the replacement on steady-state kinetics of the oxidation of P450 2B substrates. The substrate access channel residues that were subjected to site-directed mutagenesis are shown in Fig. 3.

Most of the P450 2B4 mutants (R48A, K49A, R73K, V216W, and F220W) showed 40 ~ 80% decreased expression levels, whereas F212W and L219W had 20% increased levels (data not shown). P450 2B6 and three mutants were coexpressed with the molecular chaperones GroEL/ES in *E. coli* JM109 cells. 2B6 R48A and R73K showed 20 ~ 50% of the level of 2B6, and R49A expressed at similar levels to the 2B6 enzyme.

Steady-state kinetics of substrate access channel mutants in P450 2B4 and 2B6

The kinetic parameters obtained for 7-EFC and 7-BR oxidation by the substrate access channel mutants in P450 2B4 and P450 2B6 are shown in Table 3. R73K in P450 2B6 showed a 5-fold decrease in catalytic efficiency with 7-EFC that resulted from equal effects on both K_m and k_{cat} . The oxidation by R73K of 7-BR showed a 2-fold decrease in catalytic efficiency as a result of decreased k_{cat} .

Oxidation of 7-EFC by R48A in P450 2B4 showed a 2.5-fold lower catalytic efficiency mainly due to the changes in K_m . R73K, V216W, L219W, and F220W showed a 2-fold increase in K_m , whereas the k_{cat} values were decreased 4~8-fold, which resulted in significant decreases in catalytic efficiency (10~11-fold). With 7-BR R73K, V216W, L219W, and F220W showed significant decreases in the k_{cat} (4~8-fold) with slightly decreased K_m (1.8~3.3-fold) respectively, which resulted in a 2 to 2.8-fold decrease in catalytic efficiency. Thus with both enzymes, effects of the amino acid substitutions were more pronounced on 7-EFC oxidation than on 7-BR. It is noteworthy that the log *P* value of 7-EFC (3.3) is much lower than that of 7-BR (5.1).

It was of interest to compare these results with previous structural, mutagenesis, and computational studies in P450 2B enzymes. Photoaffinity labeling of P450 2B4 with 3-azidoadamantane identified three substrate-binding sites outside the active site [49]. The first site is located near the helices L and C on the heme proximal face, and the second and third sites are situated near channels 2*b*/2*e* and 2*f*, respectively. Recently, a study of potent mechanism-based inhibition of P450 2B4 by 9-ethynylphenanthrene (9EP) indicated an allosteric site for 9EP located near the β_1 - β_2 loop, B-C loop, F' helix, and β_4 loop [50]. The identified region was similar to the pathways 2*a* and 2*f* in the structure of P450 2B4 in complex with amlodipine [28]. With regard to other P450 enzymes, channel 2*a* has been suggested to be one of the routes for substrate access in P450 2C9 and P450 3A4, and this channel has been proposed as the main ligand egress channel in several bacterial P450s [19, 51]. In an alignment with P450 BM3, R73 in P450 2B4 is analogous to R47 in the bacterial enzyme. This residue plays an important role in substrate recognition via interaction with a proposed hydrogen-bonded water network [52]. In addition, structural analysis of P450 24A1 showed that multiple detergent molecules occupied the membrane-directed substrate access channel 2*a*, which is the main access route [53]. From computational predictions F104 in P450 24A1, which corresponds to R73 in P450 2B4, is at the entrance to channel 2*a*.

Different gating mechanism in several P450 isoforms have been suggested previously with different substrate access channels for each P450 enzymes mainly involving phenylalanine (Phe) or arginine (Arg) residues. F108 and F241 are the putative gating residues in the channel 2c of P450 3A4 [54]. F115 in P450 2B1 may act as a gate keeper in channel 2e for testosterone egress [55], while F87 and F193 are the gating residues in channel 2a in P450cam [19]. Based on the current study, R73 and F220 in P450 2B4 located at the entrance of substrate access channel 2a and 2f may be responsible for substrate recognition as gatekeepers.

In conclusion, we have demonstrated that the interactions of P450 2B6 and 2B4 with amlodipine involve two individual ligand-binding events and result in the formation of the enzyme complex with two drug molecules. The results of our steady-state absorbance titration experiments are consistent with a random order (parallel) binding mechanism where the full-amplitude Type-II spectral transition is observed in the ternary complex only. In addition, steady-state kinetic studies of the putative access channel residues demonstrated that residues R73, V216, L219, and F220 play an important role in the turnover of P450 2B substrates. These results encourage further investigation involving site-directed mutagenesis based on crystal structures of P450 2B enzymes and provide insight into the role in substrate recognition and specificity of multiple access channels resulting from intrinsic structural differences among the P450s as well as physicochemical properties of the substrates.

