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7,8-Benzoflavone Binding to Human Cytochrome P450 3A4 Reveals Complex Fluorescence Quenching, Suggesting Binding at Multiple Protein Sites

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Abstract

Human cytochrome P450 (P450) 3A4 is involved in the metabolism of one-half of marketed drugs and shows cooperative interactions with some substrates and other ligands. The interaction between P450 3A4 and the known allosteric effector 7,8-benzoflavone (α -naphthoflavone, α NF) was characterized using steady-state fluorescence spectroscopy. The binding interaction of P450 3A4 and α NF effectively quenched the fluorescence of both the enzyme and ligand. The Hill Equation and Stern-Volmer fluorescence quenching models were used to evaluate binding of ligand to enzyme. P450 3A4 fluorescence was quenched by titration with α NF; at the relatively higher [α NF]/[P450 3A4] ratios in this experiment, two weaker quenching interactions were revealed (K_d 1.7–2.5 μ M and 6.5 μ M). A range is given for the stronger interaction since α NF quenching of P450 3A4 fluorescence changed the protein spectral profile: quenching of 315 nm emission was slightly more efficient (K_d 1.7 μ M) than the quenching of protein fluorescence at 335 and 355 nm (K_d 2.5 and 2.1 μ M, respectively). In the reverse titration, α NF fluorescence was quenched by P450 3A4; at the lower [α NF]/[P450 3A4] ratios here, two strong quenching interactions were revealed (K_d 0.048 μ M and 1.0 μ M). Thus, four binding interactions of α NF to P450 3A4 are suggested by this study, one of which may be newly recognized and which could affect studies of drug oxidations by this important enzyme.

Keywords

P450 3A4; α -Naphthoflavone; Fluorescence Quenching; Cytochromes P450

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Supplementary Material

Supplementary material dealing with derivations of Stern-Volmer quenching models is available from

Introduction

Cytochrome P450 (P450) is a widely-distributed superfamily of hemoproteins that are usually membrane-bound in eukaryotic organisms. The malleability of some P450s is extensive, particularly for P450 3A4 (Ekroos & Sjogren, 2006; Guengerich, 2006; Sevrioukova & Poulos, 2013b). Collectively the P450s catalyze a rich suite of reactions and many involve the oxidation of xenobiotics, e.g. drugs and carcinogens (Guengerich, 2001, 2015; Guengerich & Munro, 2013). Oxidations occur in a cycle, in association with NADPH-P450 reductase, with changes in oxidation state of the iron of the heme prosthetic group. The P450s are involved in the metabolism of almost three-quarters of all marketed drugs and the Subfamily 3A P450s, largely expressed in the liver, are involved in the metabolism of one-half of those (Guengerich, 2001, 2015).

P450s can exhibit complex non-Michaelis-Menten kinetics (Atkins, 2004; Denisov, Frank, & Sligar, 2009; Denisov & Sligar, 2012; Galetin, Clarke, & Houston, 2002; Witherow & Houston, 1999). Although P450 3A4 consists of a single polypeptide chain, it can exhibit both homotropic and heterotropic cooperativity, depending on the ligand (Atkins, 2004; Denisov, Grinkova, Baylon, Tajkhorshid, & Sligar, 2015; Guengerich, 1999, 2015; Roberts et al., 2011; Shimada & Guengerich, 1989; Ueng, Kuwabara, Chun, & Guengerich, 1997). There is growing evidence that multiple ligands can be bound to P450 3A4 at the same time, both in the active site and at peripheral sites. This laboratory previously reported evidence that ligand binding to P450 3A4 occurs in a three-step process (Isin and Guengerich, 2006) and that multiple substrates, including small peptides, can simultaneously reside in the enzyme active site (Hosea, Miller, & Guengerich, 2000; Isin & Guengerich, 2006).

Kinetic studies of α NF and pyrene binding to P450 1A2 (Isin, Sohl, Eoff, & Guengerich, 2008) and of bromocriptine and α NF binding to P450 3A4 showed multi-step kinetics using stopped-flow methods, with the first step limited only by diffusion (Isin & Guengerich, 2006, 2007). This first step did not perturb the heme absorption spectrum, suggesting that the first binding step is not near the heme. Atkins and co-workers reported that P450 3A4 has two binding sites for the dye Nile Red (Lampe, Fernandez, Nath, & Atkins, 2008; Nath, Fernandez, Lampe, & Atkins, 2008), one of which is peripheral, and cannot bind Nile Red if the inhibitors/substrates α NF and testosterone are present. They also presented evidence that more than one pyrene molecule can be bound in the active site (Dabrowski, Schrag, Wienkers, & Atkins, 2002). Halpert and his associates reported that 1-pyrenebutanol affected the cooperativity of steroid hydroxylation (Fernando, Rumfeldt, Davydova, Halpert, & Davydov, 2011) and that Fluorol-7GA dye binding sites could be localized to two positions on the P450 3A4 molecule (Davydov et al., 2012), one in the active site and one in a peripheral site that appears to be similar to that of progesterone bound to P450 3A4 in a crystal structure (Williams et al., 2004). Whether the progesterone bound at this site functions as either effector or substrate is perhaps doubtful (Sevrioukova & Poulos, 2015).

P450 3A4 has been crystallized without and with ligands, including metyrapone, erythromycin (Williams et al., 2004; Yano et al., 2004), and the inhibitors ritonavir and its analogues (Sevrioukova & Poulos, 2012, 2013a, 2014). The promiscuity of P450 3A4 ligand binding and ability of this protein to bind multiple molecules in the substrate pocket is

considered to be due to a large and flexible active site (Ekroos & Sjogren, 2006; Yano et al., 2004). Several structure motifs unique to P450 3A4 can be gleaned from structural data. A cluster of phenylalanine residues in P450 3A4 helices F and G comprises a hydrophobic cluster, and upon binding of ketoconazole this cluster is disrupted to form a hydrophobic patch on the protein surface (Ekroos & Sjogren, 2006). Two Phe to Trp mutations in the Phe cluster eliminated the cooperativity of 1-pyrenebutanol binding to P450 3A4 but did not affect multisite binding or the binding affinities to those sites (Fernando et al., 2011). Several other structure motifs are unique to P450 3A4: a new helix A'', a G and G' helix hydrophobic region, a shortened F helix, and a region of ordered polypeptide chain with no recognizable secondary structure (Williams et al., 2004; Yano et al., 2004). Using such structural knowledge, the construction of P450 3A4 'pharmacophores' should enhance drug design and optimize drug-P450 3A4 interactions (Kaur, Chamberlin, Poulos, & Sevrioukova, 2016). How P450 structure in general (including P450 3A4 structure) influences function has been recently summarized (Guengerich, Waterman, & Egli, 2016).

In the past, small molecules like acrylamide were used to develop models of fluorescence quenching of proteins in order to learn about protein structure (Eftink, 1991; Eftink & Ghiron, 1981; Lakowicz, 2006; Lehrer & Leavis, 1978). In this study, α NF was used as a quencher of P450 3A4 tryptophan fluorescence to probe structure and study binding heterogeneity. α NF is oxidized in a cooperative manner (Hosea et al., 2000; Ueng, Kuwabara, Chun, & Guengerich, 1997) and can either enhance or inhibit binding or oxidations of other P450 3A4 substrates (Koley et al., 1995; Ueng, Kuwabara, Chun, & Guengerich, 1997). As an allosteric effector, α NF regulates the kinetics (Woods, Fernandez, Kunze, & Atkins, 2011) and increases the oxidation rate of Nile Red to the products M1 and M2 (Nath et al., 2008). The binding interaction of α NF to P450 3A4 is already known to be complex; three binding interactions have been previously detected, one probably a peripheral low-affinity site (Tsalkova, Davydova, Halpert, & Davydov, 2007).

In this study, a P450 3A4 construct was used that includes residues 13-503 of the native sequence and that contains three tryptophan residues (Gillam, Baba, Kim, Ohmori, & Guengerich, 1993; Menard et al., 2012). P450 3A4 fluorescence is heterogeneous, and its quenching by α NF was complex. The Hill Equation and several Stern-Volmer (SV) quenching models were used to evaluate fluorescence quenching, including modifications of SV models that can distinguish three classes of fluorescence quenching that occur during the α NF - P450 3A4 interaction. It was found that four quenching interactions, suggesting four binding interactions, characterized the binding of the allosteric effector α NF to P450 3A4.

Experimental Procedures

Reagents

Human P450 3A4 was expressed and purified as described elsewhere (Gillam et al., 1993; Hosea et al., 2000) and quantified using the Fe^{2+} -CO vs. Fe^{3+} assay (Guengerich, Martin, Sohl, & Cheng, 2009; Omura & Sato, 1964). α NF was purchased from Sigma-Aldrich (St. Louis, MO) and recrystallized from hot $\text{C}_2\text{H}_5\text{OH}$ (mp 157-158 °C, lit 158-160 °C (Yoshida, Fujino, Saito, & Doi, 2011)) (melting points uncorrected). H_2O was purified using a Milli-Q

system (Millipore, Billerica, MA), and the CH₃OH solvent used to dissolve P450 3A4 substrates was spectrophotometric or luminescence grade (Sigma-Aldrich).

