Time-Resolved Fluorescence Studies of Heterotropic Ligand Binding to Cytochrome P450 3A4

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ABSTRACT: Cytochrome P450 3A4 (CYP3A4) is a major enzymatic determinant of drug and xenobiotic metabolism that demonstrates remarkable substrate diversity and complex kinetic properties. The complex kinetics may result, in some cases, from multiple binding of ligands within the large active site or from an effector molecule acting at a distal allosteric site. Here, the fluorescent probe TNS (2-p-toluidinylnaphthalene-6-sulfonic acid) was characterized as an active site fluorescent ligand. UV–vis difference spectroscopy revealed a TNS-induced low-spin heme absorbance spectrum with an apparent $K_d$ of 25.4 ± 2 μM. Catalytic turnover using 7-benzyloxyquinoline (7-BQ) as a substrate demonstrated TNS-dependent inhibition with an IC$_{50}$ of 9.9 ± 0.1 μM. These results suggest that TNS binds in the CYP3A4 active site. The steady-state fluorescence of TNS increased upon binding to CYP3A4, and fluorescence titrations yielded a $K_d$ of 22.8 ± 1 μM. Time-resolved frequency-domain measurement of TNS fluorescence lifetimes indicates a testosterone (TST)-dependent decrease in the excited-state lifetime of TNS, concomitant with a decrease in the steady-state fluorescence intensity. In contrast, the substrate erythromycin (ERY) had no effect on TNS lifetime, while it decreased the steady-state fluorescence intensity. Together, the results suggest that TNS binds in the active site of CYP3A4, while the first equivalent of TST binds at a distant allosteric effector site. Furthermore, the results are the first to indicate that TST bound to the effector site can modulate the environment of the heterotropic ligand.

The heme-containing hepatic and intestinal cytochrome P450s (CYPs) control the metabolism of most toxins and drugs (1–3). CYP3A4 is the major component of CYP-dependent clearance, although others also contribute. An emerging paradigm concerning these microsomal CYPs is their tendency to exhibit complex non-Michaelis–Menten kinetic profiles in vitro. Although the extent of in vivo allosteric kinetics remains in question, the in vitro complexity hinders the practical prediction of clinical outcomes based on in vitro experiments. Both homotropic and heterotropic effects have been well documented (4–9). This allosteric behavior appears to result from the simultaneous binding of multiple drugs to a single CYP. Recently, published crystal structures of several CYPs, including CYP3A4, suggest that the active site is sufficiently large to accommodate multiple ligands (10, 11).

It has been difficult to determine the spatial relationship of various ligand binding sites within the CYP3A4 scaffold by steady-state kinetic methods. Thus, it remains unknown whether homotropic and heterotropic interactions occur because of simultaneous binding by multiple ligands within the large active site or due to remote, spatially distinct binding sites. For example, an effector ligand could bind to peripheral sites on the protein surface and alter the kinetic properties of substrates within the active site via long-range effects. A crystal structure of the [CYP3A4+progesterone] complex indicates that the steroid is not bound at the active site but rather lies in a hydrophobic patch near the protein surface, known as the “phenylalanine cluster” due to the propensity of phenylalanine residues at this location (11). Alternatively, the effector could bind within the active site and directly alter the active site physical properties such as volume, hydration, and hydrogen bonding (12). Furthermore, both scenarios may be relevant, depending on the specific combination of ligands. Clearly, new methods are required to efficiently distinguish between the possible mechanisms of allosterism for a wide range of ligand combinations.

Here we report results from steady-state and frequency domain fluorescence lifetime measurements using TNS as a probe (Figure 1). The unique photophysical properties of TNS result in a nonfluorescent compound in aqueous
solution, but with a high quantum yield when bound to hydrophobic regions of a protein such as the CYP3A4 active site. Therefore, we concluded that TNS should function well as a probe for direct assessment of binding of a ligand to CYP3A4. The results obtained here from UV–vis titrations indicate that TNS binds within the active site. Moreover, data with TNS binding in the presence of testosterone (TST) demonstrate that the highest-affinity TST binding site is remote from the protoporphyrin heme. Together, these results support previous evidence for a TST binding site distant from the heme iron (13), and they provide the first indication that TST modulates the environment of the active site from its high-affinity effector site.

