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Pyrene·Pyrene Complexes at the Active Site of Cytochrome P450 3A4: Evidence for a Multiple Substrate Binding Site

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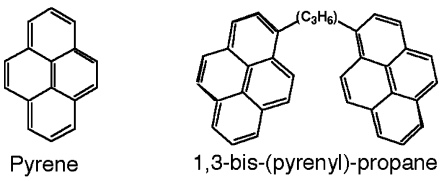
The cytochrome P450s (CYPs) catalyze the oxidative metabolism of nearly all drugs and toxins.[1] It has become appreciated recently that CYPs exhibit allosteric kinetics, including homotropic cooperativity.[2] The allosteric mechanism remains uncertain, but actively debated. Contrasting models include multiple substrate binding sites[2b,c,e] versus ligand-dependent conformational ensembles.[3] A possible mechanism includes binding of multiple substrates within a single, "fluid", active site, wherein each substrate has access to the enzymatic intermediate $[FeO]^{3+}$.[2b,c] Alternatively, individual substrates may bind within distinct, static, "subsites".[4] Here we provide the first direct spectroscopic evidence for multiple substrates within a single CYP active site, with formation of substrate·substrate complexes.

CYP3A4 hydroxylates pyrene (**1**) with positive cooperativity, requiring multiple substrate binding.[5] However, the kinetic results do not reveal the spatial relationship of bound substrates. Therefore, we exploited the "classic" fluorescence traits of **1**, wherein pyrene·pyrene complexes are nonfluorescent or yield excimer emission distinct from monomer emission.[6] In contrast to **1**, CYP3A4 did not metabolize 1,3-(bis)pyrenyl propane (**2**, data not shown).



UV−vis difference spectra of 6 μM CYP3A4 (Panvera, Inc.) in lipid-free buffer (20 mM K_2HPO_4/KH_2PO_4, pH7.4, 15% glycerol) revealed a **1**-induced conversion from low-spin (417 nm) to the high-spin CYP3A4 (391 nm) (Figure 1). This ensures that **1** binds at the active site under conditions of subsequent fluorescence experiments. Analogue **2** (1−20 μM) afforded neither a spin-state shift nor inhibition of testosterone metabolism, indicating that it does not access the CYP3A4 active site.

Fluorescence emission spectra of monomer **1** exhibit vibronic bands near 370−390 nm, whereas excimers yield a broad featureless band at 480 nm.[6] An emission spectrum corresponding to the excimer must be due to direct diffusional encounter between two **1** molecules, or excitation of a ground-state complex.[6] In the former case, excimer intensity depends on rates of diffusional encounter for an excited-state monomer and a ground-state monomer. In solution, free **1** will not be in equilibrium with CYP3A4-bound-**1** on the time scale of the photoexcited state (200 ns for pyrene in conditions used here).

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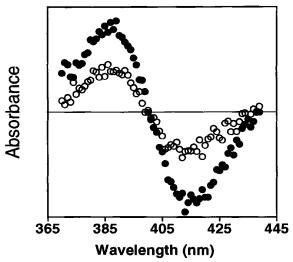

Figure 1. UV−vis difference spectra. High-spin ferric heme is produced upon addition of 2 μM **1** (○) or 8 μM **1** (●) to 6 μM CYP3A4. Full scale absorbance is −0.03 to 0.03. Only two concentrations are shown for clarity.

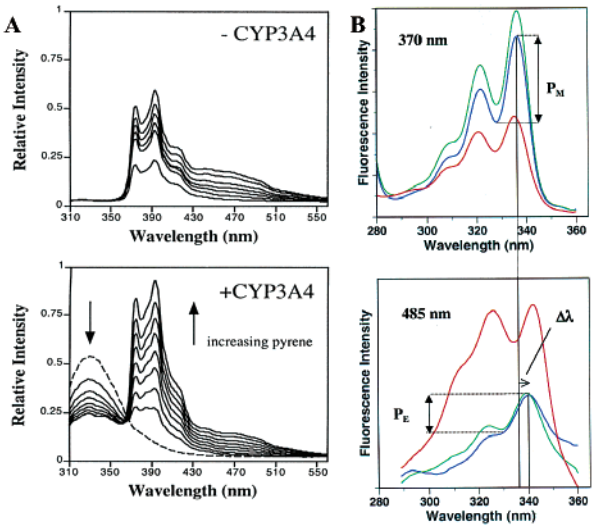

Figure 2. (A) Emission spectra of **1** in the absence (top) or presence (bottom) of CYP3A4 (exc 295 nm). The *E*/*M* ratio is decreased in the presence of CYP3A4. The dashed spectrum is Trp emission in the absence of **1**. (B) Excitation spectra of **1** (monomer, top or excimer, bottom) in the presence of CYP3A4 (red), Cyt b_5 (blue), or in the absence of protein (green). The relevant parameters, indicated for the blue spectra, are summarized in Table 1.

Therefore, two populations of excited fluorophores will be created, one complexed to the protein and one in bulk solution. Each may contain excimers and monomers. Regardless of this complexity, which precludes quantitation of each state, the excitation spectra of **1** demonstrate the presence of ground-state complexes bound to CYP3A4 (vide infra).