Spectroscopy

Absorption Spectroscopy— α NF was dissolved in a CH₃OH/H₂O mixture (4/1, v/v), and concentrations of stock solutions were calculated using an extinction coefficient (ϵ_{290}) of 23,700 M⁻¹ cm⁻¹ (Ueng, Kuwabara, Chun, & Guengerich, 1997). Absorption measurements were recorded with various spectrophotometers, including a Lambda-40 UV-visible spectrometer (Perkin-Elmer, Norwalk, CT), a Cary 14/OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA), and an Aminco DW-2a/OLIS spectrophotometer (On-Line Instrument Systems). UV-visible spectra of P450 3A4 were acquired in 100 mM potassium phosphate buffer (pH 7.4).

Fluorescence Spectroscopy—All steady-state excitation and emission fluorescence spectra were recorded using either a Fluorolog-3 spectrometer in a 111 Configuration (JY Horiba, Edison, NJ) or an OLIS DM-45 fluorescence spectrometer (On-Line Instrument Services). Fluorolog-3 slit widths were adjusted from 1 to 5 nm bandpass, depending on fluorophore concentrations and the excitation energies. Three of the four DM-45 slits were set at a 5 nm bandpass, and the slit preceding the emission monochromator was set to a 13 nm bandpass. Spectra were obtained at room temperature (23 °C) and solutions were not degassed. The slit from the spectrometer light source was set to close when spectra were not being acquired.

All solutions were frequently checked for fluorescent contaminants. Emission spectra were acquired for buffer solutions and solvents (with no protein or α NF) by exciting with light at 275, 285, 295, and 330 nm and collecting emission from $\lambda_{\text{exc}} + 10$ to $\lambda_{\text{exc}} + 200$ nm. The first three excitation wavelengths were used to generate spectra of P450 3A4, and the latter was used to generate fluorescence spectra of α NF. If the signal was <1/10 the intensity of the water Raman band, then the solutions were determined to have little or no fluorescent contaminant.

Titration of α NF to P450 3A4—Additions of α NF (0–20 μ M) to 250 nM P450 3A4 in 100 mM potassium phosphate buffer (pH 7.4) were done at room temperature (four replicates for statistical reasons). Spectra of protein fluorescence were acquired at each titration point; the excitation wavelength was 290 nm, and the fluorescence emission was collected for 0.25 s at each 0.5 nm increment from 305 to 450 nm. The CH₃OH concentration was 2% (v/v) by the end of the titration (P450 3A4 is known to retain activity at this concentration of CH₃OH) (Busby, Ackerman, & Crespi, 1999; Easterbrook, Lu, Sakai, & Li, 2001).

Titration of P450 3A4 into α NF—P450 3A4 (0 to 2.33 μ M) was added to 250 nM α NF in 100 mM potassium phosphate buffer (pH 7.4) at room temperature, and α NF emission spectra were acquired at each concentration of P450 3A4. The excitation wavelength was 330 nm, and α NF fluorescence emission was collected for 0.25 s at each 0.5 nm increment from 340 to 550 nm.

Corrections to Fluorescence Spectra—Four issues were addressed before binding parameters could be calculated from the titration curves: i) contributions to the reporter fluorescence from the titrant fluorescence; ii) photobleaching of both α NF and P450 3A4 during the titrations; iii) inner-filter corrections due to fluorophore absorption; and iv) baseline subtractions due to Raman scattering of water and light scattering in protein solutions.

Contributions to the Reporter Fluorescence from the Titrant Fluorescence—

Because α NF and P450 3A4 are both fluorescent, a large contamination to P450 3A4 fluorescence could result as α NF is added to the P450 3A4 solutions, and a large contribution to α NF fluorescence from enzyme fluorescence could result as P450 3A4 is added to α NF solutions. In the latter case, the tryptophans in P450 3A4 have minimal absorbance at 330 nm and heme is not fluorescent; it was found that very little tryptophan emission was produced by 330 nm excitation, even at high concentrations of P450 3A4 titrant (Figure 1B). Thus, the enzyme did not appreciably contribute to the α NF emission spectra as it was titrated to ligand.

There is not much spectral overlap between P450 3A4 and α NF fluorescence emissions. However, there was enough that when α NF was added to P450 3A4 solutions, α NF fluorescence contaminated enzyme fluorescence at high concentrations of α NF titrant, because the excitation wavelength of 290 nm efficiently induced transitions to excited state in both α NF and P450 3A4. The absorption of tyrosine is minimal at 290 nm but is significant for tryptophan (Weber, 1960). Control titrations were done in which α NF was added to 100 mM potassium phosphate (pH 7.4, no enzyme present) and α NF emission spectra were acquired at each α NF concentration as described above. These were subtracted from spectra of P450 3A4 obtained when α NF was titrated into P450 3A4, isolating the spectral contribution due to enzyme alone.

Photobleaching Corrections

During titrations, the fluorescence from both enzyme and ligand decreased due to photo-induced decomposition of the fluorophores. For example, if α NF was titrated into solutions of P450 3A4, then the fluorescence of P450 3A4 reporter in the absence of quencher, F_0 , decreased with time due to photobleaching. In addition, the concentration of α NF titrant was less than what was ostensibly added because ligand added earlier in the titration was photobleached over the course of the titration.

The first part of the photobleaching correction consisted of experiments determining how quickly the fluorescence from either α NF or P450 3A4 decayed when the solutions were exposed to the spectrometer light source. Experiments measuring photobleaching of either P450 3A4 or α NF were done by adding 100 mM potassium phosphate (pH 7.4) blank solutions to either an α NF or a P450 3A4 solution (both in 100 mM potassium phosphate at pH 7.4) and collecting emission spectra of the fluorophore exactly as was done in the α NF to P450 3A4 titrations (and the reverse). From these experiments decay constants for α NF and P450 3A4 photobleaching could be determined.

Modifying Reporter Fluorescence Due to Photobleaching—In Stern-Volmer models, either F_0/F or F_0/F is calculated, where F_0 is the reporter fluorescence in the absence of quencher, and F is the reporter fluorescence with quencher present. F is the amount the fluorescence quenched with a given concentration of quencher. To correct for photobleaching decay, the reporter fluorescence in the absence of quencher at each titration point k (corresponding to a given light exposure time) was denoted $F_{0,k}$, and because of photobleaching it was less than the fluorescence at the beginning of the titration ($F_{0,0}$).

At each titration point k , F_0/F becomes $F_{0,k}/F_k$. Then F_k , the amount of quenching at titration point k due to just the enzyme-ligand interaction, is expressed as a fraction of the total fluorescence that could be quenched at a given titration point. That is, for each titration point,

$$\Delta F_k = \frac{F_{0,k} - F_k}{F_{0,k}}, \quad \text{Eq. [1]}$$

in which $F_{0,k}$ is the reporter fluorescence in the absence of quencher at titration point k and F_k is the fluorescence of the reporter at titration point k that decreased due to both quencher and photobleaching.

Correcting Titrant Concentrations Due to Photobleaching—Titrant added at the initial phases of the titration photobleached during the course of the titration, so that the actual concentrations of titrant were less than what was added to the solution. For example, the titration α NF to P450 3A4 was initiated by adding 100 nM α NF at the first titration point. The residual α NF from that addition remaining at each titration point k was calculated from its photobleaching decay half-life (see Figure 2 for more information). By the end of the titration, little α NF remained from the first addition of α NF. At the second titration point, more α NF was added and it also decayed exponentially over the course of the experiment. The concentrations of α NF at each titration point are governed mainly by the amount of α NF following the last additions, little of which had decayed. For the first few titration points, the residual α NF is almost equal to the added α NF. By the end of a typical titration, the α NF concentration should have been 20 μ M. However, applying the α NF photobleaching decay constant, it was actually 13 μ M.

Inner-Filter Corrections—At higher concentrations of α NF or P450 3A4, fluorescence emission intensities were adjusted due to the inner-filter effect of the chromophore absorption according to the equation

$$F_{\text{corr}} = F_{\text{obs}} \text{antilog} \left(\frac{A_{\text{exc}} + A_{\text{em}}}{2} \right) \quad \text{Eq. [2]}$$

where F_{corr} is the corrected fluorescence intensity, F_{obs} is the observed fluorescence, and A_{exc} and A_{em} are the respective absorbances at the excitation and emission wavelengths.

For example, as α NF was added to a solution of P450 3A4, α NF absorption (A_{exc}) at the 290 nm excitation wavelength (which is near λ_{max} of the α NF spectrum) increased, yielding an increasingly significant inner filter effect as α NF was added. In these titrations P450 3A4 fluorescence emission was measured at 315, 335, and 355 nm. At these wavelengths, absorbance of α NF (A_{em}) was weaker but still significant, and inner-filter corrections for α NF absorbance were made when $A_{\text{exc}} + A_{\text{em}} > 0.05$.