**MATERIALS AND METHODS**

**Chemicals.** All chemicals were analytical grade and obtained from commercial sources. The potassium salt of 2-p-toluidinylnaphtalene-6-sulfonic acid (TNS) was obtained from Markem Gene Technologies (Eugene, OR). Testosterone was obtained from Steraloids (Newport, RI). Erythromycin and all other chemicals used were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

**Protein Expression and Purification.** Recombinant CYP3A4 was expressed and purified from *Escherichia coli* as described previously, except that a French press was used to lyse the cells (14). To ensure complete lysis, cells were passed twice through a French pressure cell (Thermo IEC, Needham Heights, MA) at 10,000 psi. When purification was complete, the concentration of CYP3A4 was quantified by the method of Omura and Sato, using an extinction coefficient of 91 mM⁻¹ cm⁻¹ (15). The protein was judged to be greater than 95% pure by SDS–PAGE. The purified CYP3A4 was divided into 1 mL aliquots and stored at −80 °C until further use. Each aliquot underwent no more than five freeze–thaw cycles.

**Determination of IC₅₀ Values for TNS Inhibition.** IC₅₀ values were determined according to the method of Cheng and Prusoff (16), using a reconstituted enzyme system as described previously (17). The CYP3A4-mediated O-debenzylation of 7-benzoyloxyquinoline (7-BQ) was monitored as a function of TNS concentration. Steady-state emission spectra were recorded to ensure no interference from background TNS fluorescence. Reconstituted enzyme mixes were incubated with a substrate concentration equivalent to the Km of the substrate (70 μM for 7-BQ) (18) and TNS (concentration range of 0–200 μM) under the conditions described previously (17). Briefly, 30 pmol of purified CYP3A4, 60 pmol of rat NADPH-P450 reductase, and 30 pmol of rat cytochrome b₅ were resuspended in a buffer containing 0.1 mg/mL CHAPS, 20 μg/mL liposomes [1-α-dilauroyl-sn-glycero-3-phosphocholine, 1-α-dioleoyl-sn-glycero-3-phosphocholine, 1-α-dilauroyl-sn-glycero-3-phosphoserine, at a 1:1:1 (w/w/w) ratio per milliliter], 600 μM GSH, and 10 mM potassium HEPES (pH 7.4). Reaction buffer and MilliQ NanoPure H₂O were added to a final concentration of 40 mM potassium HEPES (pH 7.4), 2.4 mM GSH, and 30 mM MgCl₂. This enzyme mixture was then allowed to preincubate on ice for 10 min. Reaction mixtures were then preincubated at 37 °C for 5 min, after which the reaction was initiated by addition of 1 mM NADPH. The final reaction volume was 1 mL. The reaction was allowed to proceed for 10 min at 37 °C, at which time product formation was assessed using an AB2 SLM-Aminco luminescence spectrometer set at an excitation wavelength of 409 nm and an emission wavelength of 530 nm with a band-pass of 4 nm for both wavelengths. The photomultiplier tube was set to 550 V. A (−) NADPH control was completed for each sample to correct for background fluorescence. Since the formation of 7-hydroxyquinoline (7-HQ) by CYP3A4 was determined to be linear over an incubation period of 20 min under these conditions, an incubation time of 10 min was employed for all kinetic determinations. Product formation was calculated by comparison to a standard curve for 7-HQ fluorescence and converted to nanomoles per minute per nanomole of CYP3A4. IC₅₀ values for TNS inhibition were determined by fitting the data using GraphPad Prism (GraphPad Software, San Diego, CA) to the variable slope sigmoidal dose–response equation (eq 1):

\[
v = \frac{100}{1 + 10^{\beta (\log IC_{50} - [L])}}
\]

where B is a factor that varies the slope of the curve to achieve the best fit.

**TNS Kₐ Determination using Optical Difference Spectroscopy.** To determine the equilibrium binding constant (Kₐ) for binding of TNS to CYP3A4, UV–vis difference spectra were acquired using a single-beam Agilent (Palo Alto, CA) model 8453 UV–vis spectrophotometer. Because of the strong UV absorbance of TNS below 380 nm, spectra were obtained using the absolute difference method, as described previously (13). The instrument was referenced against a buffer containing 100 mM KF (pH 7.4), 10% glycerol, and 1 mM EDTA. Subsequently, an initial spectrum was obtained for a 1 mL solution of 1 μM 3A4 diluted into the same buffer. The titration was begun by adding 1 μL aliquots of 1 mM TNS dissolved in 50% ethanol to the cuvette and recording the full spectrum after a 1 min equilibration time. The titration continued until the peak at ~425 nm reached apparent saturation (~30 μM TNS). At the conclusion of the titration, the final concentration of ethanol did not exceed 1.5%. However, to take into account any possible effect of solvent on the heme spin state, this titration was normalized to a separate control titration of CYP3A4 conducted with ethanol to a final concentration of 1.5%. All spectra were recorded at 22 °C (room temperature). After the initial spectrum was subtracted from each subsequent spectrum, a Kₐ value for the binding of TNS was obtained by comparing the absolute change in absorbance in the peak at 425 nm versus the trough at 398 nm and fitting these values to the Michaelis– Menten equilibrium binding equation (eq 2):

\[
\frac{\Delta_{ABS}}{ABS_{max}} = \frac{[S]}{K_d + [S]}
\]


