Peripheral Ligand Binding Site in Cytochrome P450 3A4 Located with Fluorescence Resonance Energy Transfer (FRET).

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Supplemental Data

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Analysis of substrate binding with a two-step sequential model

According to the sequential model, the binding at one of the two substrate binding sites in the enzyme requires a prior substrate association at another, higher affinity site:

$$\begin{array}{ccccc} & & K_{D1} & & K_{D2} \\ \mathrm{E} & \longleftrightarrow & \mathrm{ES} & \longleftrightarrow & \mathrm{SES} \\ & & \uparrow & & \uparrow \\ & & \mathrm{S} & & \mathrm{S} \end{array}$$

This property results in cooperativity in the formation of the final ternary complex (SES). In the general case the two-step sequential mechanism may be described with the system of non-linear equations first introduced in our earlier publication[1]:

$$\begin{cases} 2\cdot[SES]^2-([E]_0-[SES]+K_{D1})\cdot[ES]-([S]_0+2\cdot[E]_0+K_{D2})\cdot[SES]+[S]_0\cdot[E]_0=0 \\ [ES]=\dfrac{[S]_0}{2}-[SES]-\left([SES]^2-([S]_0+K_{D2})\cdot[SES]+\dfrac{[S]_0^2}{4}\right)^{1/2} \end{cases} \quad \text{(S1)}$$

In contrast to the Adair equation, which describes the behavior of this system at [S]>>[E], the above system of equations is valid at any concentrations of [S] and [E]. Although analytical solution of this system with respect to *[ES]* and *[SES]* is intricate, its roots may be found by numerical means[1] . In our implementation of this model in the SpectraLab package we used the algorithm of the golden section search in one dimension[2].

[1] Davydov, D.R., Botchkareva, A.E., Davydova, N.E., and Halpert, J.R. (2005) *Biophys. J.* **89**, 418-432.
[2] Press, W.H., Teukolsky, S.A., Vetterling, W.T., and Flannery, B.P. (1992) Numerical Recipes in C. The Art of Scientific Computing. 2nd. Ed., Cambridge University Press. (see pages 397-400)

Calculation of efficiencies of FRET specific to each of the two bound ligand molecules

These calculations were performed using the known formalism for overall efficiency of FRET in a system with two acceptors and a single donor (41). In these calculations we used the canonical expression that defines the overall efficiency of FRET in a system with two acceptors and a single donor (41):

$$E = \frac{k_{D\to A_1} + k_{D\to A_2}}{k_{D\to A_1} + k_{D\to A_2} + \frac{1}{\tau_D}} \qquad \text{(S2)}$$

In this expression $k_{D\to A1}$ and $k_{D\to A1}$ stand for the rate constants of the energy transfer to each of the two acceptors and τ_D is the lifetime of the excited state of the donor. Taking into account that the efficiency of FRET in each particular donor/accepror pair (E_1 and E_2) is defined as

$$E = \frac{k_{D\to A}}{k_{D\to A} + \frac{1}{\tau_D}}$$

((38), eq. 13.11), we may obtain the following relationship between E_T , E_1 and E_2 :

$$E_T = \frac{E_1 - 2\cdot E_1 \cdot E_2 + E_2}{1 - E_1 \cdot E_2} \qquad \text{(S3)}$$

According to our definition of F_{ES} (3), the efficiency of FRET in the complex *ES* may be determined as $E_1 = E_T \cdot F_{ES}$. Combining this relationship with equation (S3) we may now calculate the efficiency of FRET to the second bound molecule of the acceptor, E_2:

$$E_2 = \frac{E_T - E_1}{1 - 2\cdot E_1 + E_T \cdot E_1} \qquad \text{(S4)}$$

Determination of the quantum yield of CYP3A4-attached PIA labels.