Similar corrections were conducted when P450 3A4 was added to α NF. At high concentrations of P450 3A4, substantial P450 3A4 absorbance existed at both the excitation (330 nm) and monitored emission (440 nm) wavelengths; the inner-filter effect was adjusted using Eq. [2].

Baseline and Scattering Corrections—Spectra of buffer blanks, which include the Raman scattering band from H_2O , were subtracted from all spectra containing fluorophores. However, a blank spectrum of 100 mM potassium phosphate (pH 7.4) could not be simply subtracted from subsequent P450 3A4 or α NF emission spectra to make an adequate correction. For example, as α NF was added, the P450 3A4 fluorescence intensity and the Raman scattering band were increasingly suppressed because of the inner filter effect. Subtracting a spectrum of a buffer blank from such spectra would over-subtract the baseline, yielding a significant trough at the position of the Raman band. Accordingly, at higher concentrations of α NF ($>2 \mu\text{M}$) the Raman scatter spectral intensities were corrected using Eq. [2], and then the baseline could be subtracted.

Model Fitting

Derivations of Stern-Volmer fluorescence quenching models for protein-ligand interactions are given in the Supplementary Materials. Here we present the equations used to analyze the fluorescence quenching resulting from the protein-ligand interaction. In a Stern-Volmer analysis, quenching can be accomplished through dynamic quenching (collisions) or through formation of static complexes (non-covalent binding). Mathematically the following models are the same for either static or dynamic quenching (Lakowicz, 2006).

P450 3A4 and α NF form strong non-covalent (“static”) complexes ($K \sim 2 \times 10^5 \text{ M}^{-1}$, $K_d \sim 5 \mu\text{M}$) (Hosea et al., 2000; Tsalkova et al., 2007). Collisional quenching constants are many orders of magnitude lower (Lakowicz, 2006), and probably undetectable at the very low concentrations of quencher used in this study. Thus, in the models that follow, all fluorescence quenching constants K are assumed to characterize the strong non-covalent interaction of α NF with P450 3A4. In the text and tables binding affinities are expressed as dissociation constants ($K_d = 1/K$), as is common in equilibrium binding studies of ligand to macromolecules.

Stern-Volmer Analysis—If only one fluorophore is present or if equally quenchable and independent multiple fluorophores exist, then the quenching of fluorescence can be described by a linear Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + K [Q] = 1 + \frac{[Q]}{K_d} \quad \text{Eq. [3]}$$

, where $[Q]$ is the concentration of quencher Q , K is the static quenching constant, and K_d is the dissociation constant for the interaction of α NF with P450 3A4, both of which are strong fluorophores.

Modified Stern-Volmer Analysis—To better quantify the heterogeneous fluorophore environment in P450 3A4, a modified Stern-Volmer treatment was applied to the fluorescence quenching by α NF. The modified Stern-Volmer model can be derived from the Lehrer classic treatment (Lakowicz, 2006) and can distinguish two protein fluorophores, one of which is inaccessible to quencher ($K = 0$). This quenching model is linear in the inverse concentration of quencher:

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K_a} \frac{1}{[Q]} + \frac{1}{f_a} \quad \text{Eq. [4]}$$

In this expression, f_a is the fraction of fluorescence that cannot be quenched and K_a is the quenching constant for the quenching of the other class of accessible fluorescence.

One Fluorophore Quenched by Two Interactions—In the classical form of this model, one class of protein fluorescence can be quenched by both a static and dynamic interaction (collisional quenching) (Seidel, Schulz, & Sauer, 1996). In this study, since it is assumed that collisional quenching should not be detectable (*vide supra*) at the very low concentrations of quencher used in this study, this model was used to characterize one class of protein fluorescence that is quenched by the formation of two static complexes.

In this model, a plot of F_0/F is quadratic (see Supplementary Material for a complete description). To obtain the two static association constants, at each concentration of quencher Q , an apparent association constant K_{app} is calculated from the expression

$$K_{app} = \left(\frac{F_0}{F} - 1 \right) \frac{1}{[Q]} \quad \text{Eq. [5]}$$

K_{app} is also defined as

$$K_{app} = (K_1 + K_2) + K_1 K_2 [Q] \quad \text{Eq. [6]}$$

A plot of K_{app} as a function of quencher concentration (Eq. [6]) should be linear, and the quenching constants can be calculated from the slope ($K_1 K_2$) and the y-intercept ($K_1 + K_2$) (Lakowicz, 2006).

Three-State Quenching Models—In theory, fluorescence quenching from experiment can be fit to a model in which there are N distinguishable classes of fluorescence that form different-affinity static complexes with quencher (See Supplementary Material). In this work the Stern-Volmer quenching is limited to three classes of protein fluorescence, two of which are accessible to the quencher molecule with affinities K_1 and K_2 , and a third class that is inaccessible (i) to quencher; for this third class $K_3 = K_i = 0$. The equation (see Supplementary Material Eqs. [13] – [17] for derivation) has the form

$$\frac{F_0}{F} = \frac{g_{1,2}([Q])}{f_1(1+K_2[Q]) + f_2(1+K_1[Q]) + (1-f_1-f_2)g_{1,2}([Q])} \quad \text{Eq. [7]}$$

, where f_1 and f_2 are the fractions of the total fluorescence from fluorophores 1 and 2, and where

$$g_{1,2}([Q]) = (1+K_1[Q])(1+K_2[Q]) \quad \text{Eq. [8]}$$

Another three-state model (Hays, Ryan, & Pennell, 2004), similar to Eq. [7], was used in which F_0/F was measured with increasing quencher concentrations (see Supplementary Material for derivation). As before, this three-state model allows for three classes of fluorophores, two of which can be quenched with affinities K_1 and K_2 , and one class completely inaccessible to quencher (Hays et al., 2004; Lehrer, 1971). The equation is

$$\frac{F_0}{\Delta F} = \frac{(1+K_1[Q])(1+K_2[Q])}{f_1K_1[Q](1+K_2[Q]) + f_2K_2[Q](1+K_1[Q])} \quad \text{Eq. [9]}$$

, where f_1 and f_2 are the fractions of fluorescence quenched by α NF-P450 3A4 interactions defined by affinities K_1 and K_2 .

Prism software (GraphPad, La Jolla, CA) was used to perform the fitting. Both three-state quenching models have four adjustable parameters and many possible values of K_1 , K_2 , f_1 , and f_2 that can fit the models. Such models must be used circumspectly, but there is one constraint that can be obtained from Eq. [9]:

$$\lim_{[Q] \rightarrow \infty} \frac{F_0}{\Delta F} = \frac{1}{f_{\text{effective accessible}}} = \frac{1}{f_1 + f_2} \quad \text{Eq. [10]}$$

At high concentrations of quencher, this constraint yields the fraction of inaccessible fluorescence, since $f_1 + f_2 + f_i = 1$.

Hill Models—The quenching of α NF fluorescence, F , at 440 nm was used for model fitting using the Hill equation (Eq. [11]), which was done using Prism software.

$$\Delta F = \frac{\Delta F_{max} [Q]^n}{[Q]^n + K_d^n} \quad \text{Eq. [11]}$$

In this expression, F_{max} is the maximum fluorescence quenching possible in the experiment, K_d is the dissociation constant, and n is the Hill coefficient. If the Hill model converges well with the data, then maximum quenching should be near unity ($F_{max} = 1.0$, Eq. [11]).

A two-site model in which the binding sites are independent and the interaction non-cooperative at each site ($n = 1$) was also used to fit the quenching of 440 nm fluorescence emission. This equation is written as

$$\Delta F = \frac{\Delta F_{1,max} [Q]}{[Q] + K_{1,d}} + \frac{\Delta F_{2,max} [Q]}{[Q] + K_{2,d}} \quad \text{Eq. [12]}$$

In this model, F is the *fraction* of fluorescence that is quenched by each quenching interaction. Thus, $F_{1,max} + F_{2,max}$ should be near unity.

Calculation of Förster Distances (R_0) for P450 3A4 Substrates—If two chromophores are in close proximity (i.e., 1-10 nm), then the fluorescence from one chromophore (the donor) may be quenched by the other chromophore (the acceptor) through singlet-singlet energy transfer or fluorescence resonance energy transfer (FRET) (Cantor & Schimmel, 1980; Förster, 1948; Lakowicz, 2006; Stryer & Haugland, 1967). The Förster distance R_0 (in Å), is the distance between donor and acceptor transition dipoles at which half of donor fluorescence is quenched

$$R_0 = 0.211 [\kappa^2 \eta^{-4} \phi_D J(\lambda)]^{1/6} \quad \text{Eq. [13]}$$

where the orientation factor $\kappa^2 = 2/3$ is assumed for random segmental motion, η (= 1.35) is the index of refraction of an organic or macromolecular environment, ϕ_D is the quantum yield of the donor (P450 3A4 substrate) in the absence of the acceptor (P450 3A4 heme), and $J(\lambda)$ is the overlap integral, which in turn is

$$J(\lambda) = \frac{\int_0^\infty \varepsilon(\lambda) \lambda^4 f(\lambda) d\lambda}{\int_0^\infty f(\lambda) d\lambda} \quad \text{Eq. [14]}$$

In this integral, $\varepsilon(\lambda)$ is the absorption at each wavelength (λ) of the acceptor and $f(\lambda)$ is the donor fluorescence emission intensity at each wavelength. In the titration of α NF to P450 3A4, the absorption profile of α NF (the acceptor) overlaps the tryptophan fluorescence (the donor); thus the addition of α NF quenches tryptophan fluorescence. In the reverse titrations (P450 3A4 to α NF), the donor fluorescence is emitted by α NF, and the acceptor absorption

is from the P450 3A4 heme moiety, which quenches α NF fluorescence. In this work we calculated the Förster distance only in the case of the reverse titration.

