

## Fluorescent Probes for Cytochrome P450 Structural Characterization and Inhibitor Screening

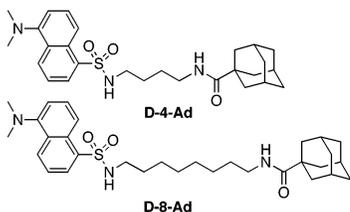
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Substrate-specific cytochromes P450 play major roles in steroid and eicosanoid biosynthesis and thus constitute important drug design targets.<sup>2</sup> In contrast, P450 isozymes expressed in the liver take part in the metabolism of nearly all drugs.<sup>3</sup> Adverse drug reactions, for instance to Prozac,<sup>4</sup> result from individual variations in hepatic P450s.<sup>5</sup> It is thus important to predict which P450s interact with a potential drug candidate and to understand the nature of these interactions.

We have developed fluorescent probe molecules for P450cam (the prototypical P450) that consist of an  $\alpha,\omega$ -diaminoalkane chain connecting a dansyl fluorophore to the P450cam substrate adamantane:

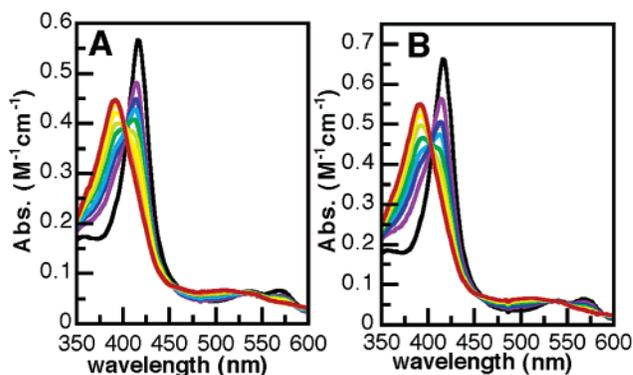


A shift in Soret absorption (Figure 1) and greatly diminished dansyl luminescence attributable to Förster energy transfer to the heme<sup>6</sup> (Figure 2) accompany probe binding. When D-4-Ad is displaced from the active site by camphor, fluorescence is restored (Figure 2a).<sup>7</sup> Because a bright signal stands out against a dark background, substrate or inhibitor binding is readily detected. This assay, which is both simple and sensitive, can be employed to screen combinatorial chemical libraries.<sup>8</sup>

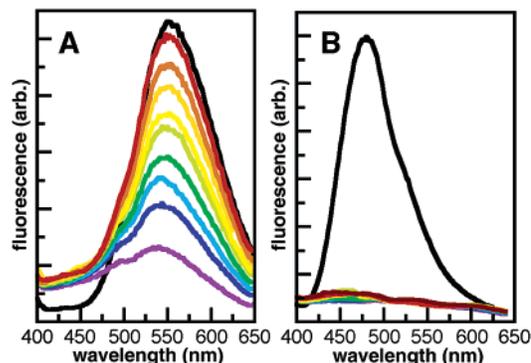
Both fluorescence and absorption spectra show that D-4-Ad binding to P450cam is competitive with camphor. The Soret shift (416–414 nm) induced by D-4-Ad indicates that it binds in the active site. With a  $K_d$  of 0.83  $\mu\text{M}$ , D-4-Ad binds twice as strongly as the natural substrate.<sup>9</sup> D-8-Ad also induces a shift in the Soret maximum from 416 to 414 nm: from the integrated D-8-Ad fluorescence in the presence and in the absence of P450cam, we estimate an upper limit  $K_d \approx 0.02 \mu\text{M}$  for this probe.<sup>10</sup>

The crystal structure of the P450cam:D-8-Ad complex shows that the probe binds in the same channel as  $\text{Ru}^{\text{II}}(\text{bpy})_3\text{-linker-Ad}$  (bpy = 2,2'-bipyridine) analogues (Figure 3).<sup>11</sup> The eight-carbon chain is nearly fully extended, allowing the dansyl moiety to bind at the surface of the protein. The good fit is attributable to conformational flexibility; that is, the F and G helices open just enough to allow the probe to enter and bind. The observed conformation is midway between the “closed” (camphor)<sup>12</sup> and “open” (Ru-linker-Ad)<sup>11d</sup> structures.

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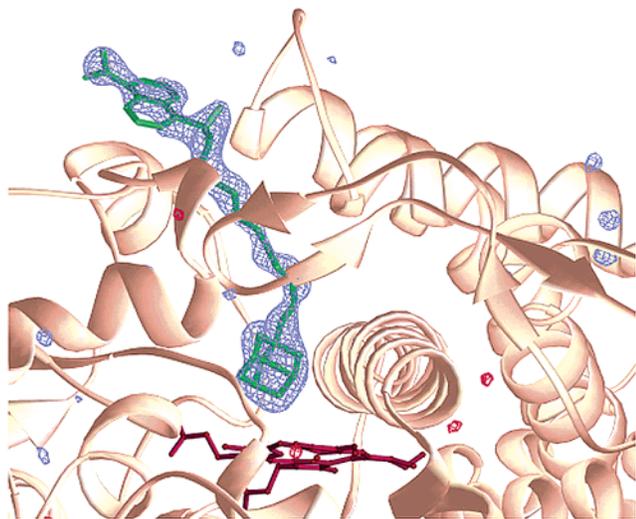
**Figure 1.** (A) Absorption spectra showing the binding of camphor to P450cam (4.9  $\mu\text{M}$ ) in the presence of 1 equiv of D-4-Ad. The initial addition of D-4-Ad to P450cam results in a shift in the Soret absorption from 416 to 414 nm. A fit of the data to a competitive binding model gives a dissociation constant of 0.83  $\mu\text{M}$ . (B) The camphor-induced shift from low- to high-spin P450cam (5.7  $\mu\text{M}$ ) in the presence of 1 equiv of D-8-Ad. Black, P450cam; purple, P450cam + 1 equiv of dansyl probe; blue to red, 0.5, 1, 2, 4, 8, 16, 32, and 64 equiv of camphor.



