

# Enantiomeric discrimination of Ru-substrates by cytochrome P450<sub>cam</sub>

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Received 7 February 2000; received in revised form 6 June 2000; accepted 12 June 2000

## Abstract

Molecules with photosensitizers attached to substrates (Wilker et al., *Angew. Chem. Int. Ed.* 38 (1999) 90–92) or cofactors (Hamachi et al., *J. Am. Chem. Soc.* 121 (1999) 5500–5506) can rapidly deliver redox equivalents to buried active sites. The structure of cytochrome P450<sub>cam</sub> (P450) co-crystallized with a prototypal sensitizer-substrate, [Ru-C<sub>9</sub>-Ad]Cl<sub>2</sub>, has been determined (Dmochowski et al., *Proc. Natl. Acad. Sci. USA* 96 (1999) 12987–12990); and, in separate UV–vis absorption and time-resolved luminescence experiments, the binding of the  $\Lambda$  and  $\Delta$  enantiomers of Ru-C<sub>9</sub>-Ad to P450 has been measured. The results,  $K_D(\Delta/\Lambda)\sim 2$ , indicate that the bipyridyl ligands of the  $\Lambda$  isomer interact more favorably with hydrophobic residues at the entrance to the substrate channel. We conclude that enantiospecific interactions may be exploited in the design of enzyme-metallosubstrate conjugates. © 2000 Elsevier Science S.A. All rights reserved.

**Keywords:** Cytochrome P450; Ruthenium; Chiral resolution; Sensitizer-linked substrates

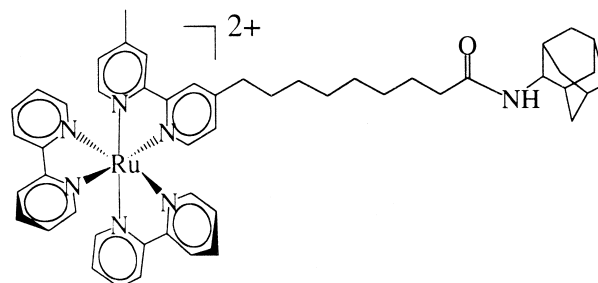
## 1. Introduction

Much attention is being given to the design, synthesis, and employment of molecular probes of enzyme structure and function [1–11], owing in part to the abundance of naturally occurring cavity proteins [12–15] and in part to the power of site-directed mutagenesis to modify existing cavities and create new substrate binding sites [16–20]. Especially appealing targets are the cytochromes P450 (an important family of monooxygenases involved in drug metabolism and steroid biosynthesis) because they possess hydrophobic pockets capable of sequestering a wide variety of substrates [21].

We have found that substrates and ligands attached via an alkyl chain to the inorganic photosensitizer [Ru(bpy)<sub>3</sub>]<sup>2+</sup> (where bpy is 2,2'-bipyridine) bind P450 reversibly [1] with high affinity ( $K_D\sim 1\ \mu\text{M}$ ) and specificity [3]. The substrate [Ru-C<sub>9</sub>-Ad]Cl<sub>2</sub> (Scheme 1) was recently crystallized with P450 and the X-ray structure determined to 1.55 Å (PDB code, 1qmq; Fig. 1) [3]. Electron density (data not shown) from the ruthenium and bipyridyl ligands appears in multiple positions near the substrate channel, thereby indicating either considerable mobility of the

[Ru(bpy)<sub>3</sub>]<sup>2+</sup> moiety or the existence of stable enzyme-Ru conjugates that could correspond to specific interactions of  $\Lambda$  and  $\Delta$  enantiomers with the protein surface. High thermal factors for the [Ru(bpy)<sub>3</sub>]<sup>2+</sup> moiety in the crystal structure prevented unambiguous assignment of either isomer. The inherent chirality of both P450 and [Ru(bpy)<sub>3</sub>]<sup>2+</sup> raises the possibility that hydrophobic interactions with aromatic residues at the channel entrance favor the binding of one isomer relative to the other. We have probed this potential enantioselectivity by resolving the  $\Lambda$  and  $\Delta$  [Ru-C<sub>9</sub>-Ad]Cl<sub>2</sub> isomers and comparing their affinities for P450.

Chromatographic techniques using SP Sephadex C-25 with chiral eluents have been developed for the separation of many enantiomeric ruthenium polypyridyl complexes [22–25]. The Sephadex ion-exchange matrix itself is



Scheme 1. Sensitizer-linked substrate, Ru-C<sub>9</sub>-Ad.

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chiral, since it is made of dextran, a 3-dimensional network of cross-linked D-glucose units. Interestingly, the ability of dextran to achieve chiral resolutions of  $d^6$  ( $\text{Re}^I$ ,  $\text{Ru}^{II}$ ,  $\text{Os}^{II}$ ,  $\text{Co}^{III}$ ,  $\text{Rh}^{III}$ ) polypyridyl compounds is greatly enhanced by the addition of tartrate salts [26]. X-ray structures of these metal complexes crystallized with aromatic tartrate counterions (i.e., (+)-*O,O'*-di-4-toluoyl-D-tartrate) show well-ordered stacking interactions between the ditoluoyl and phenanthroline groups [24]. Well-defined structures incorporating a variety of organic salts also have been observed in solution by  $^1\text{H}$  NMR [22]. Aromatic stacking has been implicated as a major factor in the mechanism of stereoisomer separation with these eluents [24], and provides a mechanism for chiral discrimination by the enzyme. In our work, sodium (–)-*O,O'*-dibenzoyl-L-tartrate was chosen for the isolation of the ( $\pm$ )-[Ru- $\text{C}_9$ -Ad] $\text{Cl}_2$  isomers because it most efficiently resolves the parent compound,  $[\text{Ru}(\text{bpy})_2(\text{Me}_2\text{bpy})]\text{Cl}_2$  ( $\text{Me}_2\text{bpy}$  is 4,4'-dimethyl-2,2'-bipyridine) [27].