The quantum yield of α NF was calculated using comparisons of α NF fluorescence with fluorescence references having known quantum yields, in this case rhodamine 6G in $\text{C}_2\text{H}_5\text{OH}$ ($\phi = 0.94$) and tyrosine ($\phi = 0.14$) and tryptophan ($\phi = 0.13$) in H_2O . Reference solutions were degassed by purging solutions with Ar and by purging the sample compartment of the fluorimeter with Ar. Solutions used for determining the α NF quantum yields were in 100 mM potassium phosphate buffer (pH 7.4) and, as is typical for many P450 studies, were not degassed; thus, the α NF ϕ value published here is not a maximum quantum yield.

The α NF quantum yield was calculated using

$$\phi = \phi_R \left(\frac{I}{I_R} \frac{A_R}{A} \frac{n^2}{n_R^2} \right) \quad \text{Eq. [15]}$$

where ϕ_R is the quantum yield of the reference, I and I_R are the integrated fluorescence intensities of the α NF and references (respectively), A and A_R are the respective absorbances of α NF and references, and n and n_R are the indices of refraction of the α NF and reference solutions, respectively. These parameters were used to calculate R_0 , and the efficiency of quenching E at a distance R from the heme group can be calculated using

$$E = \frac{R_0^6}{R_0^6 + R^6} \quad \text{Eq. [16]}$$

Results and Discussion

Excitations and Emissions Used to Monitor Titrations

Titrations of α NF into P450 3A4 were monitored by exciting the solutions at 290 nm and evaluating the P450 3A4 fluorescence emission at 315, 335, and 355 nm (Figure 1A). Light at 290 nm was strongly absorbed both by P450 3A4 and α NF. The fluorescence maximum of α NF was red-shifted 85 nm (Figure 1B) with respect to the P450 3A4 emission maximum. At 355 nm, fluorescence emission from α NF (Figure 1B) overlapped slightly with the P450 fluorescence and was subtracted from P450 3A4 emission spectra (Figure 1A).

Titrations of P450 3A4 into α NF were monitored by exciting the solutions at 330 nm and evaluating the α NF fluorescence intensity at 440 nm (Figure 1B). While the non-fluorescent heme prosthetic group of P450 3A4 absorbed light at 330 nm, the protein itself did not and P450 3A4 fluorescence was not generated with 330 nm excitation.

Photobleaching of α NF and P450 3A4—The fluorescence from both P450 3A4 (Figure 2A) and α NF (Figure 2B and 2C) decreased due to photobleaching during the

course of a titration and is shown as a function of spectrometer light source exposure time. The decay of either P450 3A4 or α NF was well-described as a first-order exponential decay of the form

$$F = F_P e^{-\frac{0.693t}{t_{1/2}}} + F_{NP} \quad \text{Eq. [17]}$$

where F is the total fluorescence at exposure time t , F_P is the maximum fluorescence that could be photobleached, $t_{1/2}$ is the half-life of the decay, and F_{NP} is the plateau fluorescence, i.e. suggesting fluorophore that was not photolabile. Photobleaching of P450 3A4 fluorescence was not highly dependent on the emission wavelength monitored. Fluorescence spectra were generated using excitation at 290 nm, and the fluorescence intensity measured at 315, 335, and 355 nm decayed exponentially with time during the course of the titrations. The estimated half-lives of the decay were 12, 11, and 12 titration points, respectively, corresponding to excitation source exposure times of 8.8×10^2 , 8.0×10^2 , and 8.9×10^2 s. Substantial enzyme was resistant to photobleaching: F_{NP} was 29, 32, and 33% of the fluorescence at 315, 335, and 355 nm, respectively. The decay of α NF was rapid and dependent on excitation energy, with $t_{1/2} = 4.3$ or 7.2 titration points (corresponding to light source exposure times of 450 and 754 s) if the excitation was at 290 nm or 330 nm, respectively (Figure 2B and 2C). Little α NF was resistant to photobleaching: F_{NP} was 12% and 8.5 % at 290 nm and 330 nm excitation, respectively.

Fluorescence Spectra of P450 3A4 in the Absence of Quencher—The photophysics of indole (the tryptophan fluorophore) has been extensively studied (Sun & Song, 1977); its fluorescence emission energy is dependent on solvent hydrogen bonding to the indole imino group (Gryczynski, Wicz, Johnson, & Lakowicz, 1988) as well as to charge transfer and the local electric field environment (Callis & Burgess, 1997; Callis & Liu, 2004). Tryptophan is thus a well-known highly solvatochromic residue; depending on its electronic environment it can have an emission λ_{max} between 308 (Trp-48 in azurin) and 355 nm (Trp-29 in glucagon) (Vivian & Callis, 2001).

Fluorescence excitation and emission spectra of P450 3A4 in 100 mM potassium phosphate (pH 7.4) are shown in Figure 3A. The excitation spectra of P450 3A4 did not depend significantly on the emission wavelength being monitored. However, the protein fluorescence emission, dominated by solvatochromic tryptophan indole groups, was affected by excitation energy: excitation at 275 nm (as opposed to 290 nm) revealed increased total fluorescence with a λ_{max} of 318 nm (Figure 3A).

Titration of α NF into P450 3A4

Effect of Binding on the Spectra of α NF and P450 3A4—An excitation wavelength of 290 nm induced excited state transitions both for α NF and P450 3A4 (Figure 1A), and therefore spectral profiles of α NF and 3A4 fluorescence emission both could be studied (Figures 1A and 3B). The titration of α NF into P450 3A4 resulted in the quenching of both α NF and P450 3A4 fluorescence (Figure 3B). The α NF fluorescence emission was centered

at 440 nm and there was no shift in the spectrum energy during the titration, even with a slight molar excess of P450 3A4 that should have bound much α NF (Figure 3B).

The P450 3A4 fluorescence profile (with no quencher present) showed two shoulders at 320 and 355 nm (top spectrum, Figure 3C). As the ratio of α NF to P450 3A4 increased, all P450 3A4 emission wavelengths were quenched, but the higher-energy fluorescence at 320 nm was quenched more efficiently (middle and lower spectrum, Figure 3C). At high α NF quencher concentrations ($> 10 \mu\text{M}$), the protein fluorescence at all wavelengths was almost completely quenched (*vide infra*).

Hill Plots for the Titration of α NF into P450 3A4—P450 3A4 fluorescence emission was efficiently quenched by the addition of α NF (Figure 4A). Difference spectra from the titration (Figure 4B) were fit to the Hill equation Eq. [11]. The binding of α NF to P450 3A4 was evaluated by quenching of P450 3A4 fluorescence at 315, 335, and 355 nm (Figure 4C). The data fit the Hill equation very well, with resulting K_d values of 2.0, 2.5, and $2.4 \mu\text{M}$, respectively (Table 1). The interaction was not cooperative: the Hill coefficients (n) calculated from quenching at 315, 335, and 355 nm were 1.1, 0.98, and 0.96. The fluorescence at 315 nm was quenched more rapidly than was emission at lower energies, in accord with the spectral analysis (Figure 3C). These data suggested different affinities for quenching of tryptophan fluorescence. There are two rationales for this behavior. One is that this reflects two binding interactions due to α NF binding at different locations on the protein. Perhaps a simpler and more satisfying explanation is that one α NF binding interaction is quenching the fluorescence of two tryptophans with different singlet-singlet energy transfer efficiencies. Fluorescence quenching of the tryptophan closer to the bound α NF would have a higher FRET efficiency than the one farther away, and this would be reflected by a higher quenching constant (lower K_d in the Hill model).

Stern-Volmer Plots for the Titration of α NF into P450 3A4—The Stern-Volmer analysis of the quenching of P450 3A4 fluorescence by α NF is shown at three wavelengths (Figure 5A). The behavior of F_0/F was linear with respect to quencher at α NF concentrations at 315 and 355 nm (Figure 5A and Table 1). Interestingly, at 335 nm the behavior of F_0/F appeared quadratic rather than linear (Figure 5A; see statistical analysis in Table 1). In the limit that α NF concentration goes to infinity, F_0/F went to infinity, suggesting that all enzyme fluorescence can be quenched when α NF binds to P450 3A4. Stern-Volmer quenching data did not fit a model that describes the quenching of three classes of fluorescence (Eq. [7] or Eq. [9]).