**Competitive Ligand Displacement Experiments.** For the competitive ligand displacement experiments, UV–vis difference spectra were acquired using a dual-beam OLIS/ Aminco DW2a spectrophotometer (OLIS, Bogart, GA). For each titration with a competitive ligand, the sample chamber contained a 1 mL volume of 5 μM 3A4 in 100 mM KF (pH
was used as an internal lifetime standard for the TNS experiments, whereas p-terphenyl was used as an internal lifetime standard for the tryptophan experiments (19). Titrations were carried out at 15 °C. Phase angle shift (φ) and modulation (m) decay data were fit to the simplest exponential decay model using the Model software package (Thermogalactic, Waltham, MA), according to the following intensity decay law (eq 4):

\[
I(t) = \sum_{j=1}^{n} \alpha_j \exp(-t/\tau_j)
\]

where \(\alpha_j\) is the time-zero amplitude due to each specific decay time (\(\tau_j\)). In terms of a single-exponential decay, the lifetime can be calculated from the phase and modulation values using

\[
\tau_{\phi} = \omega^{-1} \tan \phi
\]

\[
\tau_{m} = \omega^{-1} \left( \frac{1}{m^2} - 1 \right)^{-1/2}
\]

where \(\omega\) is the light modulation frequency in radians per second, \(\tau_{\phi}\) is the apparent phase angle shift lifetime, and \(\tau_{m}\) is the apparent modulation lifetime. Average lifetimes (\(\bar{\tau}\)) of multiexponential decay were calculated using eq 7:

\[
\bar{\tau} = \frac{1}{\sum_{i=1}^{n} \alpha_i \tau_i^2}
\]

**Kinetic Simulations.** Kinetic simulations were performed using the GEPASI Biochemical Simulation Module (20–22) based on the sequential-ordered binding model (eq 3), as described previously for CYP3A4 (13).

**RESULTS**

**General Experimental Design.** The goal of these studies was to determine whether time-resolved fluorescence methods could be used to distinguish between “allosteric” ligand interactions and simple competitive ligand interactions. Therefore, several of the experiments were performed at subsaturating concentrations of the major probe used here, TNS. This experimental design minimizes the likelihood of multiple TNS molecules binding on a single CYP3A4 molecule and, thus, simplifies the spectroscopic interpretation: only a single fluorescent species, [CYP3A4•TNS], is affected by subsequent addition of a heterotropic ligand. A shortcoming of this design is that it decreases sensitivity by limiting the concentration of the reporter species, and it also introduces thermodynamic complexity into the heterotropic interaction studies, wherein several species compete for the heterotropic ligand. For example, with TST as a heterotropic ligand, and assuming at least two TST molecules can bind, free [CYP3A4], [CYP3A4•TNS], and [CYP3A4•TST] may all compete for added TST. This precludes determination of true \(K_d\) values for the heterotropic ligand and quantitative analysis of the energetics of cooperativity with the experimental design used here. The ligand affinities reported here for TST are “apparent” \(K_d\) values only, as described within.
TNS Fluoresces When It Is Bound to CYP3A4. The fluorescence of TNS in aqueous solution is highly quenched, but the quantum yield increases in nonpolar environments such as hydrophobic protein binding sites. The fluorescence of TNS is perturbed by binding to CYP3A4, resulting in a type II spectrum. This observation is consistent with previous studies indicating that TNS binds in the active site and ligates directly to the heme iron. The only other possibility, the formation of a low-spin heme ligand, has never been observed with CYP3A4.

Tryptophan Fluorescence Lifetime Measurements. Further studies were conducted to elucidate the mechanism of TNS binding to CYP3A4. Tryptophan residues are intrinsic to the protein and are known to exhibit quenching effects upon binding to proteins. The lifetime of the tryptophan fluorescence is sensitive to changes in the environment of the Trp residues, making it a valuable probe for studying protein-ligand interactions. In the absence of TNS, the tryptophan lifetime is expected to be shorter due to quenching effects. However, in the presence of TNS, the lifetime is expected to increase due to perturbation of the Trp environment.