Time-resolved luminescence measurements precisely quantify the binding of Ru-substrates to P450 [1,3]. Laser excitation of the Ru-protein solutions yields biphasic luminescence kinetics. The faster quenching process ( $k = 4\text{--}14 \times 10^6 \text{ s}^{-1}$ , depending on substrate and chain length) has been identified as Förster energy transfer from  $\text{Ru}^{2+*}$  to the heme [3]. The slower luminescence decay process ( $\tau \sim 500 \text{ ns}$ ) is the same as that of  $\text{Ru}^{2+*}$  in deoxygenated solution. Thus, dissociation constants can be calculated from the quenched fraction of  $[\text{Ru-substrate}]^{2+*}$  luminescence. Traditional P450 substrate-binding assays rely on monitoring the low-to-high-spin shift (417→392 nm) associated with water loss from a ferric-aquo heme. Time-resolved emission profiles much more reliably assess the affinity of substrates (e.g., ( $\pm$ )-[Ru- $\text{C}_9$ -Ad] $\text{Cl}_2$ ) that displace little water from the channel and only slightly perturb the spin state of the heme.

## 2. Materials and methods

### 2.1. Protein preparation

Cytochrome P450<sub>cam</sub> was overexpressed in *E. coli* TBY cells from plasmid pUS200 [28] and purified in the presence of camphor with slight modification to standard procedures [29].

### 2.2. Synthesis of [Ru- $\text{C}_9$ -Ad] $\text{Cl}_2$

#### 2.2.1. General procedures

All manipulations were conducted under an argon atmosphere using standard Schlenk techniques. Solvents used for synthesis were dried, degassed and distilled according to standard procedures [30,31]. Reactions were

performed at room temperature unless otherwise stated. NMR spectra were recorded on a General Electric QE300.

#### 2.2.2. *bpy-C<sub>9</sub>-Ad*

Thionyl chloride (24.50 g, 206 mmol) and 8-bromooctanoic acid (5.46 g, 24.5 mmol) were combined and refluxed for 1.5 h. Excess  $\text{SOCl}_2$  was removed by vacuum to yield a brown liquid that was dissolved in ether (20 ml) and transferred to an addition funnel. The acid chloride was added over 20 min to an ether (20 ml) solution of 2-adamantanamine hydrochloride (11.97 g, 63.8 mmol) and triethylamine (22.50 g, 222 mmol) chilled in an ice bath. The resulting slurry was stirred at  $0^\circ\text{C}$  for 3 h and then overnight at room temperature. The reaction solution was added to water (75 ml) and extracted with ether (75 ml) in a separatory funnel. After washing the organic layer with 0.1 M HCl (3×75 ml), water (2×75 ml), and saturated brine (2×75 ml), the solution was dried over  $\text{MgSO}_4$  and solvent removed by rotary evaporation. The off-white solid was used directly without purification for attachment to  $\text{Me}_2\text{bpy}$ .

Diisopropylamine (8.09 g, 79.9 mmol), *n*-butyl lithium (80 mmol in hexanes), and cold THF (25 ml) were combined in a 500 ml Schlenk flask at  $0^\circ\text{C}$ . A cold solution of 4,4'-dimethyl-2,2'-bipyridine (6.41 g, 34.8 mmol) in THF (180 ml) was added by cannula over 15 min, and was stirred for an additional 15 min. The amide was dissolved in THF (120 ml) and cannulated dropwise into the bipyridine, turning the solution from burgundy to black. After 3 h on an ice bath, the reaction was allowed to proceed overnight at room temperature. The reaction solution was transferred to a separatory funnel with water (250 ml) and extracted with ether (150 ml). The organic layer was washed with saturated  $\text{NaHCO}_3$  (2×125 ml), water (3×300 ml), and saturated brine (2×200 ml). After drying with  $\text{MgSO}_4$  and vacuum, a beige solid was obtained. The product was eluted as the second band by silica gel column chromatography (3:2 ethyl acetate/hexanes). Yield was 3.40 g (30.2% based on 8-bromooctanoic acid) of a pale yellow oil.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1–2 (m/s), 2.20 (t,  $\text{CH}_2$ -amide), 2.42 (s,  $\text{bpy-CH}_3$ ), 2.59 (t,  $\text{bpy-CH}_2$ ), 4.09 (m), 5.79 (m), 7.21 (d,  $\text{bpy}$  5 and 5'), 8.23 (s,  $\text{bpy}$  3 and 3'), 8.58 (d,  $\text{bpy}$  6 and 6').

#### 2.2.3. $[\text{Ru}(\text{bpy})_2(\text{bpy-C}_9\text{-Ad})]\text{Cl}_2$

The ligand *bpy-C<sub>9</sub>-Ad* (505 mg, 1.10 mmol) and *cis*-[Ru( $\text{bpy}$ ) $_2\text{Cl}_2$ ] (538.6 mg, 1.04 mmol) were combined with 5:1 water/ethanol (18 ml) and refluxed for 12 h. Solvent was removed under vacuum and the dark red solid was dissolved in water (60 ml). This aqueous solution was combined with a solution of  $\text{NH}_4\text{PF}_6$  (1.20 g, 7.36 mmol) in water (20 ml) to yield an orange precipitate. The aqueous slurry was extracted with  $\text{CH}_2\text{Cl}_2$  (75 ml); the organic layer was washed with 1 M HCl (2×50 ml), 1 M NaOH (2×50 ml), and water (2×75 ml) prior to rotary evaporation. The  $\text{PF}_6^-$  salt of this ruthenium complex was