The SV quenching analysis at 315 and 355 nm suggested one quenching interaction at each wavelength with K_d values of 1.7 and $2.1 \mu\text{M}$, respectively. The quadratic increase in F_0/F observed at 335 nm indicates quenching in which two ligand binding events quenched one class of enzyme fluorescence (Eq. [5] and [6]; see also Supplementary Materials). This was good evidence for the formation of two static α NF-P450 3A4 complexes (multisite binding to P450 3A4). To calculate the two static quenching constants from these data, the apparent association constant K_{app} was plotted as a function of α NF concentration (Figure 5B). The scatter in the data (Figure 5B) was not trivial but both quenching constants (Table I) were obtained from the slope and y-intercept of the plot (Figure 5B; also see Eq. [6]). At 335 nm

the two K_d values were 6.5 and 2.9 μM (Table 1). The lower K_d values (1.7 and 2.1 μM) calculated from the slopes of linear SV plots (detection at 315 and 335 nm, Table 1) may reveal the same tighter binding interaction as the stronger interaction detected at 335 nm (2.9 μM). The higher K_d value calculated from enzyme fluorescence quenching at 335 nm likely revealed a separate weaker binding interaction. Thus, this analysis suggested two distinct classes of αNF binding sites to P450 3A4, one with K_d 2.5 μM (means of the three values from emission intensities at 315, 335, and 355 nm) and another with K_d 6.5 μM (analysis at 335 nm).

Modified Stern-Volmer Plots for the Titration of αNF to P450 3A4—Modified Stern-Volmer (F_0/F) data in Figure 6A appeared linear, suggesting that at each detection wavelength, one class of quenchable 3A4 fluorescence was observed. The dissociation constants from these MSV plots were 1.8, 2.5, and 2.2 μM at 315, 335, and 355 nm, respectively (Table 1). Examination of the modified SV quenching at high concentrations of αNF quencher (low $1/[\alpha\text{NF}]$) showed that the linear fit was still acceptable (inset, Figure 6B). The y-intercepts of MSV quenching of protein fluorescence at 315 and 355 nm were very close to 1.00, indicating complete quenching of P450 3A4 fluorescence at those wavelengths (fraction accessibility f_a 1.0). However, the y-intercept of the 335 nm MSV plot implied that $> 100\%$ of fluorescence at 335 nm was accessible to αNF quencher (f_a 1.2), a physical impossibility (Figure 6C and Table 1). This result suggests that a second quenching interaction was present, as suggested by the quadratic behavior of SV quenching at 335 nm.

A linear fit only to the lowest values of $1/[\alpha\text{NF}]$ was therefore attempted (Figure 6C), according to Lehrer's treatment (Lehrer, 1971), and from it the fraction of fluorescence quenched, $f_{a,\text{eff}}$, and the effective binding affinity for the interaction, $K_{d,\text{eff}}$, were determined (Lehrer, 1971). The fraction of fluorescence accessible to quencher, $f_{a,\text{eff}}$, was 0.98, 1.1, and 0.99 at 315, 335, and 355 nm, respectively (Figure 6C and Table 1). Because these values are within standard error of unity (f_a 1.0; see Table 1), these data suggest that the αNF -P450 3A4 interaction completely quenched the fluorescence of the enzyme. The dissociation constants obtained from the quenching data were lower for detection at 315 (K_d 1.5 μM), but indicated slightly weaker binding at 335 and 355 nm (K_d 2.2 and 2.0 μM , Table 1). Stronger quenching (when monitoring 315 nm fluorescence) was in accord with the optical spectra, which indicated that the peak at 320 nm became less intense relative to the other emission energies as αNF was added (Figure 3C).

Titration of P450 3A4 into αNF

Hill Analysis—P450 3A4 was an effective quencher of αNF fluorescence. Indeed, the fluorescence from 250 nM αNF could be eliminated by the addition of $\sim 3 \mu\text{M}$ P450 3A4 (data not shown). The αNF difference spectra acquired after each addition of P450 3A4 to the αNF solution are shown (Figure 7A). Substantial fluorescence quenching at sub-micromolar concentrations of P450 3A4 suggested strong binding.

Three variations of a Hill analysis were performed. Model fitting was first done assuming a one-site, non-cooperative (hyperbolic) interaction (solid line, Figure 7B and Table 2), then allowing the Hill parameter n to be unconstrained (dashed line, Figure 7B). Finally two sites

of interaction were assumed in which α NF binding to each P450 3A4 site is independent of the other and is not cooperative (dotted line, Figure 7B).

The goodness-of-fit was acceptable for all three models, but especially if the Hill parameter n was unconstrained or if a two-site binding model was assumed (see correlation coefficient R^2 and standard deviation of residuals $Sy.x$ values in Table 2). To distinguish which model best fit the data, the Hill analysis was shown at low concentrations of P450 3A4 titrant ($< 0.7 \mu\text{M}$, Figure 7C). A one-site, non-cooperative model (solid line) generated values of F/F_{max} that were too small when the P450 3A4 concentration was $< 0.2 \mu\text{M}$ and too large when the concentration of enzyme was $> 0.2 \mu\text{M}$ (compare solid line to data points, Figure 7C). If the Hill parameter n was 0.80, suggesting modest negative cooperativity for the interaction (dashed line, Figure 7C and Table 2), then a strong interaction was revealed (K_d $0.46 \mu\text{M}$) and the fit to the α NF quenching data was very good (Table 2).

The two-site Hill model (interaction not cooperative at either site) also fit the data very well (dotted line, Figure 7C and Table 2). If this model is correct, then 25% of the fluorescence was quenched in a very strong interaction (K_d $0.052 \mu\text{M}$) and 76% was quenched by a weaker but still strong interaction (K_d $0.87 \mu\text{M}$). (That the total fluorescence quenched was close to 1.00 is consistent with good model convergence to the data.)

The two-site Hill model was not much better than the model with slight negative cooperativity (see R^2 and $Sy.x$ values in Table 2), and it is not possible to distinguish the two models from a Hill analysis alone. The two-site Hill model was preferred as the better interpretation of the Hill analysis data because Stern-Volmer quenching models to render the data (three-site Stern-Volmer, Figure 8 and three-site Modified SV, Figure 9) also suggested the high-affinity site.

The weaker of these two interactions was observed during the first part of the titration, when the α NF/P450 3A4 molecular ratios were larger than at the end of the titration. This interaction quenched most of the fluorescence, but the stronger interaction quenched the rest of it by the end of the titration, at low α NF/P450 3A4 ratios. While it is difficult to conclude which interaction more efficiently quenches α NF fluorescence, these results are consistent with previous findings that weaker ligand binding occurs at a more peripheral binding site. This interaction might not quench all of the α NF fluorescence because the ligand is too far from the enzyme heme group; the stronger interaction at the active site will quench the rest of the α NF fluorescence that was not quenched earlier in the titration.

Stern-Volmer Analysis—The Stern-Volmer plot curves were concave downward (Figure 8) and did not increase quadratically, as occurred when α NF was added to P450 3A4 at high α NF/P450 3A4 ratios. A linear Stern-Volmer analysis of α NF quenching by 3A4 (dashed line, Figure 8A) (which assumes one class of quenched fluorescence) showed strong binding (K_{SV} $1.8 \mu\text{M}^{-1}$, K_d $0.56 \mu\text{M}$). The linear fit was not good and showed systematic error, suggesting the existence of more than one class of quenchable fluorescence. A better fit was obtained using a three-class SV model (solid line, Eq. [7] and Figure 8A and 8B), which showed that all the fluorescence was quenched by α NF and yielded dissociation constants of 1.0 and $0.043 \mu\text{M}$ (Table 2). The weaker (but still strong) interaction was able to quench

more α NF fluorescence (82% accessibility to quencher) than the stronger interaction (24% accessibility). The total fractional accessibility was not constrained in the model fitting but should be close to 100% for confidence in the model.

Modified Stern-Volmer Analysis—A Modified SV analysis showed a non-linear plot, indicating at least two classes of fluorescence with different efficiencies of quenching by P450 3A4 (Figure 9A). The behavior at low concentrations of P450 3A4 quencher is shown (inset, Figure 9B), which yielded the effective fractional accessibility to quencher $f_{a,eff}$ and the effective binding affinity K_{eff} . The y-intercept was 0.98 (corresponding to $f_{a,eff}$ of 1.0), showing that quenching of α NF fluorescence was complete at a high concentration of P450 3A4. The effective Stern-Volmer quenching constant K_{eff} was $2.3 \times 10^6 \text{ M}^{-1}$ (corresponding to $K_{d,eff}$ 0.59 μM).

A plot of F_0/F as a function of the P450 3A4 concentration showed an excellent fit to a three-state model (Figure 9C and Table 2), revealing a weaker (but still strong) site of binding and a very strong one ($K_{d,1}$ 1.1 μM , $K_{d,2}$ 0.048 μM). The fractional accessibilities f_1 and f_2 summed to yield a total fractional quenching accessibility $f_a = 1.1$. Thus, all α NF fluorescence could be quenched at high concentrations of P450 3A4 titrant.