Figure 3: Inhibition of catalytic turnover by TNS using 7-BQ as a substrate. The IC$_{50}$ of 9.9 ± 0.1 μM was recovered from fitting the data to eq 1.
suggest a TNS-induced change in the environment for at least one of the Trp residues present in CYP3A4, at low TNS occupancy.

Time-Resolved Fluorescence of TNS. The excited-state decay of TNS bound to CYP3A4 was determined by frequency modulation time-resolved fluorescence, using 20 frequencies ranging from 1 to 200 MHz, and multiple wavelengths. Several multiexponential decay models were compared and the data fit best to a three-component model with lifetime values near 0.3, 0.6, and 7.4 ns. The recovered parameters are included in Table 2. Only the results for the 440 nm emission are tabulated. A notable feature of the results is the recovery of a significant negative pre-exponential term at all wavelengths for the short lifetime component, but with an increased magnitude at longer wavelengths. Similar behavior has been reported with TNS and many TNS analogues, such as the related 1-anilino-8-naphthalenesulfonic acid (ANS), when in viscous solvents or bound to proteins (25). The negative pre-exponential terms result from an excited-state reaction, as reported previously with this compound (25).

Heterotropic Effects between TNS and Testosterone. On the basis of the conclusion that TNS binds at the active site of CYP3A4, its fluorescence properties can be used as a mechanistic probe of heterotropic interactions with other ligands. TST is a well-studied substrate for CYP3A4 and is known to exhibit homotropic and heterotropic behavior (13, 27). With reconstituted CYP3A4, TST exhibits complex biphasic binding, with two or three TST molecules binding each CYP3A4 molecule, possibly depending on whether the enzyme is partially aggregated or monodisperse in nanodisks (27-30). On the basis of optical difference spectra of the heme spin state and EPR, we previously observed two apparent $K_d$ values of ~22 and ~440 µM for binding of TST to CYP3A4 (13). TST binding at the high-affinity site does not perturb the heme spin state (31) and thus is likely to be in a remote corner of the active site or at a distinct peripheral site. Therefore, we examined the effects of varying the concentration of TST on the steady-state spectrum and on the fluorescence lifetime of CYP3A4-bound TNS. Figure 6A demonstrates that TNS fluorescence is quenched upon addition of TST and blue-shifted, thus demonstrating a heterotropic ligand effect. The TST concentration dependence of the TNS quenching exhibits complex behavior, consistent with multiple TST molecules binding. In fact, the steady-state quenching curve fits well to a model describing two TST binding sites (eq 3) with apparent $K_d$ values of 14 and 243 µM (Figure 6C), which is in good agreement with the biphasic binding observed previously with heme absorbance and EPR spectra (13). It should be noted, as above, that the
$K_d$ values for TST measured here are "apparent" because of the presence of ligand-free CYP3A4 in the experiment. An important feature of this fluorescence quenching curve is that the first, high-affinity, TST binding event quenches TNS fluorescence. There is no lag in the quenching versus TST concentration curve. In contrast, UV–vis difference spectra of the titration of TST into TNS-bound CYP3A4 indicate that the high-affinity TST molecule does not immediately decrease the fraction of low-spin heme (13), as already observed for TST in the absence of a heterotropic ligand. Conversion from a low-spin [CYP3A4–TNS] complex to a high-spin [CYP3A4–TST] complex occurs only at higher TST concentrations, i.e., >100 μM (data not shown). This is similar to the lag observed in the curve of the high-spin fraction versus TST concentration published previously for CYP3A4 in the absence of TNS (13).

In summary, the first equivalent of TST that binds to TNS-bound CYP3A4 quenches TNS fluorescence but does not alter the TNS-dependent spin-state equilibrium. Whether 100 μM TST would be sufficient to generate a spin-state change, knowing that the first equivalent of TST does not, is considered in more detail below. The data suggest, but do not prove, that the high-affinity TST site is distinct from the TNS site and possibly modulates TNS fluorescence through long-range effects rather than through direct displacement of TNS. Furthermore, TST not only quenches the

\[ \tilde{\tau} = \sum_{i=1}^{n} \alpha_i \tau_i^2 / \tau_i. \]