purified by silica gel flash chromatography (column dimensions 30×4.5 cm) employing an eluent of 3% methanol in CH<sub>2</sub>Cl<sub>2</sub>. Pure product PF<sub>6</sub><sup>-</sup> salt was found in elution volumes 550–1300 ml. Further product could be obtained by running a second column on the initial 200–550 ml. Volumes 550–1300 ml were combined and dried by rotary evaporation. In order to metathesize the ruthenium complex to the Cl<sup>-</sup> salt, the purified PF<sub>6</sub><sup>-</sup> salt was dissolved in MeOH (10 ml) and loaded onto a CM Sepharose cation-exchange column (2×13 cm). The column was washed with water (600 ml) and 25 mM NaCl (600 ml). The ruthenium complex was then eluted with 500 mM NaCl (300 ml) and dried by vacuum. The desired [Ru(bpy)<sub>2</sub>(bpy-C<sub>9</sub>-Ad)]Cl<sub>2</sub> was isolated from the NaCl-containing solid by repeated washings with CH<sub>2</sub>Cl<sub>2</sub>, followed by filtering and drying under vacuum. Yield of the dark red solid was 195 mg (20.0%). Yields of this procedure are generally 20–30%, and approach 60% with repeated column chromatography on the crude reaction mixture. <sup>1</sup>H NMR (CD<sub>2</sub>Cl<sub>2</sub>): δ 0.8–2 (m's), 2.21 (t, CH<sub>2</sub>-amide), 2.65 (s, bpy'-CH<sub>3</sub>), 2.78 (t, bpy'-CH<sub>2</sub>), 3.62 (m), 3.95 (m), 6.32 (m), 7.23 (m), 7.45 (m), 7.70 (m), 8.18 (m), 8.77 (s), 8.80 (s), 9.20 (m). LRMS (electrospray, positive ion) calcd for C<sub>50</sub>H<sub>57</sub>N<sub>7</sub>ORu (M+H<sup>+</sup>) *m/z* 874, found 874. UV-vis [λ (Δε), H<sub>2</sub>O]: 206 nm (74,200), 244 (26,000), 286 (80,100), 454 (14,500).

### 2.3. Chiral resolution of (±)-[Ru-C<sub>9</sub>-Ad]Cl<sub>2</sub>

Circular dichroism (CD) spectra were measured on samples dissolved in acetonitrile (50–100 μM) using an Aviv Model 62A DS spectropolarimeter. Chiral separation was achieved by cation-exchange chromatography (SP Sephadex C-25, Fluka) using 50 mM sodium (-)-*O,O'*-dibenzoyl-L-tartrate as the eluent. The aqueous tartrate solution was prepared by neutralization of the acid with two equivalents of NaOH, followed by filtration to remove insoluble impurities. Racemic [Ru-C<sub>9</sub>-Ad]Cl<sub>2</sub> (4 mg) was loaded onto a column (dimensions 120×3.5 cm) covered with aluminum foil to eliminate the possibility of photo-racemization. Eluent flow was regulated (~1 ml/min) with a peristaltic pump. The resolution of two bands occurred after traversing an effective column length (ECL) of 2 m. Upon separation, the Sephadex was expelled from the column with air, and the first and second bands were collected and soaked in acetonitrile to remove Ru from the dextran. The red solutions were rotary evaporated at room temperature, redissolved in water, and metathesized by ion-exchange to their chloride salts. Band 1 (the first eluted fraction) had a negative rotation and was assigned the Δ absolute configuration based on the CD characteristics of similar complexes [24]. CD [λ (Δε), CH<sub>3</sub>CN]: Δ(-): 227 nm (+26), 240 (+23), 260 (-10), 278 (+134), 294 (+307), 325 (+18), 365 (+11), 424 (+19), 476 (-15); Λ(+): 227 (-27), 240 (-23), 260 (+7), 278 (-126), 294 (+281), 325 (-17), 365 (-11), 424 (-19), 476 (+13).

### 2.4. K<sub>I</sub> determination

A Hewlett-Packard 8452A spectrophotometer was used to collect UV-vis data. Buffer conditions were 50 mM potassium phosphate, 100 mM potassium chloride, pH 7.4 for all protein solutions (~5 μM P450). UV-vis titrations were performed at 20°C with stirring (500 rpm) using a Hewlett-Packard 89090A stirrer/temperature controller. Λ and Δ-[Ru-C<sub>9</sub>-Ad]Cl<sub>2</sub> displace little water from the ferric-aquo heme and binding results in only 30% conversion to the high-spin species, due, presumably, to the abundance of water in Ru-bound (open) structure. Thus, affinities were determined by the ability of these complexes to inhibit the low- to high-spin transition produced by camphor. Concentrated ethanolic stock solutions of camphor titrated in small aliquots (0.5–1.0 μl) into the protein solutions gave the desired range of camphor concentrations (250 nM–2 mM). The concentration of ethanol never exceeded 1% of the total volume. Apparent dissociation constants of camphor, K<sub>S</sub>, were spectroscopically determined at three concentrations (0–20 μM, 99% bound) of both Ru-C<sub>9</sub>-Ad isomers. K<sub>S</sub> was calculated by fitting the data to 1/ΔA vs. 1/[S], the slope of which yields K<sub>S</sub>/([E]Δε) from the relationship 1/ΔA = ((K<sub>S</sub>/[S]) + 1)/([E]Δε), where ΔA is the absorbance change from the initial value, [S] is the concentration of camphor, [E] is the concentration of P450, and Δε is the difference in molar absorptivity between [Ru-C<sub>9</sub>-Ad]- and camphor-bound P450. Absorbance changes were recorded at 392 and 416 nm. Values of K<sub>I</sub>, the equilibrium constant between Ru-bound and camphor-bound P450, were determined for both isomers by plotting K<sub>S</sub> against the Ru-C<sub>9</sub>-Ad concentration. The dissociation constants, K<sub>D</sub>, of (±)-[Ru-C<sub>9</sub>-Ad]Cl<sub>2</sub> were calculated based on a single-substrate binding model (Fig. 2).