Extent of α NF Fluorescence Quenching by P450 3A4—Both Hill and Stern-Volmer analyses show that α NF fluorescence was completely quenched at high P450 3A4 concentrations (Table 2). In addition, the structure of the α NF fluorescence spectrum did not change. A fluorophore that is non-covalently bound to protein (as opposed to being free in aqueous solution) should generate a different bound-state spectral profile, probably more blue-shifted. Thus, the fact that the α NF spectral shape was unchanged as P450 3A4 was added, and that Stern-Volmer quenching analyses showed complete accessibility of quencher, suggest α NF forms a dark complex with P450 3A4.

FRET Calculations—While both charge transfer (Callis & Liu, 2004) and FRET can substantially quench proteins, efficient quenching of α NF fluorescence can be explained through singlet-singlet energy transfer to the heme moiety (FRET). In this case, α NF is the donor and the heme prosthetic group in P450 3A4 is the acceptor, and there is a large overlap between α NF donor fluorescence and heme acceptor absorption (Figure 10B).

Using tyrosine, tryptophan, and rhodamine 6G as standards and Eq. [15], the quantum yield of α NF in (non-degassed) 0.10 M potassium phosphate buffer (pH 7.4) at 23 °C was determined to be $\phi = 0.35$. Acceptor (P450 3A4) absorption spectra were collected in the absence of ligand or in the presence of either testosterone or α NF ligand; spectra with either ligand yielded a classic high-spin Type I spectral change (Figure 10A). Testosterone was in a 50-to-1 molar ratio to P450 3A4 and produced a more enhanced Type I spectrum.

Assuming that heme absorption (λ_{max} 417 nm) was unperturbed by ligand binding, the overlap integral for the α NF-P450 3A4 donor-acceptor pair, obtained under the curve (dashes) in Figure 10B, was $J(\lambda) = 1.72 \times 10^{15} \text{ M}^{-1} \text{ cm}^{-1} \text{ nm}^4$. Given these values, the Förster distance for the α NF-P450 3A4 donor-acceptor pair was 4.6 nm and the

corresponding theoretical quenching efficiency would be 99% if the distance between donor and acceptor is 2.1 nm (Eq. [16]).

Calculating the overlap integral using a Type I heme spectrum had very little effect on FRET efficiencies. In this work, the Type I heme spectrum generated by adding α NF to a P450 3A4 solution yielded an overlap integral $J(\lambda) = 1.52 \times 10^{15} \text{ M}^{-1} \text{ cm}^{-1} \text{ nm}^4$ (overlap spectrum not shown). If the “purer” Type I heme spectrum that resulted from a separate testosterone-P450 3A4 solution was used in the α NF-P450 3A4 overlap integral, the Förster distance R_0 changed very little. R_0 was still 4.5 nm using either Type I spectrum; the effect on the Förster distance was minimal because the overlap integral is taken to the one-sixth power.

Since the diameter of the enzyme molecule is greater than 3.0 nm, (Williams et al., 2004) the fluorescence from a ligand bound at a peripheral binding site would probably not be completely quenched. Indeed, P450 3A4 fluorescence at 320 nm was preferentially quenched by α NF (Figure 3C). This result implies that certain tryptophan residues are quenched preferentially and that these residues reside in hydrophobic environments, e.g., perhaps the active site near the phenylalanine cluster of the P450 3A4 molecule.

Conclusions from Quenching Studies on the Binding Interactions of α NF with P450 3A4

In this work, four quenching interactions were implied: two found by the addition of α NF to P450 3A4, and two found by the addition of P450 3A4 to α NF. Fluorescence quenching was assumed to be due to the formation of non-covalently bound α NF-P450 3A4 complexes; therefore the static quenching constants are equilibrium binding constants (expressed as dissociation constants).

In the titration of α NF into P450 3A4, the affinity measurement was wavelength-dependent. All models were in broad agreement (Table 1) that P450 3A4 fluorescence quenching by α NF at 315 nm yielded a slightly stronger quenching constant (average K_d , $1.7 \pm 0.2 \mu\text{M}$ from all models, Table 1) than did quenching at 335 and 355 nm ($2.5 \pm 0.4 \mu\text{M}$ and $2.1 \pm 0.2 \mu\text{M}$, respectively). Although the standard errors are fairly small (Table 1), it is likely that these K_d values are close enough that just one binding interaction is being revealed. However, the P450 3A4 spectra changed shape during the titrations (Figure 3C), with the fluorescence at 315–320 nm being more efficiently quenched than the fluorescence at 335 and 355 nm.

The most likely explanation for wavelength-dependent quenching of P450 3A4 fluorescence is that the K_d 1.7–2.5 μM quenching constants represent just one interaction. In this scenario, a bound α NF is closer to one or more tryptophans than the other (there are three in the protein). The fluorescence from the closer tryptophan(s) is thus completely quenched by FRET, but the fluorescence from the one more distant is not, at least not until multiple α NF molecules are bound at very high α NF/3A4 ratios. Interestingly, the higher-energy protein fluorescence emission ($\lambda_{\text{max}} = 320 \text{ nm}$, Figure 3C), presumably from tryptophan, is consistent with the residue being sequestered in a hydrophobic environment. That α NF quenched its fluorescence more efficiently suggests that the ligand is bound in the more hydrophobic active site.

It is possible that all three tryptophans in P450 3A4 can be distinguished from the quenching of protein fluorescence by α NF. One emits a higher-energy fluorescence at 315-320 nm and another emits a lower-energy fluorescence with λ_{max} 355 nm. Each of these tryptophans detect one (and probably the same) quenching interaction. The fluorescence at ~ 335 nm may represent the third tryptophan in the protein construct used in this study, and it appears to be quenched by two binding interactions. Future work using site-directed mutagenesis could establish how each tryptophan contributes to the total spectrum, allowing P450 3A4 fluorescence quenching by ligands to become a more precise probe for the location of ligand binding.

The weakest quenching constant was detected at the highest α NF/P450 3A4 ratios and was obtained from the SV model (see Eq. [5] and [6]) in which one class of fluorescence is quenched by two interactions. The first and stronger interaction revealed by this model is the one described above. The weaker one revealed by this model yielded a quenching constant of K_d 6.5 ± 0.8 μM at 335 nm (Table 1). A weak binding interaction of α NF to P450 3A4 has been previously identified, but it was weaker than the one identified in this work (K_d 18 μM , Tsalkova et al., 2007). These may indicate the same binding interaction.

For the second titration in which P450 3A4 was titrated into α NF solutions, two strong interactions were implied (K_d 0.048 ± 0.005 μM and 1.0 ± 0.1 μM). The tighter interaction has not been previously reported for α NF binding to P450 3A4. These values represent averages from three-state SV and Modified SV treatments as well as the two-site Hill analysis (Table 2). In what follows, a few observations will be addressed.

Why did the two titrations not yield the same binding affinities?—In principle, adding α NF to P450 3A4 or adding P450 3A4 to α NF would yield the same binding affinities, but this was not observed. One explanation is that the α NF/P450 3A4 concentration ratios in the titrations overlapped but overall were not the same (Figure 11). For example, when α NF was added to P450 3A4, larger α NF/P450 3A4 molecular ratios in the solution resulted, but when P450 3A4 was titrated into α NF solutions, smaller α NF/P450 3A4 molecular ratios resulted (Figure 11). The weak interaction with dissociation quenching constant $K_d = 6.5$ μM could not have been detected when P450 3A4 was titrated into α NF, because the α NF/P450 3A4 ratios were too small. Likewise, the strong interaction characterized by the 0.048 μM dissociation constant could not have been detected when α NF was titrated into P450 3A4, because the α NF/P450 3A4 ratios were too large.

α NF/P450 3A4 ratios overlapped in the two titrations (Figure 11), and it is possible that the 1.0 μM and 1.7-2.5 μM quenching constants represent the same interaction, in which case these results suggest three binding interactions, not four. Since this work and a number of laboratories have shown that P450 3A4 can bind several ligands simultaneously, adding α NF to P450 3A4 might yield a smaller quenching constant (larger K_d) because the fluorescence of one enzyme molecule was not completely quenched when one ligand molecule was added (*vide supra*). By contrast, in the reverse titration, perhaps relatively more α NF reporter fluorescence was quenched when enzyme was added because multiple α NF molecules might bind to and be quenched by each added molecule of P450 3A4, suggesting tighter binding (higher apparent dissociation constant).

Why did the Stern-Volmer plots from the two titrations not have the same shape?—In the classic SV treatment, the K_{SV} values are collisional constants but the expression for quenching by formation of static complexes is mathematically identical. In quenching by collisions, the fluorescence from the first fluorophore quenched by collisions (i.e., at the lowest concentrations of quencher) has a higher K_{SV} than the fluorescence quenching of sites quenched at higher concentrations of quencher. The higher the quencher concentration needed to collisionally quench a class of fluorescence, the lower must be its collisional constant K_{SV} . However, quenching by formation of non-covalent complexes need not follow this rule and did not appear to in this case. In fact, the shape of the SV curves should depend on the relative K_d values for the binding interactions and on the affinity of quenching due to each interaction.