**Table 1: Excited-State Parameters for Trp Decay in CYP3A4 at 330 nm**

<table>
<thead>
<tr>
<th>[TNS] (μM)</th>
<th>$\tau_1$ (ns)</th>
<th>$\tau_2$ (ns)</th>
<th>$\tau_3$ (ns)</th>
<th>$\alpha_1$</th>
<th>$\alpha_2$</th>
<th>$\alpha_3$</th>
<th>$\chi^2$</th>
<th>$\tilde{\tau}$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.182 ± 0.059</td>
<td>4.665 ± 0.233</td>
<td>10.762 ± 0.538</td>
<td>0.424</td>
<td>0.544</td>
<td>0.031</td>
<td>0.613</td>
<td>4.75 ± 0.238</td>
</tr>
<tr>
<td>10</td>
<td>1.060 ± 0.053</td>
<td>4.501 ± 0.225</td>
<td>10.072 ± 0.515</td>
<td>0.751</td>
<td>0.249</td>
<td>0.710</td>
<td>3.070</td>
<td>3.07 ± 0.154</td>
</tr>
</tbody>
</table>

**Table 2: Excited-State Parameters for TNS Bound to CYP3A4 with Varying TST Concentrations at 440 nm**

<table>
<thead>
<tr>
<th>[TST] (μM)</th>
<th>$\tau_1$ (ns)</th>
<th>$\tau_2$ (ns)</th>
<th>$\tau_3$ (ns)</th>
<th>$\alpha_1$</th>
<th>$\alpha_2$</th>
<th>$\alpha_3$</th>
<th>$\chi^2$</th>
<th>$\tilde{\tau}$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.307 ± 0.015</td>
<td>0.608 ± 0.030</td>
<td>7.42 ± 0.371</td>
<td>-0.693</td>
<td>0.969</td>
<td>0.725</td>
<td>0.551</td>
<td>6.98 ± 0.349</td>
</tr>
<tr>
<td>20</td>
<td>0.331 ± 0.017</td>
<td>3.418 ± 0.171</td>
<td>0.161</td>
<td>0.839</td>
<td>0.701</td>
<td>3.36 ± 0.168</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.576 ± 0.028</td>
<td>2.981 ± 0.149</td>
<td>0.629</td>
<td>0.371</td>
<td>0.606</td>
<td>2.38 ± 0.119</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 6:** Testosterone quenches the steady-state fluorescence and decreases the fluorescence lifetime of 3 μM TNS bound to 5 μM CYP3A4. (A) Steady-state emission spectrum of TNS bound to CYP3A4 in the presence of increasing concentrations of testosterone. The testosterone concentration increases with the downward arrow. (B) Phase modulation domain decay data for emission of TNS from CYP3A4 in the presence of increasing testosterone concentrations ([●] 0, [○] 20, and [◆] 100 μM TST; $\lambda_{em} = 318$ nm, $\lambda_{ext} = 440$ nm). Note the clear dependence of fluorescence lifetime on TST concentration. (C) Plot of TST steady-state quenching of TNS-CYP3A4 emission (○) vs TNS average fluorescence lifetime (●) at various concentrations of TST. Steady-state quenching data were fit to eq 3 to obtain the $K_d$ values for the first and second TST binding events.
TNS fluorescence intensity but significantly blue shifts it as well. This directly indicates formation of a ternary [CYP3A4•TNS•TST] complex. As described below, monitoring the excited-state lifetime provides a direct probe of competitive versus noncompetitive binding for the two ligands.

If TST binds at the same site as TNS and therefore displaces it from the active site, then the apparent average lifetime of TNS will not change with an increase in TST concentration because only bound TNS contributes to the average lifetime. Displaced TNS will not be detected because it is nonfluorescent under these solution conditions. In contrast, if TST binds at a remote site or a distinct subsite within the large active site, then the decrease in steady-state fluorescence intensity will be accompanied by a decrease in excited-state lifetime. This situation is summarized in Scheme 1.

On the basis of Scheme 1, the excited-state lifetime of TNS was determined at varying TST concentrations above and below the $K_d$ for the low-affinity TST site (Figure 6B). Higher concentrations of TST yielded poor quality phase modulation data, presumably due to the lowered fluorescent intensity of the bound TNS and the low solubility of TST. In fact, in the absence of additional cosolvent, TST begins to precipitate above 200 µM. Thus, it was technically impossible to obtain phase modulation data at high TST concentrations. However, the excited-state lifetime decreases even at low TST concentrations, and the trend is consistent with the TST concentration-dependent decrease in steady-state intensity (Figure 6C). The phase angle and modulation data clearly demonstrate a shift toward a higher-frequency response with addition of TST (Figure 6B). Interestingly, the shortest recovered lifetime contains a significant negative