### 2.5. K<sub>D</sub> determination

Emission experiments were conducted under similar conditions (20°C, buffered solutions, 5 μM in both Ru-

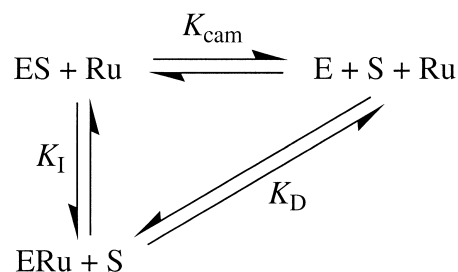


Fig. 2. Binding of a single substrate to P450: by definition,  $K_D = K_{\text{cam}}/K_I$ , where  $K_{\text{cam}}$  is the dissociation constant of camphor (in the absence of Ru-substrate), and  $K_I$  is the equilibrium constant between camphor- and ruthenium-bound P450, spectroscopically determined by measuring the dissociation constant of camphor at multiple Ru concentrations. Luminescence experiments measure  $K_D$  directly.

substrate and protein). Samples were prepared in a 1-cm-pathlength quartz cuvette with a long neck fitted with a 24/40 joint and a threaded compression seal. The samples (1.5 ml) were deoxygenated by repeated cycles of vacuum followed by argon back-filling. Bubbling of the samples was avoided to minimize protein denaturation. UV-vis spectra were measured routinely before and after each luminescence measurement to verify that the protein samples had not degraded. Nanosecond emission kinetics were fit to the sum of two exponentials ( $I(t) = c_0 + c_1 \exp(-k_1 t) + c_2 \exp(-k_2 t)$ ) using an in-house nonlinear least-squares fitting program. Dissociation constants for both isomers were determined using the ratio of the coefficients for the fast and slow phases,  $c_1/(c_1 + c_2)$ .

### 2.6. Time-resolved emission

The excitation source for all experiments was a tunable (220–2000 nm) optical parametric oscillator (Spectra Physics, MOPO) pumped by a frequency-tripled Q-switched Nd:YAG laser (Spectra Physics, 355 nm, 350 mJ/pulse, 8-ns FWHM). The OPO output power was attenuated by passage through a polarizer; laser shots with energies differing by more than 10% from the mean value (laser pulses detected by a photodiode and selected by a discriminator, Phillips Scientific Model 6930) were rejected. Deoxygenated Ru-protein samples were excited at 470 nm, typically 2 mJ/pulse at the sample. Emission was collected 180° to the incident excitation with reflective optics ( $f/10$ ), sent through a long-pass filter ( $\lambda > 600$  nm), and focused onto the entrance slit of an ISA double 0.1 meter monochromator. Luminescence was detected by a Hamamatsu photomultiplier tube (R928); the output signal passed through a high-speed (100 MHz) current to voltage amplifier, digitized (Sony/Tektronix digitizer, Model RTD710A), and recorded on a PC. Instrument response was 10 ns (FWHM). Emission kinetics data are averages of at least 250 laser shots.

## 3. Results

Resolution of racemic  $[\text{Ru-C}_9\text{-Ad}]^{2+}$  was accomplished by cation-exchange chromatography using a chiral eluent, sodium (–)-*O,O'*-dibenzoyl-L-tartrate. The CD spectra of the  $\Lambda$ - (+) and  $\Delta$ - (–)- $[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$  isomers are shown in Fig. 3. In both cases, enantiomeric excess is >90% based on the similarity of their extinction coefficients at every wavelength (<10% deviation), as well as their similarity to published values for  $(\pm)\text{-}[\text{Ru}(\text{bpy})_3]\text{Cl}_2$  [24]. Initial efforts to purify  $[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$  in larger quantities (40 mg) and with more concentrated eluent (150 mM) were unsuccessful.

Fig. 4 shows a standard low- to high-spin conversion involving the titration of camphor into the P450 active site in the presence of  $\Lambda$  and  $\Delta$ - $[\text{Ru-C}_9\text{-Ad}]$  isomers. The

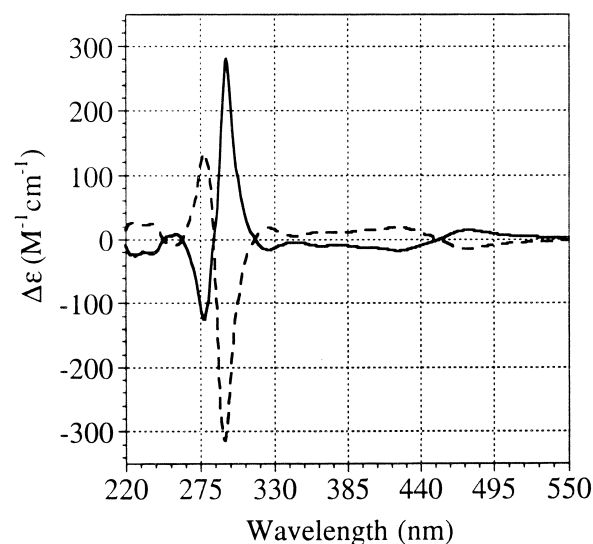


Fig. 3. CD spectra of the enantiomeric forms of  $[\text{Ru}(\text{bpy})_2(\text{bpy-C}_9\text{-Ad})]^{2+}$ :  $\Delta$  (dotted line);  $\Lambda$  (solid line).

dissociation constant for camphor alone ( $K_{\text{cam}}$ ) was found to be  $3.0 \pm 0.2 \mu\text{M}$  under the experimental conditions, in good agreement with the literature value [21]. The steric bulk of Ru-substrates appears to preclude co-occupation of the active site with camphor, an observation supported by the P450:Ru-C<sub>9</sub>-Ad crystal structure in which the adamantyl moiety binds above the heme and hydrogen

