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Fluorescent Probes for Cytochrome P450 Structural Characterization and Inhibitor Screening

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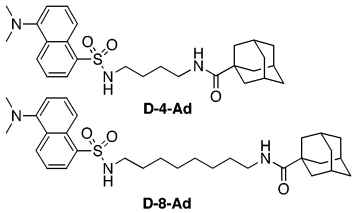
Substrate-specific cytochromes P450 play major roles in steroid and eicosanoid biosynthesis and thus constitute important drug design targets.[2] In contrast, P450 isozymes expressed in the liver take part in the metabolism of nearly all drugs.[3] Adverse drug reactions, for instance to Prozac,[4] result from individual variations in hepatic P450s.[5] 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 α,ω-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[6] (Figure 2) accompany probe binding. When D-4-Ad is displaced from the active site by camphor, fluorescence is restored (Figure 2a).[7] 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.[8]

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 μM, D-4-Ad binds twice as strongly as the natural substrate.[9] 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 $K_d \approx 0.02$ μM for this probe.[10]

The crystal structure of the P450cam:D-8-Ad complex shows that the probe binds in the same channel as $Ru^{II}(bpy)_3$−linker−Ad (bpy = 2,2′-bipyridine) analogues (Figure 3).[11] 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)[12] and "open" (Ru-linker-Ad)[11d] structures.

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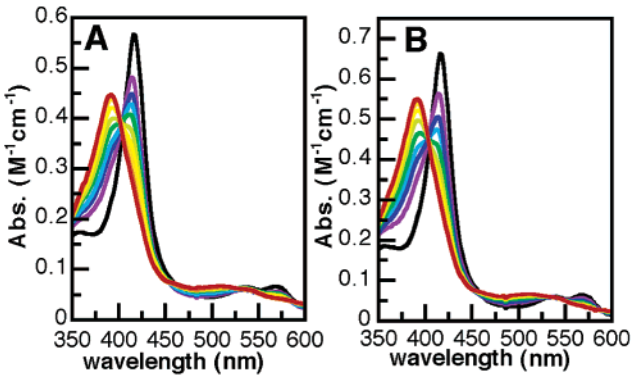

Figure 1. (A) Absorption spectra showing the binding of camphor to P450cam (4.9 μM) in the presence of 1 equiv of D-4-Ad. The initial addition of D-4-Ad to P450cam results in a shift in the Soret absorption from 416 to 414 nm. A fit of the data to a competitive binding model gives a dissociation constant of 0.83 μM. (B) The camphor-induced shift from low- to high-spin P450cam (5.7 μM) 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.

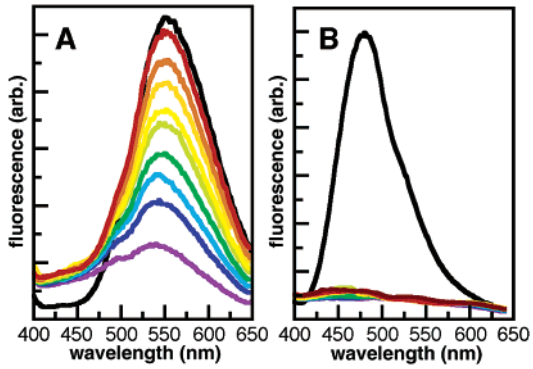

Figure 2. Fluorescence spectra of D-4-Ad (A) and D-8-Ad (B). Black, 2 μM D-8-Ad or D-4-Ad; purple, 2 μM dansyl probe + 1 equiv of P450cam; blue to red, 2 μM P450cam and dansyl probe + 0.5, 1, 2, 4, 8, 16, 32, and 64 equiv of camphor (K_d = 1.6 μM).[9]

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.[12] 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 μM corresponds to a binding energy of ∼11 kcal/mol, or ∼11 cal/(mol Å^2) (Figure 4).

The $Ru(bpy)_3^{2+}$ analogues (Ru-9-Ad and Ru-F_8bp-Ad) do not bind as tightly to P450cam, but the free energy changes per buried

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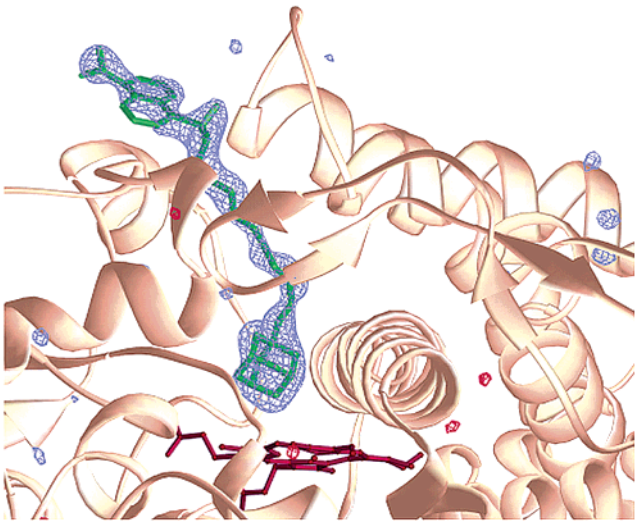
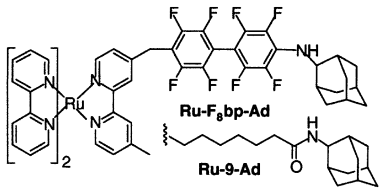


Figure 3. The 2.2 Å resolution structure of the D-8-Ad:P450cam cocrystal, with the omit electron density ($|F_{obs}| - |F_{calc}|$) contoured at 4.0 σ (blue positive, red negative).



Compound	K_d (nM)	kcal mol^{-1}	Buried SASA (Å^2)	cal mol^{-1} Å^{-2}
D-8-Ad	~20	~11	988	~11
Ru-9-Ad (Δ)	190	9.23	1097	8.4
Ru-9-Ad (Λ)	90	9.69	1042	9.3
Ru-F$_8$bp-Ad	74	9.80	1191	8.2

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$_8$bp-Ad 1.65 Å).[10b,d] (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 Δ and Λ 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.[11d] Both solution[13] and crystallographic[14] 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 (λ_{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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