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Abbreviations

P450	Cytochrome P450-dependent monooxygenases
7-EFC	7-ethoxy-4-(trifluoromethyl)coumarin
7-BR	7-benzyloxyresorufin
RNase	ribonuclease A
DNase	deoxyribonuclease I
BME	2-mercaptoethanol
PMSF	phenylmethanesulfonyl fluoride
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
EDTA	Ethylenediaminetetraacetic acid
DTT	dithiothreitol
PCA	principal component analysis

References

1. Johnson EF, Stout CD. Biochem Biophys Res Commun. 2005; 338:331–336. [PubMed: 16157296]

2. Al Omari A, Murry DJ. *J Pharm Pract.* 2007; 20:206–218.
3. Johnson EF, Stout CD. *J Biol Chem.* 2013; 288:17082–17090. [PubMed: 23632020]
4. Guengerich FP. *Chem Res Toxicol.* 2001; 14:611–650. [PubMed: 11409933]
5. Ingelman-Sundberg M. *Naunyn Schmiedebergs Arch Pharmacol.* 2004; 369:89–104. [PubMed: 14574440]
6. Poulos TL. *Drug Metab Dispos.* 2005; 33:10–18. [PubMed: 15475411]
7. Anzenbacher P, Anzenbacherova E, Lange R, Skopalik J, Otyepka M. *Acta Chim Slov.* 2008; 55:63–66.
8. Poulos TL. *Biochem Biophys Res Commun.* 2005; 338:337–345. [PubMed: 16185651]
9. Cojocaru V, Winn PJ, Wade RC. *Biochim Biophys Acta.* 2007; 1770:390–401. [PubMed: 16920266]
10. Wade RC, Winn PJ, Schlichting I, Sudarko. *J Inorg Biochem.* 2004; 98:1175–1182. [PubMed: 15219983]
11. Scott EE, He YQ, Halpert JR. *Chem Res Toxicol.* 2002; 15:1407–1413. [PubMed: 12437331]
12. Schlichting I, Berendzen J, Chu K, Stock AM, Maves SA, Benson DE, Sweet RM, Ringe D, Petsko GA, Sligar SG. *Science.* 2000; 287:1615–1622. [PubMed: 10698731]
13. Poulos TL, Finzel BC, Gunsalus IC, Wagner GC, Kraut J. *J Biol Chem.* 1985; 260:16122–16130. [PubMed: 4066706]
14. Poulos TL, Finzel BC, Howard AJ. *J Mol Biol.* 1987; 195:687–700. [PubMed: 3656428]
15. Li H, Poulos TL. *Nat Struct Biol.* 1997; 4:140–146. [PubMed: 9033595]
16. Schoch GA, Yano JK, Sansen S, Dansette PM, Stout CD, Johnson EF. *J Biol Chem.* 2008; 283:17227–17237. [PubMed: 18413310]
17. Porubsky PR, Meneely KM, Scott EE. *J Biol Chem.* 2008; 283:33698–33707. [PubMed: 18818195]
18. Strushkevich N, Usanov SA, Park HW. *J Mol Biol.* 2010; 397:1067–1078. [PubMed: 20149798]
19. Winn PJ, Ludemann SK, Gauges R, Lounnas V, Wade RC. *Proc Natl Acad Sci U S A.* 2002; 99:5361–5366. [PubMed: 11959989]
20. Schleinkofer K, Winn PJ, Lüdemann SK, Wade RC. *EMBO Rep.* 2005; 6:584–589. [PubMed: 16028306]
21. Honma W, Li W, Liu H, Scott EE, Halpert JR. *Arch Biochem Biophys.* 2005; 435:157–165. [PubMed: 15680917]
22. Scott EE, Liu H, Qun He Y, Li W, Halpert JR. *Arch Biochem Biophys.* 2004; 423:266–276. [PubMed: 15001391]
23. Scott EE, White MA, He YA, Johnson EF, Stout CD, Halpert JR. *J Biol Chem.* 2004; 279:27294–27301. [PubMed: 15100217]
24. Gay SC, Roberts AG, Maekawa K, Talakad JC, Hong WX, Zhang Q, Stout CD, Halpert JR. *Biochemistry.* 2010; 49:8709–8720. [PubMed: 20815363]
25. Gay SC, Zhang H, Wilderman PR, Roberts AG, Liu T, Li S, Lin HL, Zhang Q, Woods VL Jr, Stout CD, Hollenberg PF, Halpert JR. *Biochemistry.* 2011; 50:4903–4911. [PubMed: 21510666]
26. Scott EE, He YA, Wester MR, White MA, Chin CC, Halpert JR, Johnson EF, Stout CD. *Proc Natl Acad Sci U S A.* 2003; 100:13196–13201. [PubMed: 14563924]
27. Zhao Y, White MA, Muralidhara BK, Sun L, Halpert JR, Stout CD. *J Biol Chem.* 2006; 281:5973–5981. [PubMed: 16373351]
28. Shah MB, Wilderman PR, Pascual J, Zhang Q, Stout CD, Halpert JR. *Biochemistry.* 2012; 51:7225–7238. [PubMed: 22909231]
29. DeVore NM, Scott EE. *J Biol Chem.* 2012; 287:26576–26585. [PubMed: 22700965]
30. Zhao B, Lei L, Kagawa N, Sundaramoorthy M, Banerjee S, Nagy LD, Guengerich FP, Waterman MR. *J Biol Chem.* 2012; 287:10613–10622. [PubMed: 22262854]
31. Ekroos M, Sjogren T. *Proc Natl Acad Sci U S A.* 2006; 103:13682–13687. [PubMed: 16954191]
32. Harlow GR, He YA, Halpert JR. *Biochim Biophys Acta.* 1997; 1338:259–266. [PubMed: 9128144]

