Supplementary Information for

Symmetrized Photoinitiated Electron Flow Within the [Myoglobin:Cytochrome b₅]

Complex on Singlet and Triplet Timescales: Energetics vs Dynamics

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1. Expanded Materials and Methods

Expression and Purification of Proteins

The protocols for expression and purification of Mb(D44K/D60K/E85K) have been outlined elsewhere. Briefly, Escherichia coli strain BL321(DE3) was used for protein expression in Terrific broth medium. An excess of the desired metalloporphyrin, M (M is Mg-protoporphyrin IX = Mg or Zn-deuteroporphyrin IX = Zn; both from Frontier Scientific) was added to a solution of apo-Mb in 8M guanidine chloride, 100 mM Tris-HCl (pH 8.0). The Mb solution was kept in the dark and on ice or in the refrigerator at 4 °C while being stirred slowly. Optical spectra were recorded every 30 minutes-1 hour until a broadening of the metalloporphyrin-Mb Soret band appeared, a distinction of full porphyrin incorporation. Following dialysis and chromatography (size-exclusion and ion-exchange), the protein was flash-frozen with liquid nitrogen and stored at -80 °C. Purity was assayed by measuring the $A_{425,414}/A_{280}$ ratio (> 10) and verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Concentrations were calculated using the Soret absorbance (MgMb: $\varepsilon_{425} = 254$ mM$^{-1}$ cm$^{-1}$; ZnMb: $\varepsilon_{414} = 361$ mM$^{-1}$ cm$^{-1}$).

The tryptic fragment of bovine cyt b$_5$ was isolated and purified as described previously. Aerobic cyt b$_5$ prefers the ferric state because of its slight negative redox potential (-0.006 V vs. NHE). However, for oxidative quenching experiments in which cyt b$_5$ served as the oxidizing agent, it was further treated with excess K$_3$[Fe(CN)$_6$] and then washed thoroughly with working buffer (5 mM KPi, pH 6). For reductive quenching experiments in which cyt b$_5$ served as the reducing agent, it was treated with excess Na$_2$S$_2$O$_4$ and then washed thoroughly with working buffer.

Singlet Measurements: Samples, Instrumentation and Data Analysis
Samples were prepared in a COY anaerobic glove box. The working buffer (5 mM KPi, pH 6.0 or 70% w/w glycerol in 5 mM KPi, pH 6) was syringe-filtered and allowed to deoxygenate in the glove box for at least 24 hours before the samples were made. Protein stock solutions were exchanged into the anaerobic working buffer using Corning Spin-X UF concentrators immediately prior to the measurements. Three types of sample were prepared in 2 mm path length quartz cuvettes for each experiment: MMb by itself, cyt \( b_3 \) by itself, and the complex, [MMb:cyt \( b_3 \)]. The final volume of each sample was 500 µL. Concentrations of 100 µM Mb and 200 µM cyt \( b_3 \) and a ratio of Mb:2 cyt \( b_3 \) were used to simultaneously ensure that ≥ 90% of Mb was bound by cyt \( b_3 \) and optimize the S/N in transient absorption experiments, as guided by a combination of the previously reported binding constant for the [ZnMb(D44K/D60K/E85K):Fe\(^{3+}\) cyt \( b_3 \)] complex and known optical properties.\(^6\)

Singlet quenching was measured via femtosecond transient absorption (fs-TA).\(^7\) The ~120 fs pulses were produced with a commercial Ti:sapphire oscillator/amplifier (Tsunamic/Spitfire, Spectra-Physics), generating ~1 W at 827 nm, operating at 1 kHz. About 40% of this output was frequency-doubled and directed to a two-stage OPA producing pulses of 540 nm (ZnMb samples) or 598 nm (MgMb samples). This beam was focused to a ~200 µm spot on the sample, with an intensity of 1.0 µJ/pulse. 5% of the amplified pulse was sent up and down a motorized delay track which provided the desired time resolution, then focused on to a sapphire disk to create a white-light continuum probe with coverage from 430-850 nm. After passing through the sample, the probe beam was dispersed onto a CMOS array detector for the collection of spectral data at multiple delay times following photoexcitation of the sample. Samples were stirred to reduce the effects of photodegradation and local heating.
Transient absorption spectra were obtained by chopping the pump beam at 500 Hz and subtracting pump-on versus pump-off spectra. The total instrument response for the pump-probe experiments was ~180 fs. Transient signal at a given time delay was averaged for 3-5 seconds. Data were treated with a group-delay dispersion correction prior to analysis. Progress curves were generated at multiple wavelengths from the TA spectra and fit using an exponential (for \(^1\)MMb decay) or a stretched exponential\(^8\) (for \(^1\)MMb reaction with cyt \(b_5\)) (see Results and Discussion). The transient absorbance signal followed the same order of appearance and disappearance for every wavelength in the 450-750 nm region with each delay time spectrum for the MMb and [MMb:cyt \(b_5\)] complex samples. \(\text{Fe}^{3+}\) cyt \(b_5\) and \(\text{Fe}^{2+}\) cyt \(b_5\) samples, when excited at 540 nm had a transient absorbance-difference that was gone by 10 ps in the region monitored (450-750 nm) attributed to the decay of the excited heme. As the lifetime of the heme excitation and decay is brief relative to the singlet decays of the MMb by itself and in complex with cyt \(b_5\), it is acknowledged but not included in the analysis.

