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Allosteric Interactions in Human Cytochrome P450 CYP3A4: The Role of Phenylalanine 213

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Abstract

The role of Phe213 in the allosteric mechanism of human cytochrome P450 CYP3A4 was studied using a combination of progesterone (PGS) and carbamazepine (CBZ) as probe substrates. We expressed, purified and incorporated in POPC Nanodiscs three mutants, F213A, F213S, and F213Y, and compared them with the wild-type CYP3A4 monitoring spectral titration, the rate of NADPH oxidation and steady-state product turnover rates with pure substrates and substrate mixtures. All mutants demonstrated higher activity with CBZ, lower activity with PGS, and reduced activation of CBZ epoxidation by PGS, most pronounced in F213A mutant. Using all-atom molecular dynamics simulations we compared dynamics of WT CYP3A4 and the F213A mutant incorporated in the lipid bilayer and the effect of the presence of PGS molecule at the allosteric peripheral site and evaluated the critical role of Phe213 in mediating the heterotropic allosteric interactions in CYP3A4.

Graphical Abstract

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Introduction

Cytochromes P450 constitute a large superfamily of heme enzymes, present in almost every species in all biological kingdoms (1). Humans have 56 cytochromes P450, which perform biosynthesis of hormones and metabolism of xenobiotics. Of a dozen xenobiotic metabolizing enzymes, the most important is CYP3A4, which is involved in the liver and intestine metabolism of more than 40% of marketed drugs and pharmaceuticals (2). This cytochrome P450 can catalyze oxidative transformations of various substrates, ranging from ethanol (molecular mass 46 Da) to cyclosporine (1202 Da). Such broad substrate specificity requires a large and flexible active site, which can accommodate more than one substrate of medium size (3-8). Therefore, CYP3A4 can bind simultaneously different molecules in the presence of substrate mixtures, which results in a rich spectrum of cooperative effects, such as heterotropic activation or inhibition (9-12), allosteric properties (13-18) and drug-drug interactions (9, 19-23). Drug-drug interactions mediated by CYP3A4 and other xenobiotic metabolizing P450 enzymes are often clinically relevant, so that warnings about possible deleterious effects are usually seen on the application guidelines (22).

Despite the vast amount of information available from CYP3A4 studies, there is still considerable lack of complete understanding of its allosteric properties and the detailed mechanism of drug-drug interactions. Hence, it is very hard to predict the effect of a new potential substrate, effector, or inhibitor, on the overall turnover of multiple pharmaceuticals (24). Human cytochromes P450 are incorporated into the lipid membrane and are less stable or inactive in detergent solubilized systems, while experimental studies in lipid vesicles face other difficulties, typical for colloidal systems with restricted diffusion and phase heterogeneity. A useful alternative is provided by application of Nanodisc technology, which yields soluble homogeneous and functionally stable preparations of human cytochromes P450 incorporated into the native-like lipid bilayer (25-30).

Based on our previous studies (31-38), we recently proposed a structural model which allows one to analyze the main features of the allosteric properties of CYP3A4 monomer incorporated in a membrane (Figure 1, ref. (38)). The presence of external allosteric site formed by F-F' and G-G' loops was suggested based on pioneering mutational studies by the Halpert group (7, 8) and the first X-ray structure of CYP3A4 with progesterone (PGS) bound at this site (39). Subsequently, binding of substrates and allosteric effectors at this distal site was confirmed by multiple experimental studies and computational models (36, 38, 40). Currently this concept of an allosteric site provides a guideline for further investigations of the mechanisms of drug-drug interactions modulated by CYP3A4, and of the role played by the key residues which constitute this effector site. Previously it was shown that mutations at positions 211-215 can result in substantial changes of homotropic and heterotropic cooperativity (7, 8, 41), as well as in variations in the absolute rates of metabolism, positive or negative, depending on substrates (8, 41-44). Despite these insights, there is lack of mechanistic understanding of these changes, and hence, prediction of drug-drug interactions for new target compounds is still not feasible without extensive and expensive screening.

Recently we demonstrated that the mobility of F213 and its interaction with progesterone (PGS) at the allosteric site play an important role in mediating heterotropic interactions with carbamazepine (CBZ) as a substrate for CYP3A4 incorporated into the native-like POPC Nanodisc bilayer (36). In this work, we explored the role of this residue by making three mutants at this position, F213A, F213S, and F213Y, and systematically probed the changes in functional properties of CYP3A4, using the same pair of substrates. Significant changes in activity and both homotropic and heterotropic cooperativity in all three mutants confirmed our earlier conclusions of the critical role of the F213 residue in mediating the observed allosteric mechanism. In order to obtain more detailed information about structural and dynamic changes of CYP3A4, we initiated a set of molecular dynamics (MD) simulations and compared the effect of the F213A mutation in the presence or absence of PGS at the allosteric site using two CBZ molecules as substrates bound near the heme, with the same MD simulations of the WT CYP3A4. We showed that this mutation cancels the allosteric effect of PGS binding on the rate of CBZ epoxidation, while accelerating this rate in the absence of PGS. On the other hand, PGS hydroxylation is inhibited in this F213A mutant, indicating important role played by F213 residue in substrate recognition and specificity.

Materials and methods.

Protein expression and purification.

