Supporting information for

Ultrafast dynamics of ligand rebinding to Nitrophorin 4

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1- Determination of Photolysis Quantum Yield

In the following we will describe the method that we used to calculate the photolysis quantum yield of ferric NP4NO. This method has been previously used to determine the photolysis quantum yield of NO and O_2 in ferrous myoglobin[1] as well as that of the methionine in ferrous cytochrome c[2].

We take $I(x) = I_0 \exp(-\varepsilon_p^{HL} c_0 x)$ to be the number of pump photons per cm^2 at depth *x* within the ligated heme sample volume, where ε_p^{HL} is the molar extinction coefficient of the ligated heme at the pump wavelength and c_0 is the concentration. The number of photons absorbed in a beam of cross-sectional area *a* passing through a sample of length *l* is then given by $\Delta n = a\Delta I = aI_0\left[1 - e^{-\varepsilon_p^{HL} c_0 l}\right]$. If we take Y_0^{HL} to be the quantum yield of photolysis and *N(t)* to be the normalized survival population of photolyzed hemes, then the ratio of the number of photolyzed molecules to the total molecules in the sample volume *la* with number density n_0 is denoted by the photolyzed fraction, η, which is given by

$$\eta = \frac{N(t)Y_0^{HL}\Delta Ia}{n_0 la} = \frac{N(t)Y_0^{HL}I_0(1-10^{-\varepsilon_p^{HL}c_0 l})}{n_0 l} = \frac{N(t)Y_0^{HL}I_0(1-10^{-A_p^{HL}})\varepsilon_\lambda^{HL}}{A_\lambda^{HL}N_A} \qquad \text{(S1)}$$

Where N_A is Avogadro's number and $A_p^{HL} = \varepsilon_p^{HL} c_0 l = \varepsilon_p^{HL} n_0 l / N_A$ is the absorbance of the ligated material at the pump wavelength. The normalized survival population *N(t)* is a measure of the ligand rebinding following photolysis. Note that the quantity $n_0 l = N_A A_\lambda^{HL} / \varepsilon_\lambda^{HL}$ can be evaluated at any convenient wavelength, such as the Soret maximum.

The transient absorption signal following photolysis depends upon the mole number of dissociated molecules, $N(t)Y_0^{HL}\Delta Ia / N_A$, in the volume al, the pathlength l, and the difference in the

molar extinction coefficient, $\Delta\varepsilon_\lambda$, between the dissociated heme and the ligated heme at the probe wavelength.

$$\Delta A_\lambda(t) = \Delta\varepsilon_\lambda \left(\frac{N(t) Y_0^{HL} \Delta I a}{N_A a l} \right) l = N(t) Y_0^{HL} \Delta I \Delta\varepsilon_\lambda / N_A \qquad \text{(S2)}$$

In the experiment, the photolyzed fraction η (which depends implicitly on *N(t)*) is derived by adding the equilibrium ligand bound state heme absorption (A_λ^{HL}) to the transient difference spectrum, $\Delta A_\lambda(t)$, so that a smooth spectrum of the equilibrium product absorption band (A_λ^{H}) is generated. The scaling factor η is the photolyzed fraction:

$$\Delta A_\lambda(t) = \eta \Delta A_\lambda = \eta (A_\lambda^H - A_\lambda^{HL}) \qquad \text{(S3a)}$$

So that

$$\eta A_\lambda^H = \Delta A_\lambda(t) + \eta A_\lambda^{HL} \qquad \text{(S3b)}$$

For MbCO, $Y_0^{MbCO} = 1$ and $N(t) = 1$ at t=20 ps. So

$$\frac{\eta_{NPL}}{\eta_{MbCO}} = \frac{N(20\,ps) Y_0^{NPL} A_{\lambda_1}^{MbCO} \varepsilon_{\lambda_2}^{NPL} (1 - 10^{-A_p^{NPL}})}{A_{\lambda_2}^{NPL} \varepsilon_{\lambda_1}^{MbCO} (1 - 10^{-A_p^{MbCO}})} \qquad \text{(S4)}$$

or

$$Y_0^{NPL} = \frac{A_{\lambda_2}^{NPL} \varepsilon_{\lambda_1}^{MbCO} (1 - 10^{-A_p^{MbCO}})}{N(20\,ps) A_{\lambda_1}^{MbCO} \varepsilon_{\lambda_2}^{NPL} (1 - 10^{-A_p^{NPL}})} \frac{\eta_{NPL}}{\eta_{MbCO}} \qquad \text{(S5)}$$

Thus, by finding the relative scaling factors η_{NPL} / η_{MbCO} (to account for the photolysis fraction and other experiment-specific factors) and using *N(t)* fitted at *t* >10ps (but normalized so *N(0)* = 1, to

eliminate the early time thermal response), the quantum yield at time zero (Y_0^{NPL}) for NP4-bound ligands relative to MbCO can be found using Eq. S5

Table S1. Parameters needed to calculate the photolysis quantum of ferric NP4NO at pH 5.6.