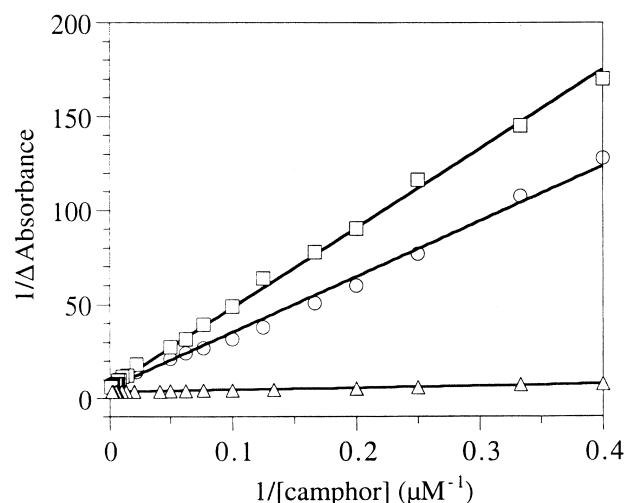


Fig. 4. Inverse absorbance changes at 392 nm as functions of inverse camphor concentration on titrating camphor into buffered (50 mM potassium phosphate, 100 mM potassium chloride, pH 7.4) solutions of ferric-aquo cytochrome P450<sub>cam</sub> (~5 μM). The triangles denote the binding of camphor to P450 in the absence of any Ru-substrate (slope =  $K_s/([\text{P450}]\Delta\epsilon)$ ;  $K_s = 3.0 \pm 0.2 \mu\text{M}$ ,  $[\text{P450}] = 5.50 \mu\text{M}$ ,  $\Delta\epsilon_{392 \text{ nm}} = 54,000 \text{ M}^{-1} \text{ cm}^{-1}$ ). Inhibition of camphor binding by  $\Lambda$ - $[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$  (squares,  $K_s = 74 \pm 10$ ,  $[\text{P450}] = 5.15 \mu\text{M}$ ,  $\Delta\epsilon_{392 \text{ nm}} = 34,000 \text{ M}^{-1} \text{ cm}^{-1}$  (difference between camphor- and  $[\text{Ru-C}_9\text{-Ad}]$ -bound P450),  $[\text{Ru}] = 4.83 \mu\text{M}$ ,  $K_1 = 15 \pm 1$ ,  $K_D = 200 \pm 50 \text{ nM}$ ) and  $\Delta$ - $[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$  (circles,  $K_s = 53 \pm 10$ ,  $[\text{P450}] = 5.29 \mu\text{M}$ ,  $[\text{Ru}] = 4.99 \mu\text{M}$ ,  $K_1 = 10 \pm 1$ ,  $K_D = 300 \pm 50 \text{ nM}$ ) is reflected by steeper slopes. Reported dissociation constants are averages of three titrations.

bonds to Tyr 96 much like camphor [3]. UV–vis absorption measurements of Ru-C<sub>9</sub>-Ad displacement show a preference for the  $\Lambda$  isomer ( $K_D$  ( $\Lambda$ )=200±50 nM;  $K_D$  ( $\Delta$ )=300±50 nM). It was found empirically that displacement of camphor (100  $\mu$ M camphor, 4.67  $\mu$ M P450, 99% bound) by Ru-substrates yields dissociation constants with higher precision. The apparent dissociation constant,  $K_D$  = 240±20 nM, of racemic Ru-C<sub>9</sub>-Ad determined by this Ru-titration method (Fig. 5) is in excellent agreement with the predicted value ( $K_D$  = 248 nM).

Time-resolved luminescence measurements also distinguish the binding of  $\Lambda$  and  $\Delta$ -[Ru-C<sub>9</sub>-Ad] isomers to P450 (Fig. 6). The monophasic emission decay ( $k$  = 2.0 × 10<sup>6</sup> s<sup>-1</sup>) of Ru-C<sub>9</sub>-Ad alone in solution is nearly identical with that of the slower phases of the two solutions containing P450. This provides strong evidence that binding can be modeled as a two-state equilibrium, and in the “free” state the Ru-substrates are completely dissociated from the protein. Virtually the same quenching rate constants ( $k$  = 4.5 × 10<sup>6</sup> s<sup>-1</sup>) from the  $\Lambda$  and  $\Delta$  “bound” states indicate comparable Ru-Fe distances for the two isomers. The proportion of the decay ( $\Lambda$ , 87.5±0.5%;  $\Delta$ , 82.5±0.5%; 5  $\mu$ M P450, 5  $\mu$ M Ru) that is attributable to this faster (quenched) phase is clearly greater for  $\Lambda$ -[Ru-C<sub>9</sub>-Ad]. The  $K_D$  values for the enantiomers determined by time-resolved emission ( $\Lambda$ , 90±20;  $\Delta$ , 190±20 nM) are in good agreement with the  $K_D$  for racemic Ru-C<sub>9</sub>-Ad (150±30 nM).

In order to test whether the single-substrate binding model (Fig. 2) accurately describes camphor displacement of Ru-C<sub>9</sub>-Ad from P450, a series of UV–vis absorption