Expression and purification of the membrane scaffold protein (MSP), cytochrome P450 CYP3A4 and rat P450 reductase, as well as assembly of CYP3A4 into POPC Nanodiscs (ND) was executed following previously described protocols (46-48). Cytochrome P450 CYP3A4 was expressed from the NF-14 construct in the PCWori+ vector with a C-terminal pentahistidine tag generously provided by Dr. F. P. Guengerich (Vanderbilt University, Nashville, TN). The CYP3A4 mutants F213A, F213S, and F213W were generated in this construct using standard methods following manufacturer's protocols. Primers encoding the mutated DNA were synthesized by IDT DNA (Coralville, IA). Modified gene fragments were generated using PCR with Pfx polymerase and then incorporated into the plasmid using the Gibson assembly procedure. The presence of the desired mutation and the absence of the others were confirmed by sequencing performed in ACGT, Inc (Wheeling, IL).

Cytochrome P450 reductase (CPR) was expressed using the rat CPR/pOR262 plasmid, a generous gift from Dr. Todd D. Porter (University of Kentucky, Lexington, KY). Incorporation of CPR into preformed and purified CYP3A4-Nanodiscs was made by direct addition of CPR at 1:4 CYP3A4/CPR molar ratio, as described (49). All experiments were performed at 37° C using a POPC Nanodisc system analogous to our earlier detailed mechanistic studies (32, 34) in order to allow direct comparison of results. This reconstitution system provides a stable well characterized preparation of CYP3A4 monomer incorporated into the model lipid bilayer effectively mimicking the native membrane.

UV-Vis spectroscopy.

Substrate titration experiments were performed using 1-2 μ M CYP3A4 in Nanodiscs with a Cary 300 spectrophotometer (Varian, Lake Forest, CA) at 37°C. For the mixed titration experiments the mixtures of steroid substrate PGS with CBZ in methanol were prepared at

1:12 molar ratio, selected to match their absolute affinities, and added to the CYP3A4-Nanodisc solution, thereby maintaining the constant substrate ratios. The final concentration of methanol was less than 1.5%.

NADPH oxidation and product formation.

CYP3A4 incorporated Nanodiscs with CPR added at a 1:4 molar ratio, together with substrate were preincubated for 5 minutes at 37°C, in a 1 ml reaction volume in 100 mM HEPES buffer (pH 7.4) containing 10 mM MgCl₂ and 0.1 mM dithiothreitol. The concentration of CYP3A4 was in the range from 60 to 100 nM. The reaction was initiated with the addition of 200 nmol of NADPH. NADPH consumption was monitored for 5 min and calculated from the absorption changes at 340 nm using the extinction coefficient 6.22 mM⁻¹ cm⁻¹. A short reaction time was used to avoid covalent modification of CYP3A4 by CBZ (50). At the end of the incubation period, 0.5 ml of the sample was removed from the cuvette, mixed with 2 ml of dichloromethane and used for product analysis. Cortisolone (2.5 nmol per sample) was added as an internal standard. The samples were thoroughly mixed; after phase separation, the organic layer was isolated and the solvent was removed under a stream of nitrogen. The dried sample was dissolved in 70 µl of methanol and 40 µl was injected onto Ace 3 C18 HPLC column, 2.1 x 150 mm (MAC-MOD Analytical, Chadds Ford, PA). The mobile phase contained 15% acetonitrile and 15% methanol in water; products of PGS hydroxylation and CBZ epoxidation were separated in linear gradient of acetonitrile and methanol rising from 15% to 37% each over 35 min at flow rate 0.2 ml/min. The calibration and method validation was performed using commercially available metabolites of PGS and CBZ. The chromatograms were processed with Millennium software (Waters).

Global analysis for deconvoluting apparent cooperative effects in P450.

Global analysis of homotropic cooperativity for metabolism of one substrate was performed, as previously described (32), by simultaneously fitting the experimental data sets to the four state linear equilibrium binding scheme:

Here E is the concentration of substrate-free CYP3A4, S is the concentration of the free substrate, and ES_i are the concentrations of the binding intermediates, i.e., complexes of CYP3A4 with i molecules of substrate bound (i = 1, 2, 3). Binding of up to three molecules of these substrates to one CYP3A4 monomer was described in previous studies (31, 33, 34, 36, 37, 51, 52). The fractions of the enzyme-substrate complexes were expressed using the standard binding polynomials (53),

$$Y = \frac{\frac{S}{K_1} + \frac{S^2}{K_1 K_2} + \frac{S^3}{K_1 K_2 K_3}}{1 + \frac{S}{K_1} + \frac{S^2}{K_1 K_2} + \frac{S^3}{K_1 K_2 K_3}}$$

with the functional properties at different substrate concentrations represented as the linear combination of the fractional contributions from binding intermediates, where K_i are stoichiometric dissociation constants. For example, the rate of NADPH oxidation by CYP3A4 as a function of substrate concentration S , V_N , is calculated as the weighted sum of the signals from the cytochrome P450 molecules with 0, 1, 2, or 3 substrate molecules bound, having v_0 , v_1 , v_2 , and v_3 rates:

$$V_N = \frac{v_0 + \frac{v_1 S}{K_1} + \frac{v_2 S^2}{K_1 K_2} + \frac{v_3 S^3}{K_1 K_2 K_3}}{1 + \frac{S}{K_1} + \frac{S^2}{K_1 K_2} + \frac{S^3}{K_1 K_2 K_3}}$$

The set of such equations for the spectral titration, NADPH consumption, and product formation have been used for the simultaneous fitting of the experimental data obtained under the same conditions, and hence with the same set of dissociation constants. In order to balance contribution of three data sets to the total root mean square deviation (RMSD) in the fitting, experimental data were scaled by dividing NADPH rates by 10 or 15, and multiplying fractions of high spin shift by 10 or 7. This scaling prevents dominant contribution of NADPH rates into the total RMSD, as described(32). The fitting program was written in MATLAB using the Nelder-Mead simplex minimization algorithm implemented in the MATLAB subroutine “fminsearch.m.”