In these experiments we first measured a series of 4 − 5 emission spectra of each of the labeled C64/C58, C377/C68, C468 and C495 mutants taken at concentrations increasing from 0.2-0.5 to 2-5 μM. The measurements were done with an Edinburgh FLS920 instrument where the excitation wavelength was set at 348 nm with 2 nm bandwidths of both emission and excitation monochromators. Such a series recorded with C468 mutant is exemplified in Fig. S1a. These measurements were accompanied by recording of the absorbance spectra of the same samples. Approximation of the absorbance in the 320 − 700 nm region with a combination of the prototypic spectra of PIA and CYP3A4 was used to determine the fraction of the optical density of the samples at 348 nm which is due to the absorbance of PIA. Based on these calculations we determined the gradients ($Grad_X$) of the plots of the integral intensity of emission (370 − 600 nm) versus the optical density of PIA (Fig. S1a, inset).

The gradient determined in this way is affected by FRET from PIA to the heme. In order to take into account this effect we determined the effect of H_2O_2-dependent heme depletion on the intensity of fluorescence of PIA-labeled CYP3A4. In these experiments we incubated the PIA-labeled enzyme with 60 mM hydrogen peroxide. This treatment results in rapid bleaching of the heme protein (Fig. S1b, inset) accompanied by an ample increase in the fluorescence of the label (Fig. S1b). The relative increase in fluorescence upon heme depletion was used to calculate the corrected values of $Grad_X$, which were assumed proportional to the quantum yield of the protein-bound PIA probes (38).

To determine the quantum yield from the corrected gradients we used an ethanol solution of the laser dye coumarin-47 as a reference (Φ=0.73, (43)). This dye has a broad maximum of excitation (λ_{max}=380 nm) and exhibits bright emission in the range of 380-600 nm when excited at 348 nm. Determination of the gradients ($Grad_{st}$) of the plots of integral intensity of emission (370 − 600 nm) vs. the optical density of the ethanol solutions of the dye (Fig. S1c) was done in a setup similar to that described above for the PIA-labeled CYP3A4.

The quantum yields of protein-bound PIA fluorophores was calculated using the canonical relationship (38):

$$\Phi_X = \Phi_{ST}\left(\frac{Grad_X}{Grad_{ST}}\right)\left(\frac{\eta_X^2}{\eta_{ST}^2}\right)$$

Here Φ_X and Φ_{ST} stand for the quantum yields of the label and the standard, respectively, $Grad_X$ and $Grad_{ST}$ are the gradients determined as described above, and η_x and η_{st} designate the refractive indices of the solutions of the labeled protein and the standard. In our calculations we used Φ_{ST} =0.73 (43). The refractive indices η_x and η_{st} were assumed to be equal to 1.333 and 1.359 for water solution of the protein and ethanol solution of the dye, respectively[3].