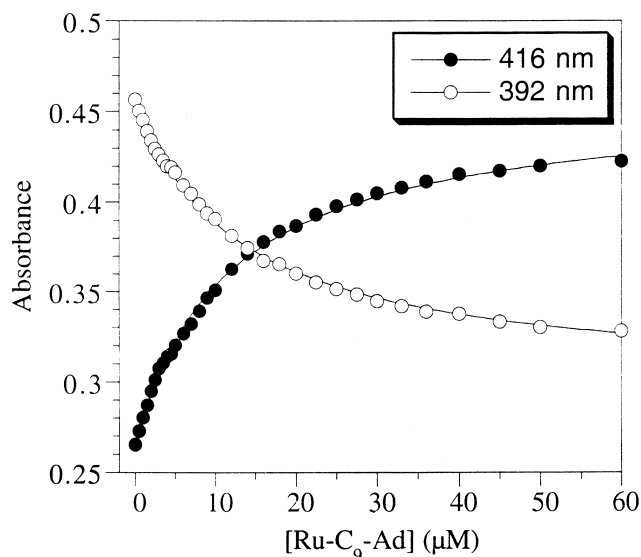


Fig. 5. Absorbance (open circles, 392 nm; filled circles, 416 nm) versus Ru-C<sub>9</sub>-Ad concentration; UV–vis data monitored the displacement of camphor from P450, and were corrected for Ru absorbance. Data are fit by the function:  $A = A_0 + \Delta\epsilon^*([P450]*[Ru])/([Ru] + K_S)$ , where  $A_0$  is the initial absorbance,  $\Delta\epsilon_{392} = 34,000$ ,  $\Delta\epsilon_{416} = 41,000$  M<sup>-1</sup> cm<sup>-1</sup>, [P450] = 4.67  $\mu$ M;  $K_S$  (392 nm) = 13.3,  $K_S$  (416 nm) = 11.7.  $K_D = K_{cam}/K_S = 240 \pm 20$  nM.

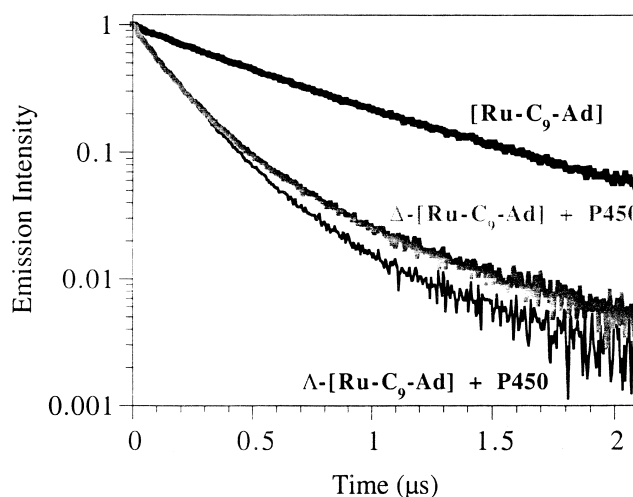


Fig. 6. Kinetics traces of [Ru-C<sub>9</sub>-Ad]<sup>2+</sup>\* emission decay. A larger fraction of  $\Lambda$ -[Ru-C<sub>9</sub>-Ad]Cl<sub>2</sub> emission (monitored at 620 nm) is quenched by P450.

and emission experiments were performed in parallel. Fig. 7 reveals that the amounts of unbound Ru<sup>2+</sup>\* (by luminescence) and low-spin P450 (by UV–vis) track closely during camphor titration into the 1:1 P450:Ru-C<sub>9</sub>-Ad complex. However, persistent quenching of Ru<sup>2+</sup>\* at high camphor concentrations suggests that a small fraction (% quenched – % low spin ~ 5%) of Ru-C<sub>9</sub>-Ad binds cooperatively in the P450 channel, presumably above the camphor binding site. Energy-transfer kinetics do not change during the titration, indicating that {Ru(bpy)<sub>3</sub>}<sup>2+</sup> remains at the surface (~20 Å from the heme) in this ternary complex.

Confirmation that  $\Lambda$ -[Ru-C<sub>9</sub>-Ad] binds P450 with

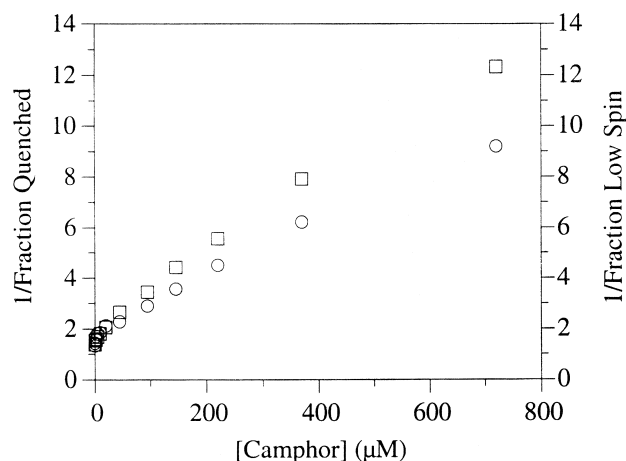


Fig. 7. 1/fraction of quenched Ru<sup>2+</sup>\* luminescence and 1/fraction of low-spin P450 as functions of camphor concentration. Luminescence data (samples at ambient conditions, monitored at 620 nm; kinetics fit to biphasic decay give fraction Ru<sup>2+</sup>\* quenched by P450) were collected subsequent to each UV–vis measurement. The spin state of the heme was calculated from changes in absorbance at 416 nm. Luminescence measurements report persistent Ru<sup>2+</sup>\* binding at high camphor concentrations.

roughly twice the affinity of the  $\Delta$  isomer comes from an experiment in which buffered P450 (20  $\mu\text{M}$ ) and  $(\pm)$ -[Ru-C<sub>9</sub>-Ad]Cl<sub>2</sub> (40  $\mu\text{M}$ ) were centrifuged together through a size-selective membrane (Centricon, YM-10). CD measurement showed that the Ru-substrate effluent (23  $\mu\text{M}$ , in good agreement with the dissociation constant) was enantiomerically enriched by 15% with the  $\Delta$  (more weakly bound) isomer. In the absence of P450, no enantiomeric enhancement was found to occur during filtration. Thus, of the 17  $\mu\text{M}$  Ru-C<sub>9</sub>-Ad remaining bound to the enzyme, 10.2  $\mu\text{M}$  corresponded to  $\Lambda$  and 6.8  $\mu\text{M}$  to the  $\Delta$  isomer. The ratio of the concentrations of bound isomers ( $\Lambda/\Delta \sim 1.5$ ) is in reasonable agreement with the corresponding  $K_D$  ratio ( $\Delta/\Lambda \sim 2$ ).