A Monte Carlo simulation was used to estimate the statistical errors of the fitted parameters as described (54). A normally distributed random error was introduced into each fitted data set, using standard deviations of 2% for spin shift, 5 nmol/nmol/min for NADPH oxidation, and 0.1 nmol/nmol/ min for product formation. A 68% confidence interval for each parameter value from the fits of 200 error-added data sets was used to estimate the error.

Molecular Dynamics methods. CYP3A4 models.

We looked at four distinct scenarios in this paper: (A) two carbamazepine (CBZ) bound to the active site of CYP3A4 in the wild type protein; (B) two CBZ bound to the active site and one progesterone (PGS) bound to the allosteric site in the wild type protein; (C) two CBZ bound to the active site in the F213A mutant; and (D) two CBZ bound to the active site and one PGS bound to the allosteric site in the F213A mutant. The initial configurations with CBZ and PGS bound to the WT CYP3A4 described in (A) and (B) were obtained from the final simulation configurations reported previously (36), with CYP3A4 molecule bound to the solvated POPC membrane (300 lipid molecules).

Similar initial configurations for the F213A mutant were obtained by utilizing the residue mutation plugin Mutator incorporated in VMD (55), which generates mutated pdb and psf files. These configurations were then minimized for 5000 steps, and equilibrated for 200 ps. During the minimization and the equilibration phase, the simulations were performed with the backbone of the protein except the the residue mutated i.e., F213 restrained with a force constant of 1 kcal/mol Å⁻². Furthermore, during the minimization and the equilibration phase the heavy atoms of the bound substrates were also restrained with a force constant of 1

kcal/mol $\text{\AA}^{-2}$ to allow for the relaxation of the protein sidechains in their vicinity. In the subsequent step, the system was simulated without restraints. These configurations with two CBZ in active site with and without PGS were used in the F213A mutant simulations. The above mentioned protocol was repeated for all configurations described in A-D. After the initial equilibration, the simulations incorporating explicit solvent, ions and lipids were extended to at least 250 ns in the production phase..

Molecular dynamics simulation protocols.

Each of the CYP3A4 structures described in (A) – (D) consisted of approximately 107,400 atoms. Molecular Dynamics (MD) simulations were performed using NAMD2 (56), by utilizing the CHARMM36 force field with cMAP corrections (57) for protein, and CHARMM36 force field for lipids (58). Parameters for PGS were obtained by utilizing the derivation from available testosterone and cholesterol CHARMM parameters, as described in previous work (36, 59, 60). Parameters for CBZ were obtained from by analogy from CHARMM General Force Field (61, 62), and incorporated in VMD. We used the TIP3P model for water (63).

MD simulations for the four distinct scenarios were performed within NPT ensemble at 1.0 atm and 310 K. The time steps used in the simulations were 2 fs. Constant pressure was maintained by incorporating the Nosé-Hoover Langevin piston method (64, 65), and constant temperature was maintained by Langevin dynamics with a damping coefficient of 0.5 ps^{-1} that was applied to all the atoms. Non-bonded interactions were cut off at 12 $\text{\AA}$, and smoothing was applied at 10 $\text{\AA}$. The particle mesh Ewald (PME) algorithm was used for long-range electrostatic interactions with a grid density $> 1 \text{ \AA}^{-3}$ (66).

Results

Functional properties of F213 mutants of CYP3A4.

In order to experimentally probe the role of F213 in the allosteric mechanism of CYP3A4, we generated several mutants at this position with various properties of side-chains. Previous studies suggested that the allosteric effect of PGS binding to the peripheral site formed by F-F' and G-G' loops is mediated by the movement of F213 side-chain (and possibly neighboring residues) and concomitant changes of size and shape of substrate binding pocket. These changes, in turn, resulted in a more productive packing of two CBZ molecules and activation of CBZ metabolism (36). The effect of the phenylalanine ring was probed by replacement of F213 by residues with small side-chains, i.e. alanine (apolar) and serine (polar, H-bonding), or tyrosine with similar size, but capable of H-bonding.

Activity of WT CYP3A4 and three mutants F213A, F213S, an F213Y was compared in a reconstituted system with CPR in Nanodiscs, as described in Methods section. Based on the approach described earlier (36), we measured the rates of PGS hydroxylation and CBZ epoxidation as function of substrate concentrations and compared the effect of mutations on activity with these two substrates. When PGS was used as a substrate, the activity of all three mutants decreased approximately to the same extent, with maximum rates half that of the WT CYP3A4 (Fig.2A). Meanwhile, affinities did not demonstrate any significant

change, indicating that the productive binding mode was not significantly perturbed. At the same time, rates of CBZ epoxidation increased in all three mutants by 2 – 2.5 fold (Figure 2B). The strongest effect is observed with F213A mutation for both substrates. In addition the midpoint of CBZ epoxidation rate is reached at lower concentration than for WT, indicating improved binding of this substrate in the F213 mutants.

These contrasting effects of a single mutation at F213 position on two reactions catalyzed by CYP3A4 with PGS and CBZ confirm an important role played by this residue, and most likely by adjacent residues in the F-F' and G-G' loops, which together assemble a "lid" covering the substrate binding pocket. The effect of these residues on the steady-state kinetics of substrate metabolism may, in principle, be due to multiple factors, including changes in substrate binding affinity, positioning of the bound substrate near catalytic iron-oxygen intermediate, as well as in modulation of non-productive decay pathways of active P450 intermediates and futile consumption of NADPH, or uncoupling (67). In order to probe the effect of F213 mutations on coupling, we compared the rates of NADPH consumption during PGS hydroxylation and CBZ epoxidation under the same conditions as used in the product turnover measurements.