33. Scott EE, Spatzenegger M, Halpert JR. *Arch Biochem Biophys*. 2001; 395:57–68. [PubMed: 11673866]
34. Mitsuda M, Iwasaki M. *Protein Expr Purif*. 2006; 46:401–405. [PubMed: 16310378]
35. Omura T, Sato R. *J Biol Chem*. 1964; 239:2379–2385. [PubMed: 14209972]
36. Davydov DR, Deprez E, Hoa GH, Knyushko TV, Kuznetsova GP, Koen YM, Archakov AI. *Arch Biochem Biophys*. 1995; 320:330–344. [PubMed: 7625841]
37. Muralidhara BK, Negi S, Chin CC, Braun W, Halpert JR. *J Biol Chem*. 2006; 281:8051–8061. [PubMed: 16439365]
38. He YQ, He YA, Halpert JR. *Chem Res Toxicol*. 1995; 8:574–579. [PubMed: 7548737]
39. Kumar S, Chen CS, Waxman DJ, Halpert JR. *J Biol Chem*. 2005; 280:19569–19575. [PubMed: 15774478]
40. Renaud JP, Davydov DR, Heirwegh KP, Mansuy D, Hui Bon Hoa GH. *Biochem J*. 1996; 319(Pt 3):675–681. [PubMed: 8920966]
41. Segel, IH. Wiley-Interscience; New York: 1975.
42. Davydov DR, Kumar S, Halpert JR. *Biochem Biophys Res Commun*. 2002; 294:806–812. [PubMed: 12061778]
43. Davydov DR, Fernando H, Halpert JR. *Biophys Chem*. 2006; 123:95–101. [PubMed: 16701937]
44. Fernando H, Rumfeldt JA, Davydova NY, Halpert JR, Davydov DR. *Xenobiotica*. 2011; 41:281–289. [PubMed: 21143007]
45. Davydov DR, Rumfeldt JA, Sineva EV, Fernando H, Davydova NY, Halpert JR. *J Biol Chem*. 2012; 287:6797–6809. [PubMed: 22194603]
46. Davydov DR, Davydova NY, Halpert JR. *Biochemistry*. 2008; 47:11348–11359. [PubMed: 18831537]
47. Cojocaru V, Balali-Mood K, Sansom MS, Wade RC. *PLoS Comput Biol*. 2011; 7:e1002152. [PubMed: 21852944]
48. Berka K, Hendrychova T, Anzenbacher P, Otyepka M. *J Phys Chem A*. 2011; 115:11248–11255. [PubMed: 21744854]
49. Hodek P, Karabec M, Sulc M, Sopko B, Smrcek S, Martinek V, Hudecek J, Stiborova M. *Arch Biochem Biophys*. 2007; 468:82–91. [PubMed: 17961497]
50. Zhang H, Gay SC, Shah M, Foroozesh M, Liu J, Osawa Y, Zhang Q, Stout CD, Halpert JR, Hollenberg PF. *Biochemistry*. 2013; 52:355–364. [PubMed: 23276288]
51. Skopalik J, Anzenbacher P, Otyepka M. *J Phys Chem B*. 2008; 112:8165–8173. [PubMed: 18598011]
52. Haines DC, Tomchick DR, Machius M, Peterson JA. *Biochemistry*. 2001; 40:13456–13465. [PubMed: 11695892]
53. Annalora AJ, Goodin DB, Hong WX, Zhang Q, Johnson EF, Stout CD. *J Mol Biol*. 2010; 396:441–451. [PubMed: 19961857]
54. Fishelovitch D, Shaik S, Wolfson HJ, Nussinov R. *J Phys Chem B*. 2009; 113:13018–13025. [PubMed: 19728720]
55. Li W, Liu H, Scott EE, Grater F, Halpert JR, Luo X, Shen J, Jiang H. *Drug Metab Dispos*. 2005; 33:910–919. [PubMed: 15821040]