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Figure 7: Erythromycin displaces TNS from the CYP3A4 active site, whereas testosterone does not. (A) Effect of increasing testosterone concentrations on the TNS-induced type II spin state of CYP3A4. Testosterone was titrated into the [CYP3A4•TNS] complex from 10 to 110 µM. Note that there is no substantial change in the spin state with an increase in TST concentration. (B) Effect of increasing erythromycin concentrations on the TNS-induced type II spin state. Note the conversion of the TNS-induced type II spectrum ($\lambda_{\text{max}} = 425$ nm, $\lambda_{\text{min}} = 398$ nm) to a type I spectrum ($\lambda_{\text{max}} = 380$ nm, $\lambda_{\text{min}} = 415$ nm), suggesting competitive displacement of TNS by ERY in the CYP3A4 active site. (C) Plot of the change in absorbance from 380 to 415 nm (ABS380-415) upon addition of TST (●) or ERY (□) to the [CYP3A4•TNS] complex. Conversion from a type II spin state to a type I state occurs only upon addition of ERY and not TST.
ERY causes no decrease in the excited-state lifetime of TNS steady-state fluorescence, with an apparent pre-exponential component results from an excited-state reaction in which the excited-state TNS dipole experiences a change in its dipolar environment during the lifetime of the excited state (25), and this reaction either is too fast to observe or does not occur in the presence of TST. Regardless, TST definitely alters the excited-state dynamics or environment of TNS. Apparently, TST does not competitively displace TNS from its low-spin binding site, as suggested by the UV–vis difference spectral titration (Figure 7A). This observation is discussed further below.

To ensure that TST alone had no effect on the fluorescence lifetime of free TNS alone, TNS fluorescence lifetime measurements were recorded in the presence of 100% glycerol and 100 μM TST, in the absence of CYP3A4. No detectable change in TNS fluorescence lifetime was observed (data not shown).

For comparison, we performed the same titration experiments with ERY, which forms a high-spin heme complex. With TNS-bound CYP3A4, ERY also causes a decrease in steady-state TNS fluorescence, with an apparent Kₐ of ~59 μM (Figure 8A). However, in marked contrast to the TST, ERY causes no decrease in the excited-state lifetime of TNS (Figure 8A,B). Thus, ERY must be competitively displacing TNS. In addition, during the optical titration experiment with ERY, a clear conversion from the TNS-induced type II spectrum (λₑₓₐ₅ = 425 nm, λₑₘᵟᵣₐᵣ = 398 nm) to a type I spectrum (λₑₓₐ₅ = 380 nm, λₑₘᵟᵣₐᵣ = 415 nm) is observed, even at low concentrations (<100 μM) of ERY, suggesting competitive displacement of TNS by ERY in the CYP3A4 active site (Figure 7B,C). These experiments provide “positive controls” for our experimental design and further demonstrate that the high-affinity TST does not compete with the TNS low-spin complex.

It is possible that TNS and each TST bind cooperatively at remote sites, affecting each other’s Kₐ values. In fact, the data suggest that there is some positive cooperativity, inasmuch as the Kₐ values for TST in the absence of TNS are 22 and 440 μM (13) versus 14 and 243 μM in the presence of TNS. In principle, the complete free energy couplings between TNS and each TST binding are available from a comparison of each TST Kₐ in the presence and absence of TNS, and from the TNS Kₐ in the presence of one or two TST molecules. However, two aspects of the experiments prevent a full analysis of the cooperativity. (1) As noted above, the titrations with TST and ERY were performed with a subsaturating TNS concentration, and (2) we do not know the quantum yield of TNS fluorescence in the presence of one TST versus two TSTs, or the relative quenching efficiencies of each TST. However, it is useful to estimate the concentration of each species present at equilibrium in the TST–TNS competition experiment based on the true Kₐ' values that are experimentally available from the current work or previous work (13), assuming no ternary complex formation, to emphasize the correlation between the different spectral effects observed and the species present in solution. Using the true Kₐ' values for TNS (25 μM) determined here in the absence of TST and the values for TST (22 and 440 μM) determined previously in the absence of TNS (13), simulations were performed to calculate the concentration of all species present at equilibrium assuming a simple competitive model (Scheme 2 and Table 3).