The results are shown in Figure 3, and indicate a significant decrease of NADPH oxidation rates in all three mutants when PGS was used as a substrate. However, as the rate of PGS hydroxylation also decreases in all F213 mutants, the overall coupling ratio turns out to be either the same as in WT CYP3A4 in case of F213Y mutation, or even lower in case of other mutants. Lower coupling means that the relative efficiency of PGS hydroxylation decreases due to the F213 mutations. The effect of the same mutations is opposite when CBZ is the substrate. Comparison of the rates of NADPH consumption shown in Fig. 3B with rates of CBZ epoxidation at Fig. 2B clearly demonstrates an increase in the coupling ratio for all three mutants, with the most pronounced effect observed with F213A and F213Y. The significant effects of mutations at position F213 on the functional properties of CYP3A4, together with the strong dependence of these effects of mutations on the coupling of reducing equivalents into productive metabolism with the various substrates, suggest the involvement of the F-F' loop in molecular recognition and selectivity.

In order to understand better the role of F213 residue in this molecular recognition, and to elucidate the functional changes in possible drug-drug interactions caused by F213 mutations, we compared the rates of PGS hydroxylation and CBZ epoxidation by WT CYP3A4 and all three mutants using a mixture of these two substrates at different concentrations while keeping their ratio constant. This approach allows one to evaluate the extent of possible preference for one substrate over another and to avoid any complications due to competitive binding because of higher molar ratio, as was described (36, 38). The results of these experiments, shown in Figure 4A for PGS hydroxylation, reveal that this reaction is strongly inhibited in WT CYP3A4 and in all the F213 mutants, as compared to the same reaction without CBZ (Figure 2A). The extent of this inhibition due to the presence of CBZ is approximately the same in F213A and in F213Y as in WT, and is even stronger in F213S. This observation shows that a more favorable binding of CBZ in the active site as compared to PGS, which has been previously described for the WT CYP3A4 (36), is retained in F213 mutants. However, comparison of the rates of CBZ epoxidation in the

mixed substrates experiments (Figure 4B) to that of CBZ only (Figure 2B) reveals that the activation of CBZ metabolism in the presence of PGS is almost eliminated in the F213A mutant, and significantly reduced in other F213 mutants. Therefore, F213 residue is involved in the communication between the active and peripheral sites and plays an important role in the mechanism of allosteric activation of CBZ epoxidation by PGS.

Spectral titration of WT CYP3A4 and F213 mutants revealed that the shift in ferric heme spin state observed with PGS is lower in all mutants, yet with CBZ it is higher than in WT (Table 1). This indicates changes in the average positioning of productive substrates in the active site near the heme iron, with more favorable position for metabolism in case of CBZ and less favorable for PGS. These changes are also consistent with measured turnover rates and coupling. Hill fits for PGS binding to F213 mutants proved spectral dissociation constants $S_{50} = 24 \mu\text{M}$ for F213A and for F213S with maximum spin shift reaching 65 – 75% in both cases, which is lower than in the wild-type protein.

Taken together, the results obtained for the F213 mutants confirmed an important role played by this residue in the regulation of substrate metabolism. Because of its positioning relatively far from the heme and from the iron-oxygen catalytically active intermediate, ($\sim 15 \text{ \AA}$), F213 is mostly involved in shaping of the substrate binding pocket for productive orientation and in communication between the allosteric effector site and productive substrate binding site. Replacing the benzene ring side chain of F213 clearly perturbs this allosteric communication and the role of PGS as an effector. Moreover, activation of CBZ metabolism by PGS in WT CYP3A4 is recovered in the F213 mutants, even without the presence of PGS, as is most evident in case of F213A. Therefore we decided to study F213A mutant in depth using global analysis approach in order to evaluate possible molecular mechanisms for this cooperative interaction.

The results of global analysis of experimental results for product turnover rates, NADPH oxidation rates and spectral titrations measured as a function of substrate concentration under identical conditions with F213A mutant are shown in Figures 5 for PGS and in Figure 6 for CBZ. The functional properties of all binding intermediates derived from these data are collected at Table 2. Comparison of these characteristics of the F213A mutant with those obtained for WT CYP3A4 provides a more detailed insight into the changes caused by this mutation. When PGS is used as a substrate, the main effect of mutation is due to the dramatically lower activity of the last binding intermediate, in which three PGS molecules are bound to the CYP3A4 monomer. While the molecular mechanism responsible for such low activity is not clear, the tendency of slow substrate metabolism at high concentrations of substrate has been observed in other human P450 enzymes as well, including CYP2E1 (68). Unlike the positive effect of PGS binding at the allosteric site observed in WT CYP3A4 (36), in F213A mutant this binding perturbs positioning of substrate PGS near catalytic iron-oxygen intermediate in unfavorable mode for catalysis.

The opposite effect of F213A mutation upon CBZ epoxidation is manifested in overall activation, as compared to the WT CYP3A4 (Figure 2B). This increase of catalytic rate is mostly due to much faster turnover from the second binding intermediate, as derived from global fitting of experimental results shown in Figure 6 and Table 2. Interestingly, inhibition

of the steady-state turnover upon binding of the third substrate molecule (see Materials and Methods for description) is also observed for CBZ in F213A mutant, but not in WT CYP3A4.