Interaction of **1** with CYP3A4 is readily apparent from the saturable decrease in intrinsic Trp fluorescence, centered at 330 nm (exc 295 nm, Figure 2A). Also, the ratio of excimer intensity (485 nm) to monomer intensity (372 nm) (*E*/*M*) is obviously

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Table 1. Effect of CYP3A4 on Pyrene Fluorescence Excitation Spectral Parameters

	parameter value		
system	$P_M - P_E$ peak-to-valley ratio	$\Delta\lambda$ (nm)	$I_{(345)}/I_{(325)}$ or $I_{(356)}/I_{338)}$ peak-to-peak ratio
1	0.23	2−3	1.74
1 + Cyt b_5	0.20	2−3	1.59
1 + CYP3A4	0.29	5	1.03
1 + CYP3A4 + testosterone	0.24	3	1.31
1 + testosterone	0.24	2−3	1.67
2	NA[a]	NA	1.18
2 + CYP3A4	NA	NA	1.12

[a] NA = not applicable. Because no monomer emission is observed with the bis-(pyrenyl)propane the $P_M - P_E$ and $\Delta\lambda$ parameters cannot be determined.

decreased in the presence of CYP3A4. In contrast, titration of Cyt *c*- or Cyt b_5-containing solutions with **1** (not shown) does *not* result in a decreased *E*/*M*. The emission of **2**, at 1−10 μM, exhibited only excimer, consistent with the increased tendency to form intramolecular complexes.

The decreased *E*/*M* of **1** could be due to a CYP-dependent sequestration of monomers within spatially distinct binding sites. However, the excitation spectra (Figure 2B) indicate an alternative based on ground state differences. Ground-state complexes of **1** yield a red-shifted absorbance with altered vibronic structure.[6c,d,h] In contrast, excimers arising from diffusional encounter yield excitation spectra that are identical to the monomer.

The excimer excitation spectra (Figure 2B, bottom) of free **1**, or with Cyt b_5, reveal obvious differences, compared to the monomer excitation spectra (Figure 2B, top). A parameter that is frequently used to identify preformed ground-state complexes is $\Delta\lambda = \lambda_{excimer} - \lambda_{monomer}$, the wavelength shift of the (0,0) transition of excitation spectrum for monomer versus excimer (Figure 2B).[6h] For excimers arising only from diffusional collision of monomers, $\Delta\lambda = 0$. In the absence of CYP3A4, $\Delta\lambda$ is 2−3 nm (Table 1), indicating ground-state complexes of free **1** in the glycerol/aqueous system. In marked contrast, $\Delta\lambda$ increased to 5 nm in the presence of CYP3A4, demonstrating that the environment or the ground-state geometry is different. Addition of the CYP3A4 substrate testosterone (100 μM) caused $\Delta\lambda$ to decrease to 3 nm, presumably due to displacement of **1**; testosterone had no effect on $\Delta\lambda$ in the absence of CYP3A4.

As a further probe of spectral perturbation due to CYP3A4, the ratio of peak-to-valley intensities for the long wavelength excitation band ((0,0) transition) and the intensity of the nearest minimum for the monomer ($IM_{peak}/IM_{valley} = P_M$) and excimer ($IE_{peak}/IE_{valley} = P_E$) are compared (Figure 2B). For ground-state complexes, $P_E < P_M$, and the difference ($P_M - P_E$) reflects extent of ground-state complex formation.[6h] Here, $P_E < P_M$ with or without CYP3A4. However, CYP3A4 markedly increases the difference (Table 1).

In addition, the vibronic structure of the excitation spectrum in the presence of CYP3A4 is different from the vibronic structure in all of the other samples, as determined by the intensity ratio of the two major excitation peaks at ∼320−323 nm and ∼335−340 nm. This ratio has been used as probe of solvent environment, as it changes with polarity.[6h] For the CYP3A4-bound excimers, the ratio of intensity at 343 nm to 327 nm is 1.03, whereas the ratio of peak intensity at 340 nm to intensity at ∼320−325 nm is 1.74 for excimers in the absence of protein or the presence of Cyt b_5 (Table 1). This further demonstrates that ground-state complexes in the presence of CYP3A4 experience a different environment than in the absence of protein, or presence of other heme proteins.

Although the spectra do not reveal the detailed structure of [excimer·CYP3A4], pyrene·pyrene ground state complexes must contribute to the fluorescence, and they are spectroscopically distinguishable from those without CYP3A4. Presumably, the CYP3A4-dependent decrease in *E*/*M* ratio of the emission spectra reflects the tendency of some ground-state complexes to be nonfluorescent.[6d,e,h] The combination of **1**/CYP3A4, uniquely yields these spectral changes. Moreover, the lack of spectral perturbation of **2** by CYP3A4 correlates with its inability to access the active site. Regardless of the structure of excimeric **1**, the results must arise from the presence of ground-state excimers bound to CYP3A4.

The inaccessibility of **2** to the active site suggests either it has low affinity relative to its low solubility, or that a restricted access channel allows individual monomers (**1**) to bind sequentially, but larger complexes are "filtered". Most importantly, the results indicate that ligands bound simultaneously within a human CYP active site may interact directly with one another as a complex, with functional implications. Possibly the "substrate" oxidized by $[FeO]^{3+}$ is a complex, rather than an individual monomer. Upon $\pi-\pi$ stacking, many planar aromatic compounds are more easily oxidized. If presented to $[FeO]^{3+}$, a [pyrene·pyrene] complex would be a "good" substrate, wherein the rate, regioselectivity, and stereoselectivity of oxidation would be *determined by the steric and electronic features of the complex, rather than the individual ligands.*

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