For the sake of completeness, the apparent Kₐ for ERY (59 μM), as determined by the displacement of TNS, was also used in the simulation. Previously described Kₐ values for binding of ERY to CYP3A4 have been reported to be somewhat lower (~52 μM) (32). The values in Table 3 indicate that 100 μM TST combined with a total of 5 μM CYP3A4 and 3 μM TNS is sufficient to decrease the concentration of the [CYP3A4-TNS] complex, with a type
II spectrum, from 0.45 μM in the absence of TST to 0.09 μM. Although 100 μM TST is sufficient for formation of only 0.73 μM high-spin, type I, [CYP3A4-TST-TST] complex, with a majority of 3.39 μM reverse type I [CYP3A4-TST] complex, this is accompanied by a decrease in [CYP3A4-TNS] by ~92% (from 1.46 to 0.09), in favor of [CYP3A4-TST], [CYP3A4-TST-TST], and [CYP3A4-TST-TNS]. Thus, although it is impossible to calculate exactly how large a spectral change might be expected upon addition of 100 μM TST as in Figure 7, it is very likely that some spectral change would have been observed if TST had displaced TNS. The complete absence of a spectral change under conditions that cause a clear change in fluorescence further suggests that such a competitive binding mechanism for TNS and TST is not operative. Note that similar calculations (Table 3) predict that the lowest concentration of ERY (25 μM) causes a decrease in [CYP3A4-TNS] of only ~25% and that is sufficient to cause an observable spectral change. This is consistent with a competitive ERY–TNS interaction.

A similar calculation for the noncompetitive case is not very informative for the reasons outlined above regarding the unavailability of true $K_d$ values. However, intuitively, the major species present would be [CYP3A4-TNS-TST], which forms with a relatively high affinity based on the apparent $K_d$ of 14 μM for TST. If, upon binding TST at a remote site, this species has a type II spectrum, then much less of the total species would be distributed to reverse type I or type I species, and a spectral change smaller than that for the competitive case is expected. Thus, the UV–vis spectral titrations do not prove a noncompetitive mechanism, but they are inconsistent with a competitive mechanism for TST and TNS. The UV–vis and fluorescence results are consistent, however.

**DISCUSSION**

CYP3A4 exhibits complex steady-state kinetics, in which substrate and effector concentration can influence the regioselectivity and kinetic profile of substrate oxidation (18, 33, 34). An understanding of the complex steady-state kinetics of CYP3A4 and other CYPs ultimately may require detailed knowledge of the spatial relationship between the binding sites for various ligands. For this reason, we have considered the utility of fluorescent probes in distinguishing discrete binding sites and monitoring changes in the environment of the active site when multiple ligands are bound simultaneously. Although many drugs fluoresce in aqueous solution, they are efficiently quenched upon binding to heme proteins and thus provide marginal utility as fluorescent probes (35, 36). On the other hand, model compounds with other spectral properties provide a possible strategy for understanding CYP ligand dynamics.

Historically, TNS and its congener ANS have been used as probes of protein–ligand dynamics for a number of proteins, including apomyoglobin, apohemoglobin, BSA, glutamine synthetase, α1-acid glycoprotein, and RUBISCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) (23, 24, 37–39). The primary utility of TNS and ANS lies in their tendency to exhibit only fluorescence when present in a highly hydrophobic environment, such as a lipid or the interior of proteins (23). Here, we have demonstrated that TNS is an excellent fluorescent probe of CYP3A4–ligand dynamics because its fluorescence is detectable only when bound to protein (Figure 4A). In addition, optical titration and kinetic inhibition studies strongly suggest its identity as a low-spin active site ligand for CYP3A4 (Figures 2 and 3). Although our data do not prove that TNS is bound in the active site, this is the most likely scenario on the basis of the numerous crystal structures of bacterial and human CYPs with ligands bound that cause type II spectral shifts, wherein the ligand forms a coordinate bond with the heme iron. There is no precedent of which we are aware in which a type II spectrum in CYP3A4 is reasonably assigned to a protein–iron coordinate bond. It is remotely possible, however, that TNS induces the spectral change and inhibition of catalytic activity from a remote binding site. If this were the case, then the data indicate that this site is distinct from the TST binding site and competitive with the ERY site. Therefore, this possibility seems unlikely. Regardless, a very clear conclusion based on these results is that the first TST is not competitive with the TNS binding, whereas ERY is.