Molecular Dynamics (MD) Simulations.—MD provides visualization of the extent of conformational dynamics on 250 ns time scale, the mobility and the multiple binding modes of substrate CBZ inside the substrate binding pocket and of PGS acting as an effector at the peripheral binding site between the shallow pocket formed by the F-F' and G-G' helices from the protein and the lipid head groups from the lipid membrane. Comparison of an initial configuration equilibrated with two CBZ, with or without PGS, for 10 ns with the substrate free X-ray structure 1TQN(69) clearly shows the flexibility and dynamics of CYP3A4. Most of flexible portions of the structure experience movements, ranging from 3-4 Å of the C α atoms of F213, to ~5 Å between C α atoms of Glu333 and ~6 Å for Thr471. Similar mobility is seen for the B-C loop, e.g. ~4.8 Å shift for C α atoms of Gly112.

In order to explore the effect of the F213A mutation on CBZ metabolism and on the allosteric effect caused by PGS binding at the allosteric site in CYP3A4, we ran four MD simulations: (A) two CBZ molecules equilibrated at the active site in the WT protein, (B) two CBZ molecules at the active site in F213A mutant protein, (C) two CBZ molecules at the active site and one PGS molecule in the allosteric site in the WT protein, and (D) two CBZ molecule at the active site and one PGS molecule in the allosteric site in the F213A mutant. We analyzed the dynamics and interactions of the allosteric residue F213 and its neighbors with CBZ and PGS, as well as the relative mobility and interactions between two CBZ molecules in the active site. In order to distinguish between two CBZ molecules, we labeled the one closest to the heme iron as CBZ1, and the distal as CBZ2.

For two CBZ molecules in the WT CYP3A4 (system A), the two CBZ molecules gradually move closer to each other, as time progresses, with the distance between their closest atoms approaching 2 - 3 Å. The side chain of F213 shows outward movement, and, as a result, the volume of the substrate binding pocket exhibits a minor increase. In the F213A mutant, the two CBZ molecules move even closer to each other, with the distance between closest atoms sometimes reaching 1 Å. The volume of the substrate binding pocket does not appear to change as a result of the mutation. The CBZ2 molecule approaches residue R212, with distance between closest atoms approaching ~1.5 Å. This is observed in both the WT and F213A mutant protein, although the contact between the residue R212 and the CBZ2 molecule is closer in the F213A mutant.

When PGS is present at the allosteric site of the WT CYP3A4 (system C), the MD simulation shows a more pronounced interaction between the CBZ1 substrate and residue R212, similar to the picture observed in the absence of PGS, although with more evident contact. Additionally, the two CBZ molecules come in closer contact, with the distance between closest atoms getting to within 1.5 Å when PGS is present in the allosteric site, as compared to 2 - 3 Å with no PGS bound. This same tendency of closer contact between two CBZ molecules is also observed when PGS is present in the F213A mutant (system D). This is in contrast to trajectory (C), where PGS is absent. PGS molecule comes closer to the CBZ2 molecule, with distance between closest atoms getting to less than 2 Å. The CBZ2

molecule does not come into direct contact with PGS in all four simulations. However, the distance between two atoms in CBZ2 and PGS is smaller in the F213A mutant protein (system D) than in the WT CYP3A4 (system C).

The dynamics of PGS within the allosteric site is distinct in the WT CYP3A4 and F213A mutant. In particular, in the F213A mutant, PGS rearranges its contacts with the residues in the allosteric pocket (Figure 1). Most of the contacts between PGS and allosteric residues are broken and re-formed except those formed with residues 213 and 214, which remain largely intact. Here, we define a ‘contact’ when the distance between two atoms in PGS and an allosteric residue is within 1.5 Å. In contrast, in the WT protein, there is less rearrangement of the PGS contacts with the allosteric residues. The contacts with R212, F213, and D214 remain intact. Comparing the dynamics of PGS at the allosteric site in WT CYP3A4 and F213A mutant protein shows a higher mobility of PGS with respect to allosteric site in the F213A mutant, and many contacts of PGS with these residues undergo significant rearrangement in the course of simulation (Figure 7).

Effect of PGS in WT CYP3A4 and the F213A mutant.

In the WT CYP3A4 protein, the effect of PGS is, in general, more pronounced, with the shift of F213 toward the heme iron being clearly observed (Figure 7). On the contrary, R212 is found at 15 – 16 Å from the heme iron in the presence of PGS, and at 12 Å without PGS. D214 and F215 are less affected by the presence of PGS. In the F213A mutant, this effect of PGS is not observed, except for D214, which moves closer to the heme in the presence of PGS. Comparing the effect of PGS in WT CYP3A4 and the F213A mutant shows that the overall effect of PGS on the dynamics of allosteric residues in the F213A mutant protein is substantially weaker, which is consistent with our experimental results.

C10-C11 bond distances from heme iron of CBZ molecules in WT and F213A CYP3A4.

In order to compare positions of CBZ substrates in the WT CYP3A4 and in F213A mutant, we calculated the distances of the C10-C11 bond of the CBZ1 substrate molecule from the heme iron in WT and F213A mutant protein (Figure 8, A-B). In the F213A mutant protein, this distance is shorter when PGS is present in the allosteric site (system D), as compared to the trajectory without PGS (system C). On the other hand, in the WT CYP3A4, this distance in average is longer in the presence of PGS in the allosteric site, due to the shift of the system to another conformational substate, as discussed below.

We also analyzed the distance of C10-C11 bond of the CBZ2 molecule docked from the heme iron (Figure 8, C-D). This CBZ2 molecule moves towards the heme in both WT and F213A mutant when PGS is present in the allosteric site. The final position of the CBZ2 molecule is somewhat closer to the heme in the WT protein, with average distance of approximately 9.5 Å compared to the corresponding average distance of approximately 10.5 Å in the F213A mutant protein.