**Triplet Measurements: Samples, Instrumentation and Data Analysis**

Working buffer (5 mM KPi, pH 6 or 70% w/w glycerol in 5 mM KPi, pH 6) and 10 mm glass cuvettes were stored open to the atmosphere overnight in an anaerobic glove box (COY). Protein stock solutions were thawed on a cold block in the glove box for several hours prior to the experiment and then exchanged into the working buffer with Corning SpinvX UF concentrators. Protein stock concentrations were determined using a Hewlett-Packard UV-Vis spectrophotometer (MgMb: \(\varepsilon_{425} = 254 \text{ mM}^{-1} \text{ cm}^{-1}\); ZnMb: \(\varepsilon_{414} = 361 \text{ mM}^{-1} \text{ cm}^{-1}\); \(\text{Fe}^{3+}\) cyt \(b_5\): \(\varepsilon_{413} = 117 \text{ mM}^{-1} \text{ cm}^{-1}\); \(\text{Fe}^{2+}\) cyt \(b_5\): \(\varepsilon_{423} = 170 \text{ mM}^{-1} \text{ cm}^{-1}\)). As with samples prepared for the singlet measurement experiments, three types of sample were constructed for each triplet experiment: MMb by itself, cyt \(b_5\) by itself, and the [Mb:cyt \(b_5\)] complex. Concentrations of 5 \(\mu\)M Mb and 50
µM cyt b\textsubscript{5} and a ratio of Mb:10 cyt b\textsubscript{5} were used to ensure that ≈ 80\% of the Mb was in complex with cyt b\textsubscript{5}.

Samples were excited with a Nd:YAG Quanta-Ray INDI laser (Spectra-Physics) tuned to 532 nm.\textsuperscript{1} The output power was set to approximately 20 mW for the MgMb samples. Triplet measurements were performed with an LKS.60 laser flash photolysis spectrometer (Applied Photophysics) fitted with a xenon lamp with pulsing capabilities as the probe source. The sub µs-ms collection mode uses an Agilent Infiniium 600 MHz digitizer with a five-stage 1P28 photomultiplier tube as the detector. The xenon lamp was pulsed for sub µs collections. The triplet decay time courses were monitored at 465 nm for samples containing MgMb, the maxima for the triplet-ground spectra difference for these samples. All kinetic experiments were performed at 20 °C. As decay traces span several orders of magnitude in time, 50-100 shots were averaged for each time-segment and then merged into single files to obtain full kinetic progress curves for analysis.
2. Determination of Driving Forces for Singlet and Triplet Charge Separation ET

Reactions between MMb (M = Mg, Zn) and cyt b₅

The driving forces (-ΔG⁰) for the oxidation and reduction charge separation reactions of the \(^1\)MMb (M = Mg, Zn) by Fe\(^{3+}\) or Fe\(^{2+}\) cyt b₅, respectively, were calculated directly from redox potentials reported for the relevant half-reactions (enumerated in Table S1) in combination with energies from the appropriate emission spectra peaks (enumerated in Table S2), which most cleanly give the energy of the excited states – either \(S_1\) for singlet or \(T_1\) for triplet – relative to the ground state, \(S_0\).