	η(20ps)	η(20ps)	*N*(20ps)	A_λ (OD)	ε_λ($mM^{-1}cm^{-1}$)	A_p(OD)
MbCO	11.5%	10%	1.0	0.64 (423nm)	207 (423nm)	0.05 (580nm)
NP4-NO	2.1%	2.2%	0.36	1.61 (419nm)	134 (419nm)	0.12 (580nm)
Y_0^{NP4NO}	90%	108%				

2- Analysis of CO rebinding kinetics to NP4 using the SRC model

As can be seen in Fig. 6, the geminate recombination of CO in NP4 is non-exponential at both pH 5 and 7. To describe this kinetic response we used a model based on a distribution of heme doming conformations, which has been described in detail by Srajer et al[3,4]. Within this model the enthalpic barrier for CO binding is separated into two parts

$$H = H_p(a) + H_0 = \frac{1}{2}Ka^2 + H_0 \ . \qquad \text{(S6)}$$

H_p represents the proximal barrier due the heme doming and, *a*, is the protein conformation-dependent generalized iron out-of-plane equilibrium position. The quantity K is the effective force constant along

the doming coordinate and H_0 represents the remaining (mostly distal) contributions to the barrier; it contains energies involving ligand docking sites and steric constraints associated with the distal pocket as well as a small *a*-independent term from the linearly coupled heme potential surface. We assume that the distribution of the iron out-of-plane displacements, *P(a)*, is Gaussian with a_0 representing the average out-of-plane displacement in the unbound state, and σ_a its variance. Using *P(a)*, a distribution for the barrier heights *g(H)* can be calculated and the survival population at time t after photolysis can be written as,

$$N(t) = \int_{H_0}^{\infty} g(H) e^{-k(H)t} dH \qquad \text{(S7a)}$$

with

$$k(H) = k_1 \exp\left(\frac{-H_p(a)}{k_B T}\right) \qquad \text{(S7b)}$$

and

$$k_1 = k_0 \exp\left(\frac{-H_0}{k_B T}\right) \quad . \qquad \text{(S7c)}$$

N(t) can be evaluated as

$$N(t) = I_g \int_0^{\infty} dx \frac{A}{2\sqrt{\pi x}} \left(e^{-\left(A\sqrt{x}-C\right)^2} + e^{-\left(A\sqrt{x}+C\right)^2} \right) e^{-k_1 t\, e^{-x}} + (1 - I_g) \qquad \text{(S8)}$$

Where the parameter I_g represents the geminate amplitude. Equation (S8) is valid when the value of the geminate amplitude I_g is near unity. The fundamental parameters $\{a_0, \sigma_a\}$ that describe the heme conformational distribution can be calculated from the fitting parameters $\{A, C\}$ as,

$$\sigma_a = \sqrt{\frac{k_B T}{K A^2}} \tag{S9a}$$

$$a_0 = \sqrt{\frac{2k_B T}{K}}\left(\frac{C}{A}\right) \tag{S9b}$$

X-ray crystallography reveals that NP4CO has two conformers, closed (c) and open (o). The relative populations of these two conformers depend on the pH. The closed conformer is dominant at low pH. We assume that the rebinding of CO in each one of these conformers can be described using Eq.S8. The normalized survival population can then be written as $N(t) = P_c N_c(t) + P_o N_o(t)$, which leads to

$$N(t) = P_c F_c(t) + P_o I_g^o F_o(t) + (1 - P_c - P_o I_g^o) \tag{S10a}$$

with

$$F_i(t) = \int_0^\infty dx \frac{A_i}{2\sqrt{\pi x}}\left(e^{-\left(A_i\sqrt{x}-C_i\right)^2} + e^{-\left(A_i\sqrt{x}+C_i\right)^2}\right) e^{-k_i^i t\, e^{-x}} \tag{S10b}$$

The P_o and P_c re the normalized initial photolyzed populations in the unbound states *(NP4:CO)$_o$* and *(NP4:CO)$_c$* respectively $(P_c + P_o = 1)$. (Since the photolysis quantum yield of CO in heme proteins is equal to unity, P_c and P_o represent also the relative populations of CO bound NP4 in the closed and open conformations respectively.) In Eq. we assumed that CO escapes to the solvent from the open conformer ($I_g^c = 1$).

References

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