In order to compare the dynamics of allosteric pocket as a whole in WT and F213A mutant, both with and without PGS, we selected representative frames from four MD trajectories and superimposed the corresponding CYP3A4 structures, using the frame of the heme mean plane, as described (45). This allows one to focus on the changes of the size and shape of the

substrate binding pocket and to ignore possible bias due to structural variations between other parts of the protein. The overlap of selected structures of F-G loops taken over 250 ns allows extracting specific information about dynamics of allosteric site and the effect of PGS binding. The coordinates of all atoms of residues 211-220 and 233-243 taken at different time points for each trajectory were combined to form a matrix, and singular value decomposition (SVD) analysis was performed as described in Materials and Methods. The results of this analysis, summarized in Figure 9, indicate that, for all four trajectories, movement of allosteric site can be well described by movement between two conformational substates. However, these conformational substates and dynamics of transitions between them are different for WT and F213A mutant, and the presence of PGS results in significant changes of this dynamics. Based on SVD analysis, we selected the most representative conformations from MD trajectories in the attempt to evaluate the main dynamic modes of allosteric pocket in CYP3A4.

Comparison of the results of SVD analysis of the four trajectories yields two main conclusions: 1) the biggest observed difference is between WT and F213A without PGS, as opposed to little difference between WT and F213A in the presence of PGS; 2) consistent with these observations, the addition of PGS to the WT protein results in a significant difference, while smaller changes are observed in F213A upon addition of PGS. In general these conclusions are in a good agreement with experimental results, showing no allosteric effect of PGS on CBZ epoxidation in the mutant, while substantial difference in CBZ turnover between WT and F213A mutant in the absence of PGS. Based on these observations, we can compare these changes in real space and identify the behavior of the main residues involved, in attempt of better understanding this allosteric mechanism in WT CYP3A4.

Comparing the simulations of WT CYP3A4 with and without PGS (Figure 10) should help in answering two main questions: Are the two conformational states observed for both simulations the same, and how do PGS and substrates behave. The first SVD component is most represented by a snapshot taken at 200 ns without PGS and with PGS (Fig. 9, red traces). Superposition of these allosteric pockets shows significant difference, i.e. in the presence of PGS both F-F' and G-G' loops shift in the directions parallel to the I-helix and towards the heme (negative Z). C α atom of F213 shifts by 7.3 Å, and the whole F-F' loop shifts almost as a rigid body, by 7 – 8 Å, when these two snapshots are overlaid using heme plane frame. However, the backbone between residues 211 and 220 does not undergo significant change with all changes due to the movements of the side chains, mostly R212 and F213, and only minor alteration of 214 – 217. The differences between starting positions are minimal, at 10 ns the positions of F213 and F215 are virtually the same in WT CYP3A4 with and without PGS, although mobility of both substrates CBZ1 and CBZ2 is more pronounced.

The mobility of the allosteric pocket (residues 211-220 and 233-243) is significantly different in WT protein in the absence and in the presence of PGS (Figure 10). Without PGS, movement of allosteric pocket is less pronounced, with the side chain of F213 staying out of the substrate binding pocket, as shown in Figure 10A (10 ns gray, 75 ns cyan, 175 ns magenta, 250 ns yellow).

When PGS is present in the allosteric pocket of WT CYP3A4, the whole pocket moves in the opposite direction, with F-F' helix slightly shifting towards the heme and away from the I-helix (Figure 10B). Both substrate molecules move in the same directions. This movement happens after the first 100 ns as a more concerted large scale transition between two distinct conformational substates (see Figure 9C). On the other hand, the more random mobility of allosteric region and no distinct transition has been observed in the absence of PGS. Interestingly, the same conclusion can be made for the F213A mutant, in which the same random mobility of the allosteric pocket is observed without PGS, while the presence of PGS induces transition between two conformational states. However, in the latter case these states are not the same as in the WT protein (see below).

In the F213A mutant the overall dynamics has much in common with the WT CYP3A4. Without PGS, the allosteric pocket, on average, remains in place, fluctuating around equilibrium position at the starting point (Figure 11A).

In the presence of PGS in the allosteric pocket, the F213A mutant undergoes a similar transition from initial position to the one shifted over the heme towards $\beta 5$ strand (Figure 11B). This shift is more pronounced and F-F' loop does not return back towards initial position, as it is observed in the WT CYP3A4.

Comparing the trajectories for WT CYP3A4 and F213A mutant in the presence of PGS shows similar movements of the F-G bundle and PGS towards the substrate CBZ2 and in the direction of the heme. Unlike the mostly translational movement of PGS in F213A simulation, PGS undergoes rotational movement in the WT protein, maintaining contact with F213 (the side chain of which is absent in the mutant). By the end of the 250 ns trajectory, the alpha carbon of F213 moves by 7.7 Å (although the same movement of F-F' backbone is observed in F213A mutant), and PGS slides deeper into the pocket between F-F' and G-G' loops, gradually turning by $\sim 90^\circ$, so that C16 atom of PGS moves by 8.7 Å (10 – 225 ns) and changes orientation from perpendicular to almost parallel to the heme plane.