Highlights

- Absorbance spectroscopy revealed two amlodipine binding events in solution with P450 2B6 and 2B4.
- The results are in agreement with recently solved X-ray structures of the amlodipine complexes.
- The function of access channel residues was determined by site-directed mutagenesis and steady-state kinetics.
- R73 in P450 2B6 and R73, V216, L219, and F220 in 2B4 are important for catalytic efficiency.

Fig. 1. P450 2B4 interactions with amlodipine

(A) The series of absorbance spectra obtained in the presence of increasing concentrations of amlodipine. The inset shows normalized spectra of the first (solid line) and the second (dashed line) principal components. (B) Approximation of the spectrum of the first principal component (circles) with the absorbance spectrum of amlodipine combined with a set of the prototypical absorbance spectra of the high-spin, low-spin and the Type-II substrate-bound P450 2B4. The thin solid line represents the overall approximation, whereas the amlodipine- and P450-specific spectral contributions are shown in dashed and thick solid lines, respectively. (C) The series of absorbance spectra obtained after subtraction of the

amlodipine absorbance. The inset shows the respective differential spectra and the first principal component (empty circles). (D) The changes in the fractions in the low-spin (circles), high-spin (inverted triangles), and type-II complex (squares) of the enzyme versus the concentration of amlodipine. The results of the fitting of the data set to equation 1 (dashed line) and to equation 3 (solid line) are shown.

Fig. 2. P450 2B6 interactions with amlodipine

(A) The series of absorbance spectra obtained after suppression of the amlodipine absorbance. The inset shows normalized spectra of the first principal component (filled circles) and the second principal component (empty circles) scaled to correspond to a transition in 1 μM enzyme. (B) The changes in the low-spin (circles), high-spin (inverted triangles), and type-II complex fraction (squares) of the enzyme versus the concentration of amlodipine. The results of the fitting of the data set to equation 1 (dashed line) and to equation 3 (solid line) are shown.

Fig. 3. Substrate access channels $2f$ and $2a$ in P450 2B4 and 2B6 as computed by CAVER, showing residues located at the channel entrances that were subjected to site-directed mutagenesis

Heme and amlodipine are colored red and cyan, respectively. (A) Substrate access channels $2f$ and $2a$ observed in the P450 2B4-amlodipine complex (green) and the residues (yellow sticks) located at the entrance of the channel. The density for R48 and for the side chains of K49 and R73 were missing in the P450 2B4-amlodipine structure. (B) Substrate access channel $2f$ found in the P450 2B6-amlodipine complexes (yellow) and the residues (yellow sticks) located at the entrance of the channel.

Table 1

Parameters of amlodipine-induced spectral changes in P450 2B4 and 2B6

Formation of the Type- II complex				Decrease in the high-spin content		
Protein	K_{D1} (μ M)	K_{D2} (μ M)	F_B	K_{D1} (μ M)	K_{D2} (μ M)	F_B
2B4	0.08 ± 0.03	12.4 ± 5.5	0.30 ± 0.04	N/A ^a	N/A	N/A
2B6	0.45 ± 0.24	30.0 ± 2.0	0.37 ± 0.04	0.51 ± 0.27	27.3 ± 3.5	0.25 ± 0.05

Results are the average $\pm$ confidence interval calculated for $p = 0.05$ of 5 ~ 7 independent experiments. The values were determined from the fitting of titration curves for the type- II complex fraction with a parallel binding model.