To determine the effect that the binding of a known heterotropic effector might have on the TNS fluorescence signal, a titration with TST was conducted in both steady-state and fluorescence lifetime experiments (Figure 6). As seen in Figure 6C, both the average fluorescence lifetime of bound TNS and the magnitude of the steady-state fluorescence signal decrease at TST concentrations expected to populate the high-affinity site, but not the second low-affinity site. This is in stark contrast to the result obtained upon titrating ERY, a much larger CYP3A4 active site ligand that is not known to exhibit allosteric effects, under the same conditions. In fact, the steady-state fluorescence signal is quenched while the average fluorescence lifetime remains unchanged (Figure 8). These results demonstrate that high-affinity TST binding does not compete with TNS, which we propose is bound directly to the sixth axial heme position within the active site. The first equivalent of TST that binds

<table>
<thead>
<tr>
<th>predominant spin state</th>
<th>0 μM TST</th>
<th>20 μM TST</th>
<th>100 μM TST</th>
<th>25 μM ERY</th>
</tr>
</thead>
<tbody>
<tr>
<td>[CYP3A4] low-spin, reverse type I</td>
<td>4.56</td>
<td>2.59</td>
<td>0.79</td>
<td>3.32</td>
</tr>
<tr>
<td>[TNS]</td>
<td>2.55</td>
<td>2.73</td>
<td>2.92</td>
<td>2.66</td>
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<td>[CYP3A4-TNS] low-spin, type II</td>
<td>0.45</td>
<td>0.28</td>
<td>0.09</td>
<td>0.35</td>
</tr>
<tr>
<td>[CYP3A4-TST] low-spin, reverse type I</td>
<td>2.08</td>
<td>3.39</td>
<td></td>
<td></td>
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<tr>
<td>[CYP3A4-TST-TST] high-spin, type I</td>
<td>0.08</td>
<td>0.73</td>
<td></td>
<td></td>
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<tr>
<td>[CYP3A4-ERY] high-spin</td>
<td></td>
<td></td>
<td></td>
<td>1.32</td>
</tr>
<tr>
<td>[ERY]</td>
<td></td>
<td></td>
<td></td>
<td>23.7</td>
</tr>
</tbody>
</table>

*Based on 3 μM TNS total, 5 μM CYP3A4 total, a $K_d$ for TNS of 25 μM, a $K_d$ for TST of 22 μM, and a $K_d$ for TST of 440 μM in the absence of TNS (ref 13). Initial concentration of heterotropic species.
change involving one of the Trp residues may occur upon binding of TNS. Further studies explicitly focused on Trp fluorescence may clarify this observation.

For the TNS fluorescence, it is striking that a negative pre-exponential term is recovered for the TNS bound to CYP3A4 and that this negative pre-exponential term is eliminated upon addition of TST. As suggested in previous studies with TNS and ANS (25), the negative pre-exponential term reflects an excited-state reaction in which a state initially populated upon excitation is changed to a different species before relaxing to the ground state and emitting fluorescent light. In this case, the excited-state reactions causing this negative pre-exponential term could include solvent dipole reorganization, protein—dipeptide interactions, or movement of the TNS probe on the time scale of the excited state. Additional experiments are required to further distinguish between these mechanisms, but these data indicate that the active site dynamics of CYP3A4 include solvent, protein, or TNS reorganization on the same time scale as the TNS lifetime (nanoseconds). Most importantly, the data further suggest that TST bound at a distinct site alters these dynamics. The dipole reorganization (negative pre-exponential) observed in the absence of TST is not observed in its presence; the first equivalent of TST changes the rate at which solvent, protein residues, or TNS reorganizes within the active site. This could have implications for homotropic and heterotropic effects on absolute turnover rate, the extent of uncoupling to peroxides, and regioselectivity. For example, if solvent reorganization is responsible for the negative pre-exponential term in the TNS decay, then addition of TST with loss of the negative pre-exponential term could reflect a decreased level of hydration of the TNS, and a more “crowded” active site. In fact, this proposal is supported by the TST-induced blue shift in the spectrum of TNS, which directly indicates that TNS is in a more hydrophobic environment upon addition of TST. To our knowledge, no previous data have demonstrated a change in the dynamics of an active site CYP ligand and its environment upon occupancy of a distinct effector site. Additional fluorescence studies may further elucidate these possibilities.

In summary, we have established the utility of the solvent-quenched fluorescent probe TNS in directly monitoring protein—ligand dynamics in CYP3A4 through steady-state and time-resolved fluorescence spectroscopy. Both optical titration and metabolic inhibition demonstrate that TNS is a probable active site ligand. Recovered TNS fluorescence lifetimes in the presence of the heterotropic ligand TST suggest a peripheral allosteric binding site, consistent with previous crystallographic and thermodynamic results. Future studies utilizing this probe in conjunction with other allosteric effector—substrate combinations or protein cofactors may further elucidate the allosteric mechanisms in CYP3A4.

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REFERENCES


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