Titration of P450 3A4 into a solution of α NF yielded a concave-downward SV curve (Figure 8). This behavior suggests two classes of fluorescence that was quenched with different efficiencies (there was no third class revealed in the SV three-class models because all of the fluorescence was quenched—there was no class of inaccessible fluorescence). When enzyme was first titrated into the α NF solution (lowest concentration of enzyme), the α NF/P450 3A4 ratio was higher, favoring the site of weaker binding (K_d 1.0 μ M), but at the end of the titration the α NF/P450 3A4 ratio was much lower, favoring the site of stronger binding (K_d 0.048 μ M; Figure 8 and Table 2). However, more of the fluorescence ($f_a \sim 0.8$, Table 2) was quenched by interaction with the site of weaker binding, so F_0/F increased rapidly in the beginning of the titration of P450 3A4 into α NF. By the end of the titration, the strongest quenching interaction predominated at the lowest α NF/P450 3A4 ratios. Far less fluorescence remained to be quenched by that interaction ($f_a \sim 0.2$, Table 2), because most of it had already been quenched during the first part of the titration (i.e., by the less strong binding interaction). Thus the curve is concave-downward.

When α NF was titrated into a solution of P450 3A4, the resulting Stern-Volmer plot made using detection at 335 nm was concave-upward (Figure 5A). Three-class SV and Modified SV models did not fit these data. Rather, the quadratic behavior at this wavelength was a signature that another model of quenching is required to explain this quenching interaction. It is one in which one class of fluorescence is quenched by two separate and independent binding interactions (Lakowicz, 2006; Seidel et al., 1996) (K_d 2.9 μ M and 6.5 μ M, Table 1; see Eqs. [5] and [6] for details). Each of the interactions obeys a one-class linear SV relationship; both together must be mathematically quadratic if we assume similar quenching efficiencies for each interaction. Accordingly, this model is a good test for multisite binding, or at least of multiple interactions.

The Hill analysis in general agrees with the Stern-Volmer models—There was broad agreement when a Hill analysis and Stern-Volmer models were used to evaluate fluorescence quenching. Three-class Stern-Volmer analyses of α NF fluorescence quenching by P450 3A4 yielded very similar results to the Hill analysis (Table 2). A strong and a very strong site were revealed by both the three-class Stern-Volmer model (K_d 1.0 and 0.043 μ M) and the three-class Modified Stern-Volmer model (K_d 1.1 and 0.048 μ M). These values were similar to those found (K_d 0.87 and 0.052 μ M, Table 2) with a Hill analysis assuming two, non-cooperative binding sites on the protein (Eq. [12]).

However, two quenching interactions were not revealed by a Hill analysis when α NF was titrated into a solution of P450 3A4. Rather, we found that at each detection wavelength, the fluorescence quenching was satisfactorily fit using a single dissociation constant. The difference spectra data did not fit a two-site Hill model (Eq. [12]), nor did the fluorescence quenching fit either three-site SV model (Eqs. [7] and [9]). Only the quadratic behavior of SV quenching (F_0/F as a function of α NF concentration, Figure 5A) indicated two interactions with dissociation constants that were different by a factor of 6 (2.9 μ M and 6.5 μ M, Table 1). However, the reverse titration (P450 3A4 into α NF) yielded dissociation constants that were different by a factor of 21 (0.048 and 1.0 μ M). Perhaps the greater difference in binding affinities observed in the reverse titration afforded a much easier separation of the binding interactions by Hill analysis and SV quenching models.

Another (and perhaps better) reason why the Hill analysis at 335 nm revealed one quenching interaction but the SV treatment yielded two is that in the Hill analysis quenching is calculated by $F/F_{\max}$, which at high concentrations of titrant approaches unity; i.e., it reaches a plateau. A second binding interaction occurring at high α NF/P450 3A4 ratios will not quench much more fluorescence than has already quenched in the titration, and the interaction will be undetectable in the Hill analysis. On the other hand, in the SV treatment F_0/F is calculated; at high α NF/P450 3A4 ratios, F becomes very small, so that F_0/F increases rapidly. Thus, the weaker quenching interaction can be more easily observed.

Conclusions and Relevance to Function

These results confirm earlier work that α NF exhibits a complex binding pattern to P450 3A4 (Tsalkova et al., 2007). Tsalkova et al. showed three binding interactions of α NF to P450 3A4, two involving Fe spin shifts plus a weaker binding interaction (S_{50} 18 μ M). The latter could be similar to the weakest interaction detected here (K_d 6.5 μ M). The extremely strong binding site found in this work (K_d 0.048 μ M) has not previously been found for a P450 3A4 interaction with α NF, but a similar very strong site of P450 3A4 binding has been found for Nile Red (Lampe et al., 2008). The very strong affinity suggests that the ligand is tightly bound to a site in the substrate pocket, if not complexed to the heme prosthetic group.

There are several explanations for why four α NF-P450 3A4 binding interactions should be detected. One is that a single P450 3A4 molecule conformer has four interactions with α NF, which is both effector and substrate. While this kind of plasticity is possible, it may not be the only explanation for the complex binding behavior of P450 3A4.

Instead, there is strong evidence that membrane-bound P450 3A4 exists as multiple conformers (Koley et al., 1995; Koley, Robinson, & Friedman, 1996). Indeed, P450 3A4 aggregates into an oligomeric form in microsomes (and perhaps in the solution conditions similar to those employed here, 100 mM potassium phosphate, pH 7.4), and P450 monomers in the oligomer can have different conformations (Davydov et al., 2005). P450 3A4 can also form mixed oligomers with other P450s, affecting the catalytic activities of each P450 species (Davydov, Davydova, Sineva, & Halpert, 2015). In our solution conditions, P450 3A4 is not membrane-bound, but if the enzyme can aggregate to oligomers then it is possible that each monomer could have different substrate affinities, yielding the complex binding

behavior seen here. If P450 3A4 does not aggregate in our solution conditions, then these data suggest that single monomers in solution exhibit very complex binding behavior.

Thus, a second explanation for the four binding interactions is that in oligomeric form, P450 3A4 exists as multiple conformers with different α NF binding affinities (Tsalkova et al., 2007). Given that a peripheral binding site for many ligands has already been identified (Williams et al., 2004; Davydov et al., 2012), it is possible that there are two distinguishable P450 3A4 conformers in the oligomer, each with a strong α NF binding site in the substrate pocket and one weaker, peripheral site on the enzyme molecule. Complex binding by α NF to P450 3A4 oligomers has repercussions in allosteric activation by α NF; it is known that α NF affects P450-P450 interactions and can increase catalysis of substrates (Davydov et al., 2013).

A further possibility entertained by Davydov and co-workers (Tsalkova et al., 2007) is that a ligand binding site may be created at the interface between two P450 3A4 subunits in the oligomer. The very strong interaction found here could be to such a molecular surface.

Collectively the results provide physical evidence for multiple modes of binding of α NF, a classical modulator of P450 3A4 catalytic function (Shimada & Guengerich, 1989; Ueng, Shimada, Yamazaki, & Guengerich, 1995; Ueng, Kuwabara, Chun, & Guengerich, 1997). α NF is involved in heterotropic cooperativity; it can stimulate rates of some reactions but inhibit others (Ueng et al., 1995). It is also a substrate for P450 3A4, producing the 5,6-epoxide (Ueng et al., 1997). Although we are still unable to assign specific binding modes of α NF to its quenching of individual classes of quenched fluorescence, our results implicate four binding sites as a basis of the observed effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

α NF	7,8-benzoflavone or α -naphthoflavone
FRET	fluorescence resonance energy transfer
MSV	Modified Stern-Volmer

P450 cytochrome P450
SV Stern-Volmer

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Figure 1.

Experimental parameters for fluorescence spectroscopy. (A) Spectroscopic parameters for α NF titrations to P450 3A4. The P450 3A4 excitation and emission spectra are shown in dotted and solid lines, respectively, and the α NF absorption spectrum is shown as dashes. (B) Spectroscopic parameters for P450 3A4 titrations to α NF. The P450 3A4 excitation and emission spectra are represented by the dotted and solid lines, respectively. The P450 absorption spectrum, which includes heme absorption, is shown in dashes, while the α NF fluorescence emission is shown as a dotted plot. In this scheme, λ_{det} is the wavelength at which the fluorescence was detected and λ_{exc} is the excitation wavelength.

Figure 2.

Photolability of P450 3A4 and α NF during the course of a titration. (A) Decay of P450 3A4 fluorescence (average of six experiments) during titration was monitored at 355 nm. (B) Decay of α NF fluorescence (average of three experiments) during titration (λ_{exc} 290 nm) was detected at 440 nm. (C) Decay of α NF fluorescence during titration (λ_{exc} 330 nm) was monitored at 440 nm. In all cases the data were fit to a single-exponential decay.

Figure 3.