^aNot applicable. In the case of 2B4 the dependencies of the high-spin heme protein content were virtually indistinguishable in shape from the titration curves reflecting the changes in the type-II complex of the enzyme.

Table 2

Oligonucleotides used for construction of P450 2B4 and 2B6 mutants. Altered nucleotides of the 2B6 and 2B4 mutants are indicated in bold italics.

Mutants	Oligonucleotide
2B6 R48A	5'-G GGA AAC CTT CTG CAG ATG GAT <i>GCA</i> AGA GGC CTA CTC-3'
2B6 R49A	5'-G GGA AAC CTT CTG CAG ATG GAT AGA <i>GCA</i> GGC CTA CTC AAA-3'
2B6 R73K	5'-G GTA CAC CTG GGA CCG AAG CCC GTG GT-3'
2B4 R48A	5'-CTT CTG CAG ATG GAC <i>GCG</i> AAG GGC CTG CTC CG-3'
2B4 K49A	5'-G CAG ATG GAC AGG <i>GCG</i> GGC CTG CTC CGC-3'
2B4 R73K	5'-TG TAC CTG GGA TCC AAA CCC GTG GTC GTG C-3'
2B4 F212W	5'-C TTC TCC CTC ATC AGC TCC <i>TGG</i> TCC AGC CAG GT-3'
2B4 V216W	5'-C TCC TTC TCC AGC CAG <i>TGG</i> TTC GAG CTC TTC TCG-3'
2B4 L219W	5'-TCC AGC CAG GTG TTC GAG <i>TGG</i> TTC TCG GGC TTC CTA AAG-3'
2B4 F220W	5'-AGC CAG GTG TTC GAG CTC <i>TGG</i> TCG GGC TTC C-3'

Table 3

Steady state kinetics of substrate oxidation by P450 2B6 and 2B4 mutants

Protein	7-EFC				7-BR			
	k_{cat} (min ⁻¹)	K_m (μM)	k_{cat}/K_m	k_{cat} (min ⁻¹)	K_m (μM)	k_{cat}/K_m		
2B6 WT	1.9 ± 0.60 ^a	4.2 ± 1.6	0.44 ± 0.02	0.8 ± 0.00	0.5 ± 0.08	1.7 ± 0.28		
R48A	1.7 ± 0.34	6.9 ± 0.8	0.24 ± 0.05	0.8 ± 0.04	0.6 ± 0.26	1.4 ± 0.48		
R49A	1.5 ± 0.19	3.6 ± 0.9	0.44 ± 0.07	0.8 ± 0.04	0.6 ± 0.11	1.5 ± 0.28		
R73K	0.8 ± 0.13	8.1 ± 2.0	0.09 ± 0.01	0.3 ± 0.01	0.5 ± 0.31	0.8 ± 0.41		
2B4 WT	1.8 ± 0.65	17.5 ± 7.2	0.10 ± 0.01	2.5 ± 0.40	2.0 ± 0.97	1.4 ± 0.72		
R48A	1.4 ± 0.65	28.0 ± 14.7	0.04 ± 0.02	1.3 ± 0.19	1.4 ± 0.17	0.9 ± 0.26		
K49A	1.5 ± 0.29	14.2 ± 1.2	0.11 ± 0.02	2.0 ± 0.49	1.2 ± 0.33	1.8 ± 0.72		
R73K	0.5 ± 0.08	33.3 ± 9.1	0.01 ± 0.00	0.5 ± 0.06	1.1 ± 0.37	0.5 ± 0.23		
F212W	2.3 ± 0.67	20.3 ± 6.9	0.11 ± 0.01	2.5 ± 0.75	1.4 ± 0.41	1.9 ± 0.46		
V216W	0.5 ± 0.01	37.8 ± 9.5	0.01 ± 0.00	0.6 ± 0.08	1.0 ± 0.27	0.7 ± 0.23		
L219W	0.3 ± 0.05	36.1 ± 12.5	0.01 ± 0.00	0.3 ± 0.02	0.6 ± 0.07	0.5 ± 0.06		
F220W	0.3 ± 0.02	41.3 ± 9.0	0.01 ± 0.00	0.4 ± 0.02	0.7 ± 0.09	0.5 ± 0.09		

Results are the mean ± standard deviation of three independent experiments done in duplicate.