Discussion.—In this study we explored the critical role of the F213 residue in the allosteric regulation of CYP3A4 metabolism. We created three mutants where the benzene ring of F213 was replaced by a phenol ring (F213Y), hydroxyl group (F213S), and methyl group (F213A). Functional properties of these three mutants were compared with WT CYP3A4 using PGS and CBZ as substrates, and the effect of these mutations on the observed heterotropic cooperative interactions between these substrates was studied using mixed substrate approach, developed in our previous works (33, 34, 36, 38). Results of this effort confirmed an essential role played by this residue in CYP3A4 catalyzed metabolism. Despite significant differences between the two substrates, strong effects were observed for both when F213 was mutated. Moreover, inhibition was observed for PGS hydroxylation, while activation was documented for CBZ epoxidation. In addition, the allosteric effect of PGS upon CBZ metabolism was almost lost in the F213 mutants, although apparent homotropic cooperativity was still observed in spectral titration experiments and in turnover with pure PGS as substrate.

All four trajectories obtained by all-atom MD simulations of WT CYP3A4 and the F213A mutant with CBZ as substrate, with or without PGS as an allosteric effector, show a relatively stable configuration of the allosteric site and no drastic displacement of substrates. Both the F-F' and G-G' loops, which form the allosteric pocket and directly interact with the effector PGS, undergo collective displacements of backbones as a whole with minimal bending, while the side chains of several residues, from R212 to F219, display significant motions. Some of these motions reconfigure the shape of the substrate binding pocket on the opposite side of the heme and take part in controlling interactions with the second CBZ in the active site, CBZ2. The position of the productive substrate molecule CBZ1 is in turn perturbed by direct contacts with CBZ2. This sequence of steric contacts provides connection between the binding of an effector PGS molecule outside the substrate binding pocket and positioning of the productive substrate molecule CBZ1. Clearly, for efficient transmission of the structural perturbation caused by effector binding to the substrate near heme iron along ~20Å distance, the presence of the second substrate CBZ2 inside the substrate binding pocket is necessary. This is not true for larger substrates, such as dronedarone and atorvastatin, which can fill the whole substrate binding cavity in CYP3A4 and demonstrate sensitivity to the presence of effector at the allosteric site (38).

Activation of CBZ epoxidation by PGS bound in the allosteric site formed by residues 210-219 and 233-243 of the WT CYP3A4 and the lipid headgroups is not directly explained by positioning of CBZ substrate against heme iron, as seen in Fig. 8B. Other factors, such as overall better CBZ packing (Fig. 8D) and slower dynamic transitions between conformational substates in the presence of PGS (Fig. 9C) may be responsible for positive allosteric effect of PGS. Packing changes in the presence of PGS are less pronounced for CBZ2 in MD simulations of F213A mutant, as seen in Fig. 8B. In addition, significantly longer trajectories could have provide more complete sampling and better insight into the allosteric effect of PGS on position and dynamics of CBZ molecules in the active site of CYP3A4.

Analysis of aligned sequences of CYP3A4 and similar P450 enzymes as provided at <https://cyped.biocatnet.de/> (70) shows that the whole F-F' loop (residues 210-221), and particularly F213, is highly conserved in all primates, while the G-G' loop is more variable. The lack of variability at F213 and neighboring positions indicates a possible evolutionary importance of this region and suggests that potentially harmful effects may be caused by F213 mutations. Our limited set of data provides support to this hypothesis, and may be considered as the first step towards a more general understanding of the role of F and G helices in the P450 fold, and their contribution to the catalytic mechanism and allosteric properties.

In conclusion, we described an experimental and theoretical study of the effect of mutation at the highly conserved F213 position on the observed heterotropic cooperativity and allosteric properties of CYP3A4 using two probe substrates PGS and CBZ. Strong dependence of absolute rates of metabolism, together with elimination of heterotropic activation of CBZ epoxidation by PGS as effector support a critical importance of F213 residue in the regulation of substrate metabolism by CYP3A4, and demonstrate that mutations at this position may inhibit steady-state catalytic transformations for some substrates, but improve rate of metabolism and coupling for others. These observations

provide useful insight into multifaceted mechanism of CYP3A4 catalysis, but also present a new challenge for future studies of drug-drug interactions

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Figure 1. Schematic model of cytochrome P450 CYP3A4 inserted in the membrane (reproduced from (38) with permission). CYP3A4 structure with progesterone (orange sticks) bound at the peripheral site (pdb file 1W0F (39)) is in cartoon representation with heme shown in red sticks, Membrane insertion is schematically depicted according to MD simulations ((36, 45)). Amino acids of F-F' (magenta) and G-G' (green) loops in direct contact with progesterone are highlighted as surfaces. The substrate binding cavity near the catalytic site is shown as a blue surface.

Figure 2. Rates of PGS hydroxylation (A) and of CBZ epoxidation (B) measured for WT CYP3A4 (blue circles) and mutants F213A (black diamonds), F213S (green crosses) and F213Y (red triangles).

Figure 3. Rates of NADPH consumption during PGS hydroxylation (A) and CBZ epoxidation (B) measured at the identical conditions as in Figure 1 for WT CYP3A4 (blue circles) and the mutants F213A (black diamonds), F213S (green crosses) and F213Y (red triangles).

Figure 4. PGS and CBZ are mixed at 1:12 molar ratio, and the rates of PGS hydroxylation (A) and of CBZ epoxidation (B) are measured for WT CYP3A4 (blue circles) and the mutants F213A (black diamonds), F213S (green crosses) and F213Y (red triangles).

Figure 5. Simultaneous fitting of three data sets for PGS binding (monitored by spin shift), the NADPH oxidation rate, and PGS hydroxylation by the F213A mutant of CYP3A4. The data are scaled in order to balance their contribution in the global fitting calculations as described in Materials and Methods. The fitted parameters are shown in Table 2.