Table S1. Half-reaction redox potentials (expressed as reductions by convention) used to estimate the \(\Delta G^0\)'s for the oxidation/reduction of \(^1,3\)MMb by Fe\(^{3+}/Fe^{2+}\) cyt b₅. All redox potentials are in terms of NHE.

| Half-reaction                  | Redox Potential vs. NHE |
|-------------------------------|-------------------------|
| MgP⁺Mb/MgPMb                  | 0.9 V \(^a\),*          |
| MgPMb/MgP⁺Mb                  | -1.3 V \(^b\),#         |
| ZnD⁺Mb/ZnDMb                  | 1.0 V \(^a\),†          |
| ZnDMb/ZnD⁺Mb                  | -1.2 V \(^c\),‡         |
| Fe\(^{3+}\) cyt b₅/Fe\(^{2+}\) cyt b₅ | -0.006 V \(^d\)       |

\(^{a}\)Cowan, J.A., et al. *Inorg. Chem.* 1989, 28, 2074-2078; \(^{b}\)Louati, A., et al. *Electrochim. Acta* 1976, 21, 1149-1153; \(^{c}\)Kakutani, T., et al. *Bull. Chem. Soc. Jpn.* 1973, 46, 3720-3723; \(^{d}\)Wang, Y., et al. *J. Electroanal. Chem.* 1997, 428, 39-45; \(^{*}\)This value was obtained from the redox potential of MgMPMb⁺Mb/MgMPMb vs. NHE; \(^{#}\)This value was obtained from the redox potential MgP(dme)/MgP⁺(dme) vs. SCE and adjusted by +0.24 V to be in terms of NHE; \(^{‡}\)This
value was obtained from the redox potential of ZnMPMb\textsuperscript{+}Mb/ZnMPMb vs. NHE; \textsuperscript{1}This value was obtained from the redox potential of ZnP(dme)/ZnP\textsuperscript{-}(dme) vs. SCE and adjusted by +0.24 V to be in terms of NHE. Previous work has demonstrated that the similarity between redox potentials for heme proteins reconstituted with metalloporphyrins and the redox potentials of simple metalloporphyrins warrants this substitution.\textsuperscript{9}

**Table S2.** Excited-State Energies for *MMb (*M=\textsuperscript{1}Mg, \textsuperscript{3}Mg, or \textsuperscript{1}Zn)

| *M      | Excited-State Energies (eV) |
|---------|-----------------------------|
| \textsuperscript{1}MgMb | 2.1\textsuperscript{*}       |
| \textsuperscript{3}MgMb | 1.7\textsuperscript{#}       |
| \textsuperscript{1}ZnMb | 2.1\textsuperscript{†}       |

\textsuperscript{*}Energy of S\textsubscript{1} band obtained from MgPMb emission band at 598 nm; \textsuperscript{#}Energy of T\textsubscript{1} band estimated from two values: ZnPMB emission band at 735 nm (Papp, S., et al. *Biophys. J.* 1990, 58, 177-186) and energy of T(0,0) emission band obtained for MgMPMb (Cowan, J.A., et al. *Inorg. Chem.* 1989, 28, 2074-2078); \textsuperscript{†}Energy of S\textsubscript{1} band obtained from ZnDMb emission band at 581 nm.

The driving forces (-\textit{∆G}\textsubscript{0}) for the charge separation reactions generated by the oxidation (1.2 eV) or reduction (0.8 eV) of \textsuperscript{1}MgMb by ferric cyt \textit{b}_5 or ferrous cyt \textit{b}_5 are presented within the ET cycle of **Scheme 1** and in **Table 1**.

The driving forces (-\textit{∆G}\textsubscript{0}) for the charge separation reactions generated by the oxidation and reduction of the \textsuperscript{1}ZnMb by ferric (1.1 eV) or ferrous (0.9 eV) cyt \textit{b}_5 are presented within the ET cycle of **Scheme S1**.
The driving forces (\(-\Delta G^0\)) for the charge separation reactions generated by the oxidation and reduction of the \(^3\)MgMb by ferric (0.8 eV) or ferrous (0.4 eV) cyt \(b_5\) are presented within the ET cycle of Scheme S2 and in Table 1.

Scheme S2.
3. Difference Spectra and Kinetic Traces for $[^1]{\text{ZnMb:cyt} \ b_5}$ on the Singlet Timescale

Figure S1 displays the difference absorbance spectra collected subsequent to laser excitation, in the 450-600 nm region, over the timescale of $S_1$ decay for (i) ZnMb by itself (top panel, Figure S1), and for complexes with (ii) Fe$^{3+}$ cyt $b_5$ (middle, Figure S1) and (iii) Fe$^{2+}$ cyt $b_5$ (bottom, Figure S1). As can be seen, the difference absorbance spectra at $t = 10$ ps for the three states of ZnMb are essentially the same. In all complexed and uncomplexed $[^1]{\text{ZnMb}}$’s the difference spectra were collected out to 5.2 ns; the absorbance difference remaining after this time is due to the triplet-ground difference ($\mu$s-$\text{ms}$ lifetime).

It can be clearly seen in Figure S1 that the $[^1]{\text{ZnMb}}$ excited state is strongly quenched by the presence of the cyt $b_5$ partner protein in both its Fe$^{3+}$ and Fe$^{2+}$ oxidation states, with the absorbance difference decaying much faster for both complexes.