#### 4. Discussion

Sodium  $(-)$ -*O,O'*-dibenzoyl-L-tartrate proved much less efficient at resolving  $(\pm)$ -[Ru-C<sub>9</sub>-Ad]Cl<sub>2</sub> (ECL=200 cm) than reported for the model compound [Ru(bpy)<sub>2</sub>(Me<sub>2</sub>bpy)]Cl<sub>2</sub> (ECL=70 cm) [24], presumably due to interference from the long alkyl substituent. Chiral ruthenium polypyridyl compounds have been synthesized directly by starting with one of the enantiomers of [Ru(bpy)<sub>2</sub>(CO)<sub>2</sub>]<sup>2+</sup> [24]. Addition of the third bipyridyl ligand occurs with stereoretention if the temperature, solvent, and ligand concentration are carefully controlled [23]. This method would seem preferable to chiral separations of functionalized [Ru(bpy)<sub>2</sub>(bpy')]Cl<sub>2</sub> compounds, which in our hands required 2–3 weeks for purification of milligram quantities of material.

That  $K_D(\text{energy transfer}) < K_D(\text{UV-vis})$  reflects subtle, method-dependent differences in the quantification of Ru-substrate binding. Nonspecific binding of Ru-C<sub>9</sub>-Ad to the enzyme will affect the Ru<sup>2+</sup>\* emission decay profile, owing to energy transfer to the heme, but will not perturb the UV-vis spectrum if camphor is in place at the active site. Interestingly, when the Ru-C<sub>9</sub>-Ad concentration exceeds that of P450, the luminescence results begin to deviate from predictions based on a single-substrate binding model. Energy-transfer experiments reveal that when [Ru]  $\gg$  [P450], at least four equivalents of Ru-C<sub>9</sub>-Ad associate with the enzyme (5  $\mu\text{M}$  P450, 50  $\mu\text{M}$  Ru; 20  $\mu\text{M}$  Ru quenched at  $\sim 20$  Å from the heme). In fact, the P450 interior is greatly expanded in this open form (Fig. 1) and should permit orientations of Ru-C<sub>9</sub>-Ad different from that found in the crystallized complex.

An electrostatic map of the protein surface indicates that the entrance to the substrate channel is neutral, favoring hydrophobic rather than electrostatic interactions in recruiting {Ru(bpy)<sub>3</sub>}<sup>2+</sup> to this region, especially at high ionic strengths. Evidence of the dominance of hydrophobic interactions is the finding that bipyridyl-substituted adamantane itself, before ruthenation, strongly binds P450

(data not shown). It also is of interest that hydrophobic interactions appear to play a role in certain stereoselective bimolecular electron-transfer reactions between metalloproteins and inorganic complexes [32–34].

Experiments with other Ru-linked moieties [1,3] indicate that the terminal group moderately influences the overall affinity of the Ru-substrate for P450. (Ru-adamantane compounds bind with 3-fold higher affinity than Ru-(ethyl benzene) analogs, and 9-fold higher affinity than unsubstituted Ru-alkyl chains.) A comparable effect ( $K_D$  increases 9-fold) is observed when short linkers connecting the ethyl benzene to the photosensitizer prohibit optimal positioning of the substrate within the active-site pocket [3]. Sufficient chain length is especially critical for imidazole-terminated compounds, where the ability to bind the iron is requisite for association. The modest 2-fold discrimination of  $\Lambda$  and  $\Delta$ -[Ru-C<sub>9</sub>-Ad] provides strong evidence that interactions near the protein surface are of lesser importance than the shape complementarity and hydrophobicity of the Ru-substrate in binding to the enzyme.

Enantiospecific binding indicates that noncovalent interactions over 10 Å from the active site impact substrate selection even when the channel is open, as must occur during entrance and egress of natural substrates. Similar long-range secondary interactions also influence the binding of benzenesulfonamide ligands to carbonic anhydrase [35]. Based on the P450:Ru-C<sub>9</sub>-Ad crystal structure [3], which confirms the ability of P450 to accommodate large substrates, and identifies hydrophobic interactions of the bipyridyl groups with Phe 193 and Tyr 29 (Fig. 1), we infer that aromatic stacking plays an important role in chiral discrimination. Aromatic residues at the mouth of the P450 channel have been implicated previously in the recognition of hydrophobic substrates [36].

Submicromolar affinities, protein specificity, reversible binding, and synthetic versatility make sensitizer-linked substrates ideal for probing P450 active sites. Employing UV-vis and time-resolved luminescence measurements, we have found a 2-fold preference of P450 for  $\Lambda$ -[Ru-C<sub>9</sub>-Ad]Cl<sub>2</sub>. Emission experiments, especially with highly luminescent {Ru(bpy)<sub>3</sub>}<sup>2+</sup> complexes, are particularly sensitive and convenient for measuring substrate binding. It is well known that the chirality and shape of substrate pockets promote enantio- and regioselective P450 catalysis. We have demonstrated that specific long-range interactions with a pendant metal complex also affect substrate binding at the active site of the enzyme.

#### 5. Nomenclature

bpy	2,2'-bipyridine
Ad	adamantane
*	excited state
ECL	effective column length
CD	circular dichroism

## Acknowledgements

We thank J.J. Wilker for experimental work, J.H. Dawson, B.R. Crane, E.D.A. Stemp, and A.G. Lappin for helpful discussions, and S.G. Sligar for providing a P450<sub>cam</sub> vector. I.J.D. is an NIH predoctoral trainee (GM08346). This work was supported by the NIH Metalloprotein Program Project Grant (P01 GM48495) and the National Science Foundation (CHE9807150).