[3] Scott, T.A. Jr. (1946) *J. Phys. Chem.*, **50**, 406–412

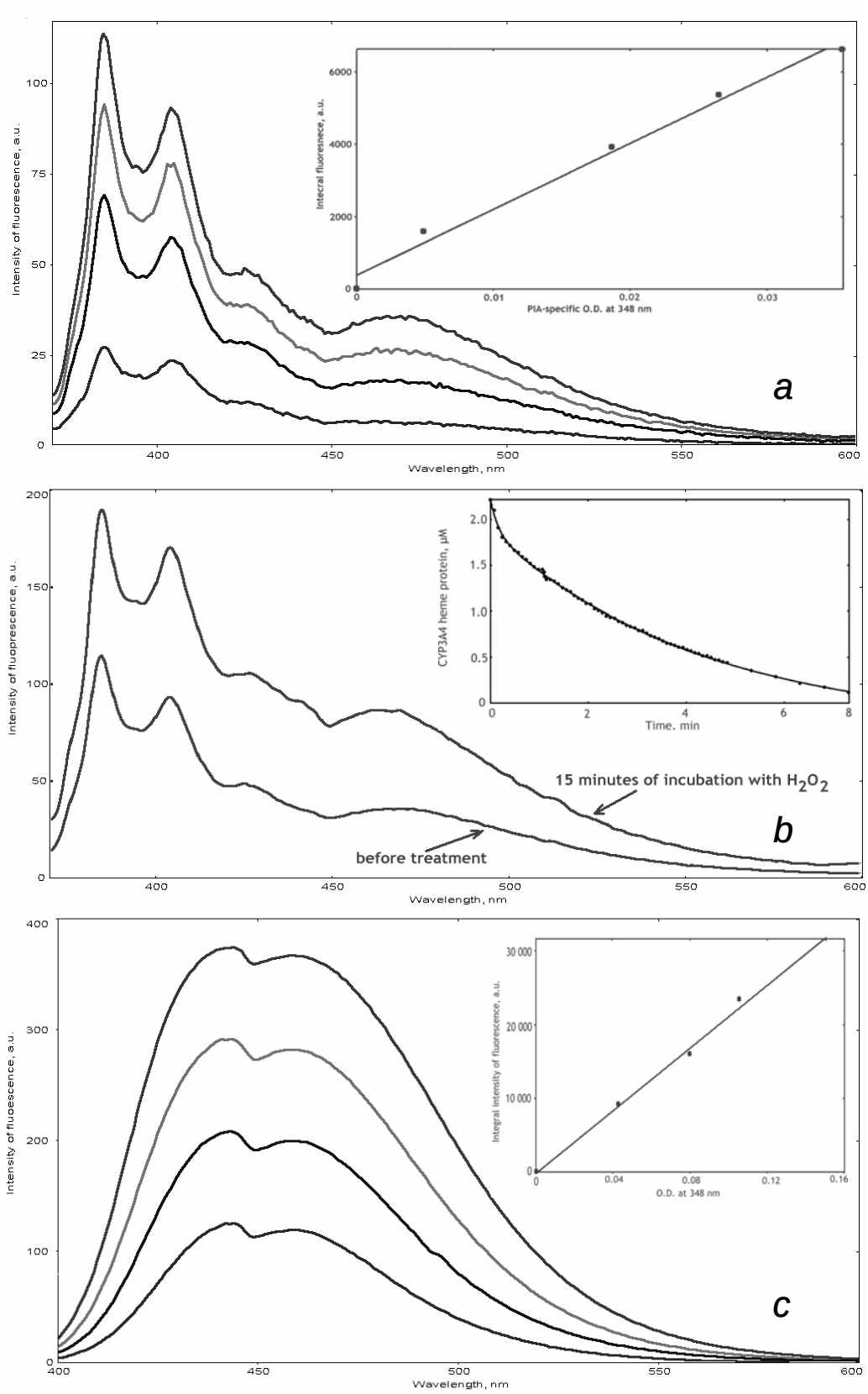

Fig. S1. Determination of the quantum yield of CYP3A4-bound PIA label. Panel ***a*** shows a series of emission spectra of CYP3A4(C468)-PIA taken at increasing concentrations of the enzyme. The inset shows the dependence of the integral fluorescence on the optical density of PIA. The effect of treatment of CYP3A4(C468)-PIA with hydrogen peroxide is illustrated in panel ***b.*** Panel ***c*** shows a series of emission spectra of coumarin-47, a quantum yield standard. The inset illustrates the dependence of the integral fluorescence on the optical density at the wavelength of excitation (348 nm).

<u>Table S1.</u> Parameters of interactions of F7GA with PIA-labeled single-cysteine mutants of CYP3A4 incorporated into proteoliposomes.

Position of the label	Fitting to the Hill equation		Approximation with the sequential two-step model (2)				FRET efficiencies for each of the two binding event	
	S_{50}	Hill coeff.	K_{D1}, μM	K_{D2}, μM	F_{ES}	E_T, %[a]	E_1, %	E_2, %
Cys-64	10.9 ± 0.9	1.9 ± 0.5	5.0 ± 1.7	11.7 ± 0.4	0.17 ± 0.15	0.94 ± 0.03	0.49 ± 0.13	0.93 ± 0.04
Cys-468	8.8 ± 3.9	1.5 ± 0.3	5.9 ± 1.0	9.4 ± 2.4	0.26 ± 0.18	0.84 ± 0.04	0.14 ± 0.13	0.83 ± 0.04

[a] Overall efficiency of FRET in the enzyme completely saturated with F7GA

* The values given in the table represent the averages of 2-4 individual measurements, and the "±" values show the confidence interval calculated for $p = 0.05$.