Fluorescence spectra of P450 3A4 with and without α NF showing multiple fluorescence contributions from enzyme. (A) Normalized fluorescence excitation spectra (left two curves) and emission spectra (right three curves) of P450 3A4 in the absence of α NF. Excitation spectra were collected using emission detection at 310 nm (solid) and 355 nm (dashes), and emission spectra were collected using excitation wavelengths of 275 nm (solid) and 290 nm (dashes). The dotted spectrum shows the fluorescence contribution at 320 nm generated by using the higher energy 275 nm excitation (solid minus dashes). (B) Emission spectra of 250 nM P450 3A4 (solid), 300 nM α NF (dashes) and 250 nM P450 3A4 + 300 nM α NF (dotted) in 100 mM potassium phosphate buffer (pH 7.4). (C) Spectra of 250 nM P450 3A4 with no α NF (solid) and in the presence of 750 nM α NF (dashes). The lower spectrum (dotted) shows what is quenched by 750 nM α NF; it is the solid spectrum minus the dashed spectrum.

Figure 4.

Hill analysis of P450 3A4 fluorescence quenching by α NF. α NF (0–20 μM before photobleaching adjustment) was titrated into a 250 nM solution of P450 3A4 in 100 mM potassium phosphate (pH 7.4). (A) P450 3A4 spectra (λ_{exc} 290 nm) measured after α NF quencher was added. (B) P450 3A4 difference spectra, obtained by subtracting the initial P450 3A4 spectrum ($[\alpha \text{NF}] = 0 \mu\text{M}$) from the 3A4 spectrum obtained at a given concentration of P450 3A4. (C) Hill plots generated from the difference spectra and fitted to the Hill equation (Eq. 11). The extent of quenching ($\Delta F / F_{\max}$) at 315 (●), 335 (■), and 355 nm (▲) is shown in solid, dashed, and dotted plots, respectively.

Figure 5.

Stern-Volmer analysis for titration of α NF into P450 3A4. P450 3A4 fluorescence quenching F_0/F measured at 315 (●), 335 (■), and 355 nm (▲); each point is average of four experiments. (A) Linear fit to P450 3A4 F_0/F quantified at each concentration of α NF quencher. Quenching at 315, 335, and 355 nm is shown as solid, dashed, and dotted plots, respectively. F_0/F at 335 nm was also fit to a quadratic function (solid line). (B) Two association constants determined from fluorescence quenching at 335 nm and distinguished by plotting the apparent association constant K_{app} as a function of α NF concentration.

Figure 6.

Panels (A)-(C) Modified Stern-Volmer plots for titration of α NF into P450 3A4. (A) P450 3A4 fluorescence quenched (F) was quantified at each concentration of α NF quencher and was fit to the Modified Stern-Volmer equation (Eq. [4]). (B, inset) Expansion of the plot at low values of $[\alpha\text{NF}]^{-1}$. (C) Linear fit done only to high concentrations of α NF quencher (low values of $[\alpha\text{NF}]^{-1}$). Quenching (average of 4 experiments) at 315 (●), 335 (■), and 355 nm (▲) is shown in solid, dashed, and dotted plots, respectively.

Figure 7.

Difference spectra and Hill analysis for titration of P450 3A4 into *a*NF. (A) Difference spectra (λ_{exc} 330 nm) obtained by subtracting the *a*NF spectrum acquired without quencher from the *a*NF fluorescence emission obtained at higher concentrations of P450 3A4 quencher. (B) F/F_{max} values from two experiments ($\blacktriangledown$ and $\bullet$) are shown and were calculated using quenching at 440 nm. Hill analysis was done three different ways: assuming a non-cooperative hyperbolic fit (solid line), assuming no constraint of the Hill coefficient (dashed line), and assuming a two-site Hill model in which each site is independent and non-cooperative (dotted line). (C) Data from Figure 7B focused on low concentrations of P450

3A4 titrant ($0 - 0.7 \mu\text{M}$). Data points represent the mean of $F/F_{\max}$ values from the two quenching experiments. Hill analysis was done and plotted as in Figure 7B.

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Figure 8.

Stern-Volmer analysis for titration of P450 3A4 into α NF. Data from two quenching experiments are shown; SV analysis was done through the mean of the two experiments. (A) Stern-Volmer quenching showing fits to all concentrations of P450 3A4. (B) SV plot showing fits at low concentrations at P450 3A4. The linear fit to the data (dashed line) assumes one class of fluorescence; the three-class SV model that was fit to the data is shown as a solid plot. Excitation was at 330 nm and the fluorescence emission was detected at 440 nm.

Figure 9.

Modified Stern-Volmer analysis for titration of P450 3A4 into α NF. (A) Linear fit to the data. Data from two quenching experiments are shown; MSV analysis was done through the mean of the two experiments. (B) Linear relationship at high concentrations of P450 3A4 quencher (low $[P450\ 3A4]^{-1}$). (C) Three-state model fit to the data. Excitation was at 330 nm and fluorescence emission was detected at 440 nm.

Figure 10.

A) Absorption spectra of 2 μM P450 3A4 in the absence of ligand (solid) and in the presence of 10 μM αNF (dashed line) and 100 μM testosterone (dotted line) ligand. B) Overlap integral for αNF fluorescence quenching by heme. The fluorescence spectrum of αNF donor (dotted) and the absorption spectrum of the heme acceptor (solid) yield the overlap spectrum (dashes), in units of fluorescence intensity $\text{M}^{-1} \text{cm}^{-1} \text{nm}^4$.

Figure 11.

Ratios of α NF to P450 3A4 concentrations for the two titrations: α NF into P450 3A4 solutions (triangles) and P450 3A4 into α NF solutions (circles). Dissociation constants for each titration are averages from analyses using Stern-Volmer and Hill models of fluorescence quenching (see Tables 1 and 2).

Table 1
Dissociation Constants K_D and Solvent Accessibilities f_a for α NF Quenching of P450 3A4 Fluorescence.

fluorescence emission quantitation method	K_D , μ M, fit to Hill Plot	n , fit to Hill Plot	K_D , μ M Linear one class SV Model,	$K_{D,app}$, μ M One class, two constants	$K_d = 1/K_{d,MSV}$ μ M MSV model	f_a , MSV model	$K_{d,eff} = 1/K_{d,MSV}$ μ M MSV Model ([α NF] $\rightarrow \infty$)	$f_{a,eff}$ MSV Model ([α NF] $\rightarrow \infty$)
fluorescence intensity, 315 nm	2.0 ± 0.3	1.1 ± 0.1	1.9 ± 0.1 $R^2 = 0.952$ $S_{y,x} = 0.301$	Quadratic fit not required	1.8 ± 0.1	0.98 ± 0.06	1.5 ± 0.2	0.98 ± 0.04
fluorescence intensity, 335 nm	2.5 ± 0.4	0.98 ± 0.07	1.1 ± 0.1 $R^2 = 0.918$ $S_{y,x} = 0.614$	Quadratic fit $R^2 = 0.956$ $S_{y,x} = 0.454$ Yields two $K_{D,s}$ 2.9 ± 0.1 6.5 ± 0.8	2.5 ± 0.5	1.2 ± 0.2	2.2 ± 0.3	1.1 ± 0.1
fluorescence intensity, 355 nm	2.4 ± 0.2	0.96 ± 0.06	1.9 ± 0.1 $R^2 = 0.939$ $S_{y,x} = 0.345$	Quadratic fit not required	2.2 ± 0.4	1.1 ± 0.2	2.0 ± 0.2	0.99 ± 0.04

Values are means $\pm$ SE from four titrations. MSV: Modified Stern-Volmer.

Hill Parameters, Dissociation Constants, and Solvent Accessibilities for P450 3A4 Quenching of α NF Fluorescence.^a

Table 2

A. Hill parameters							
Model	n	K_{d1} , μM	K_{d2} , μM	f_1	f_2	Goodness of Fit ^b	
						R^2	$S_{y,x}$
Hill, $n = 1$, one site	1.0	0.32 ± 0.03	N/A ^c	N/A	N/A	0.985	0.034
Hill, n unconstrained, one site	0.80 ± 0.07	0.46 ± 0.10	N/A	N/A	N/A	0.993	0.023
Hill, $n = 1$, two site	1.0	0.87 ± 0.35	0.052 ± 0.04	0.76 ± 0.09	0.25 ± 0.12	0.995	0.021

B. Stern-Volmer Parameters					
Model	K_{d1} , μM	K_{d2} , μM	f_1	f_2	R^2
linear Stern-Volmer	1.5 ± 0.2	N/A	N/A	N/A	0.945
modified Stern-Volmer (all data)	0.15 ± 0.02	N/A	0.67 ± 0.06	N/A	0.925
modified Stern-Volmer ([3A4] $\rightarrow \infty$)	0.59 ± 0.13	N/A	1.02 ± 0.03	N/A	0.79
Stern-Volmer (three class)	1.02 ± 0.96	0.043 ± 0.16	0.82 ± 0.26	0.24 ± 0.36	0.947
modified Stern-Volmer (three class)	1.1 ± 0.4	0.048 ± 0.010	0.84 ± 0.09	0.26 ± 0.05	0.999

^aValues are means from duplicate titrations $\pm$ the standard errors obtained from linear or nonlinear analysis of the mean.

^bGoodness of fit parameters R^2 (correlation coefficient) and $S_{y,x}$ (standard deviation of the residuals)

^cN/A: not applicable