## References

- [1] J.J. Wilker, I.J. Dmochowski, J.H. Dawson, J.R. Winkler, H.B. Gray, *Angew. Chem. Int. Ed.* 38 (1999) 90–92.
- [2] I. Hamachi, S. Tsukiji, S. Shinkai, S. Oishi, *J. Am. Chem. Soc.* 121 (1999) 5500–5506.
- [3] I.J. Dmochowski, B.R. Crane, J.J. Wilker, J.R. Winkler, H.B. Gray, *Proc. Natl. Acad. Sci. USA* 96 (1999) 12987–12990.
- [4] R.N. Atkinson, L. Moore, J. Tobin, S.B. King, *J. Org. Chem.* 64 (1999) 3467–3475.
- [5] R.A. Tschirret-Guth, K.F. Medzihradzky, P.R. Ortiz de Montellano, *J. Am. Chem. Soc.* 121 (1999) 4731–4737.
- [6] K. DiGleria, D.P. Nickerson, H.A.O. Hill, L.-L. Wong, V. Fülöp, *J. Am. Chem. Soc.* 120 (1998) 46–52.
- [7] Y. Murthy, V. Massey, *Meth. Enzymol.* 280 (1997) 436–460.
- [8] M. Newcomb, M.H. Letadic, D.A. Putt, P.F. Hollenberg, *J. Am. Chem. Soc.* 117 (1995) 3312–3313.
- [9] J.K. Atkinson, K.U. Ingold, *Biochemistry* 32 (1993) 9209–9214.
- [10] K.E. Liu, C.C. Johnson, M. Newcomb, S.J. Lippard, *J. Am. Chem. Soc.* 115 (1993) 939–947.
- [11] R.A. Tschirret-Guth, K.F. Medzihradzky, P.R. Ortiz de Montellano, *J. Am. Chem. Soc.* 120 (1998) 7404–7410.
- [12] J.A. Tainer, E.D. Getzoff, K.M. Beem, J.S. Richardson, D.C. Richardson, *J. Mol. Biol.* 160 (1982) 181–217.
- [13] T.L. Bigler, W. Lu, S.J. Park, M. Tashiro, M. Wieczorek, R. Wynn, M. Laskowski, *Prot. Sci.* 2 (1993) 786–799.
- [14] J. Badger, I. Minor, M.J. Kremer, M.A. Oliveira, T.J. Smith, J.P. Griffith, D.M.A. Guerin, S. Krishnaswamy, M. Luo, M.G. Rossmann, M.A. McKinlay, G.D. Diana, F.J. Dutko, M. Fanher, R.R. Rueckert, B.A. Heinz, *Proc. Natl. Acad. Sci. USA* 85 (1988) 3304–3308.
- [15] T.L. Poulos, B.C. Finzel, A.J. Howard, *Biochemistry* 25 (1986) 5314–5322.
- [16] S.K. Wilcox, C.D. Putnam, M. Sastry, J. Blankenship, W.J. Chazin, D.E. McRee, D.B. Goodin, *Biochemistry* 37 (1998) 16853–16862.
- [17] J.O. Goldsmith, B. King, S.G. Boxer, *Biochemistry* 35 (1996) 2421–2428.
- [18] G.D. DePillis, S.M. Decatur, D. Barrick, S.G. Boxer, *J. Am. Chem. Soc.* 116 (1994) 6981–6982.
- [19] M.M. Fitzgerald, M.J. Churchill, D.E. McRee, D.B. Goodin, *Biochemistry* 33 (1994) 3807–3818.
- [20] A.E. Eriksson, W.A. Baase, J.A. Wozniak, B.W. Matthews, *Nature* 355 (1992) 371–373.
- [21] E.J. Mueller, P.J. Loida, S.G. Sligar, Twenty-five years of P450<sub>cam</sub> research, in: P.R. Ortiz de Montellano (Ed.), *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd ed., Plenum Press, New York, 1995, pp. 83–124.
- [22] N.C. Fletcher, F.R. Keene, *J. Chem. Soc., Dalton Trans.* 5 (1999) 683–689.
- [23] N.C. Fletcher, P.C. Junk, D.A. Reitsma, F.R. Keene, *J. Chem. Soc., Dalton* 1 (1998) 133–138.
- [24] T.J. Rutherford, P.A. Pellegrini, J. Aldrich-Wright, P.C. Junk, F.R. Keene, *Eur. J. Inorg. Chem.* 11 (1998) 1677–1688.
- [25] T.J. Rutherford, M.G. Quagliotto, F.R. Keene, *Inorg. Chem.* 34 (1995) 3857–3858.
- [26] Y. Yoshikawa, K. Yamasaki, *Coord. Chem. Rev.* 28 (1979) 205–229.
- [27] F.R. Keene, personal communication.
- [28] B.P. Unger, I.C. Gunsalus, S.G. Sligar, *J. Biol. Chem.* 261 (1986) 1158–1163.
- [29] D. Nickerson, L.-L. Wong, Z.H. Rao, *Acta Crystallogr. D* 54 (1998) 470–472.
- [30] D.D. Perrin, W.L.F. Armarego, *Purification of Laboratory Chemicals*, 3rd ed., Butterworth-Heinemann Ltd, Boston, 1988.
- [31] A.J. Gordon, R.A. Ford, *The Chemist's Companion. A Handbook of Practical Data, Techniques, and References*, John Wiley and Sons, New York, 1972.
- [32] S. Sakaki, Y.-i. Nishijima, H. Koga, K. Ohkubo, *Inorg. Chem.* 28 (1989) 4061–4063.
- [33] S. Sakaki, Y.-i. Nishijima, K. Ohkubo, *J. Chem. Soc., Dalton Trans.* 4 (1991) 1143–1148.
- [34] J.R. Pladziewicz, M.A. Accola, P. Osvath, A.M. Sargeson, *Inorg. Chem.* 32 (1993) 2525–2533.
- [35] P.A. Boriak, D.W. Christianson, J. Kingerywood, G.M. Whitesides, *J. Med. Chem.* 38 (1995) 2286–2291.
- [36] R. Raag, H. Li, B.C. Jones, T.L. Poulos, *Biochemistry* 32 (1993) 4571–4578.