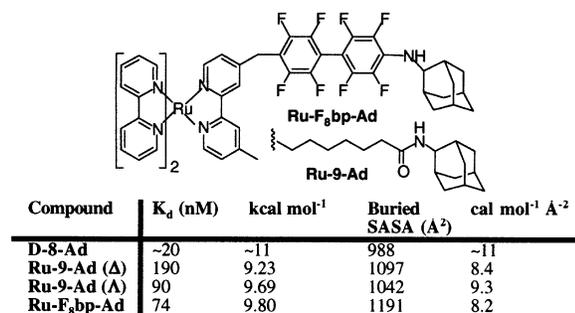
**Figure 2.** Fluorescence spectra of D-4-Ad (A) and D-8-Ad (B). Black, 2  $\mu\text{M}$  D-8-Ad or D-4-Ad; purple, 2  $\mu\text{M}$  dansyl probe + 1 equiv of P450cam; blue to red, 2  $\mu\text{M}$  P450cam and dansyl probe + 0.5, 1, 2, 4, 8, 16, 32, and 64 equiv of camphor ( $K_d = 1.6 \mu\text{M}$ ).<sup>9</sup>

The structure reveals a hydrogen bond between the amide carbonyl of the probe and Tyr96 in P450cam:D-8-Ad, mimicking the hydrogen bond between camphor and Tyr96 in the P450cam: substrate complex.<sup>12</sup> In addition, there are a great many hydrophobic interactions between the probe molecule and the enzyme; analysis of these contacts shows that much of the solvent-accessible surface area is buried. The estimated  $K_d$  of 0.02  $\mu\text{M}$  corresponds to a binding energy of  $\sim 11$  kcal/mol, or  $\sim 11$  cal/(mol  $\text{\AA}^2$ ) (Figure 4).

The  $\text{Ru}(\text{bpy})_3^{2+}$  analogues (Ru-9-Ad and Ru-F<sub>8</sub>bp-Ad) do not bind as tightly to P450cam, but the free energy changes per buried



**Figure 3.** The 2.2 Å resolution structure of the D-8-Ad:P450cam cocrystal, with the omit electron density ( $|F_{\text{obs}}| - |F_{\text{calc}}|$ ) contoured at 4.0  $\sigma$  (blue positive, red negative).



**Figure 4.** (Top) Ruthenium tris-bipyridyl photosensitizers known to bind P450cam. The crystal structures of both compounds bound to P450cam have been determined to high resolution (Ru-9-Ad 1.55 Å, Ru-F<sub>8</sub>bp-Ad 1.65 Å).<sup>10b,d</sup> (Bottom) Dissociation constants, binding energies, buried solvent accessible surface areas (SASA), and the binding energy per square angstrom of buried surface area for the P450cam:probe complexes. The Ru-9-Ad:P450cam crystal contains both  $\Delta$  and  $\Lambda$  stereoisomers.

surface area are comparable. Thus, even though P450cam has evolved for a single, relatively small substrate, it has the ability to bind much larger molecules more tightly. The key to this ability is the mobility of the B', F, and G helices.<sup>11d</sup> Both solution<sup>13</sup> and crystallographic<sup>14</sup> studies of other P450s suggest that this feature is common to the P450 superfamily.

The two probes described herein illustrate the usefulness of our methodology. D-4-Ad can be employed to screen potential P450 inhibitors, as it is easily displaced by other molecules with comparable or lower dissociation constants. In contrast, D-8-Ad binds extremely tightly: the conformational flexibility of the P450 fold allows the enzyme to close around the probe, thereby making a great many productive hydrophobic contacts. The insight gained from the D-8-Ad:P450cam structure could potentially lead to a more rational design strategy for P450 inhibitors.

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**Supporting Information Available:** Syntheses of compounds; spectroscopic data; and crystallographic experimental details (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (7) The fluorescence of D-8-Ad ( $\lambda_{\text{max}} = 480$  nm) is blue shifted from that of D-4-Ad (550 nm), indicating that the D-8-Ad environment is less polar [Li, Y.-H.; Chan, L.-M.; Tyer, L.; Moody, R. T.; Himel, C. M.; Hercules, D. M. *J. Am. Chem. Soc.* **1975**, *97*, 3118–3126]. One possible explanation is that the hydrophobic tail of D-8-Ad folds back in solution to partially cover the dansyl fluorophore, effectively lowering the local dielectric. Although the fluorescence maximum is not concentration dependent (data not shown), D-8-Ad aggregation cannot be ruled out.
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- (10) As with D-4-Ad, titration of a 1:1 mixture of P450cam and D-8-Ad with camphor results in conversion to high-spin ferric P450cam (Figure 1b). However, the fluorescence spectra indicate that D-8-Ad is not displaced from the enzyme by even a large excess of camphor (250  $\mu\text{M}$ ; see also Figure 2b). These data are consistent with simultaneous binding of camphor and D-8-Ad. Simultaneous binding of camphor and ruthenium tris-bipyridyl probes has been previously observed [Dmochowski, I. J. *Probing cytochrome P450 with sensitizer-linked substrates*; California Institute of Technology: Pasadena, CA, 2000].
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