Figure 6. Simultaneous fitting of three data sets for CBZ binding (monitored by spin shift), the NADPH oxidation rate, and CBZ epoxidation by the F213A mutant of CYP3A4. The data are scaled in order to balance their contribution in the global fitting calculations as described in Materials and Methods. The fitted parameters are shown in Table 2.

Figure 7.

Time dependences of distances from the heme iron to the center of mass of residues F213 (A), or A213 (E), R212 (B, F), D214 (C, G), and F215 (D, H) in the WT CYP3A4 (A-D) and F213A mutant (E-H) with PGS (green) and without PGS (blue) bound at the allosteric site.

Figure 8. Time dependences of distances between heme iron and C10-C11 bond of CBZ1 (A,B) and CBZ2 (C,D) molecules in F213A mutant (A,C) and in the WT CYP3A4 (B,D) with PGS (green) and without PGS (blue) bound at the allosteric site

Figure 9.

First two singular vectors calculated by SVD of coordinates of all atoms of allosteric residues 211 – 220 and 233-243 taken from the snapshots at selected time points for WT CYP3A4 (A,C) and F213A mutant protein (B,D) without PGS (A,B) and with PGS bound at the active site (C,D). The first vector (black lines) indicates contribution of the average position, and the second vector (red) shows contribution of the main mode of deviation from the mean position.

Figure 10. Snapshots of allosteric pocket for WT CYP3A4 at various time points. (A) WT CYP3A4 without PGS, snapshots at various times. F213 movement is changing from grey (10 ns) to cyan (75 ns), magenta (175 ns) and back to yellow (250 ns). (B) In WT CYP3A4 with PGS, the movement of F213 is shown progressing from green (10 ns) and yellow (75 ns) to pink (125 ns) and back to cyan and magenta (200 and 250 ns),

Figure 11.

Snapshots of allosteric pocket for the F213A mutant at various time points. (A) F213A without PGS, F215 moves back and forth from green (10 ns) to yellow (25 ns), gray (100 ns), cyan (175 ns), magenta (225ns) to pink (250 ns). (B) F213A with PGS, direct motion of F215 side chain from green (10 ns), yellow (25 ns), magenta (125 ns), to pink (175 ns) and cyan (225 ns).

Table 1.

Summary of the titrations with PGS and CBZ with WT CYP3A4 and the three F213 mutants, fitted using the Hill equation (n_H – Hill coefficient). Confidence intervals corresponding to 68% limits are shown in brackets.

CYP3A4	PGS		
	S_{50} , μM	n_H	Spin Shift
WT	31 [29.2-31.8]	1.49 [1.41-1.55]	84% [82 - 87%]
F213Y	21.8 [20.9-22.5]	2.50 [2.30-2.71]	66% [63 - 69%]
F213S	24.2 [23.3-25.2]	1.67 [1.57-1.77]	64% [61 - 68%]
F213A	24.3 [23.5-25.1]	1.62 [1.53-1.70]	75% [72 - 79%]
CYP3A4	CBZ		
	S_{50} , μM	n_H	Spin Shift
WT	803 [732-934]	1.21 [1.10-1.45]	23% [21 - 26%]
F213Y	557 [503-769]	1.37 [1.10-1.61]	41% [37 - 43%]
F213S	800 [718-1008]	1.39 [1.27-1.70]	44% [38 - 49%]
F213A	872 [642-1040]	1.27 [1.17-1.51]	46% [38 - 49%]

Parameters derived for F213A mutant from single substrate global analyses of PGS hydroxylation and CBZ epoxidation. Stoichiometric dissociation constants, high spin fractions and rates of NADPH consumption and product formation are shown for CYP3A4 with N substrate molecules bound. Corresponding parameters derived for the WT CYP3A4 (36) shown for comparison. Confidence intervals for 68% limit are shown in the brackets.

Progesterone									
K ₀ , μM	High Spin Fraction, %			NADPH rate (nmol/nmol/min)			Product Forming Rate (nmol/nmol/min)		
	WT	N	F213A	WT	F213A	WT	F213A	WT	F213A
F213A		0	17	21	80	44	0	0	0
15 [4 – 10]	7 [9–20]	1	17 [2– 53]	22 [15–40]	283 [213–327]	171 [126–220]	0 [0.01]	0 [0.01]	0 [0.01]
31 [20–45]	14 [11–18]	2	85 [52–100]	37 [26–47]	231 [178–258]	388 [343–450]	14.4 [12.5–18.4]	14.7 [13.8–15.8]	
119 [56–187]	36 [27–48]	3	75 [42–94]	92 [88–97]	63 [36 – 92]	108 [100–114]	4.1 [1.2 – 5.7]	16.6 [16.3–16.9]	
Carbamazepine									
K ₀ , μM	High Spin Fraction, %			NADPH rate (nmol/nmol/min)			Product Forming Rate (nmol/nmol/min)		
	WT	N	F213A	WT	F213A	WT	F213A	WT	F213A
F213A		0	14	21	49	41	0	0	0
127 [80–210]	100 [73–134]	1	22 [13–38]	22 [12–31]	212 [190–267]	195 [155–227]	0 [0.01]	0 [0.01]	
490 [370–610]	210 [113–340]	2	12 [7–20]	22 [8–33]	38 [18 – 45]	124 [90–163]	14 [11.8–14.9]	1.7 [0.9 – 2.4]	
505 [390–640]	570 [330–900]	3	71 [55–83]	59 [54–64]	85 [80 – 90]	56 [46 – 66]	2.5 [1.5 – 3.7]	3.0 [2.7 – 3.4]	