Figure S1. Difference spectra for $[^1]{\text{ZnMb}}$ by itself (top), in complex with Fe$^{3+}$ cyt $b_5$ (middle), and in complex with Fe$^{2+}$ cyt $b_5$ (bottom).
The kinetic trace for the decay of $^1\text{ZnMb}$ as assembled from the absorbance-difference spectra slices at 452.5 nm is shown in Figure S2. The decay is described by an exponential function, (Eq. 1, parameters in Table 3). The $^1\text{ZnMb}$ decay traces for the photoexcited $[^1\text{ZnMb}:\text{Fe}^{3+}\text{cyt} b_3]$ and $[^1\text{ZnMb}:\text{Fe}^{2+}\text{cyt} b_3]$ complexes are also in Figure S2. In both complexes, the presence of the cyt $b_3$ partner quenches the $S_1$ state for the ZnMb and also leads to progress curves that are best described by augmenting the intrinsic decay with quenching portrayed by a stretched exponential, with average decay constant, $^1k_f$, and distribution exponent, $n$ (Eq. 2). The parameters obtained by globally fitting the progress curves at multiple wavelengths to Eq. 2 are presented in Table 2. As with the $[^1\text{MgMb}:\text{cyt} b_3]$ complexes, we attribute the distribution of quenching constants represented by the stretched-exponential behavior as signifying: 1) the presence of an ensemble of bound complexes with a distribution of quenching constants; and 2) that these complexes do not experience conformational interconversion on the timescale of the decay of the quenched $^1\text{ZnMb}$.

Figure S2. Progress curves for singlet to ground decay for $^1\text{ZnMb}$ (green), reductive quenching of $^1\text{ZnMb}$ in the presence of $\text{Fe}^{2+}\text{cyt} b_3$ (cyan), and oxidative quenching of $^1\text{ZnMb}$ in the presence of $\text{Fe}^{3+}\text{cyt} b_3$ (pink). The $^1\text{ZnMb}$ trace is described by Eq. 1 while the $[^1\text{ZnMb}:\text{Fe}^{2+}\text{cyt} b_3]$ and $[^1\text{ZnMb}:\text{Fe}^{3+}\text{cyt} b_3]$ traces are best described by Eq. 2. Fit parameters are in Table 2.
4. Förster Resonance Energy Transfer Consideration

As described in the text, the enhanced decay of $^1$MgMb/$^1$ZnMb in complex with Fe$^{3+}$ or Fe$^{2+}$ cyt $b_5$ can be assigned to ET quenching. In this section we demonstrate that resonance energy transfer (RET) from the excited $^1$MgMb/$^1$ZnMb to Fe$^{3+}$ or Fe$^{2+}$ cyt $b_5$ cannot be a significant component of the quenching.

RET is a radiationless process in which the energy from an excited electron of the donor in the singlet state is transferred to a ground state electron of the acceptor because the dipoles of the donor and acceptor resonate at the same frequency. Empirically, this equi-resonance is evident in spectral overlap between the emission spectrum of the donor and the absorbance spectrum of the acceptor. RET calculations$^{10}$ (Eq. S1) were carried out using accepted values from the literature for the dipole-dipole orientation factor, $\kappa^2$ (assumed to be 2/3 for dynamic random averaging of the donor and acceptor); the refractive index of the solution, $n$ (1.4 for aqueous solutions); and the quantum yield of the donor, $Q_D$ (0.02 for MgMb; 0.05 for ZnMb).$^{11}$ The value for the lifetime of the donor, $\tau_D$, is simply the inverse of the $^1k_D$ for $^1$MMb. Donor to acceptor distances were taken to be in the range of 15-20 Å, which is the range of ET reactivity distances expected for the [Mb:cyt $b_5$] complexes discussed here based on Brownian Dynamics (BD) simulations.$^{12}$ The spectral overlap, $J(\lambda)$, was calculated from the absorbance spectrum of the acceptor (cyt $b_5$) and the emission spectrum of the donor (MMb) and taken as the integrated product over the 500-800 nm spectral range of these three components: $F_D(\lambda)$, the fluorescence intensity of the donor in the wavelength $\lambda$ to $\lambda+\Delta\lambda$, with the total intensity normalized to unity; $\varepsilon_A(\lambda)$, the extinction coefficient of the acceptor at $\lambda$; and $\lambda^4$. 


\[
k_T = \frac{8.8 \times 10^{-28} \kappa^2 Q_D}{n^4 \tau_D R^6} \int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda
\]

\[
\int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda = J(\lambda)
\]

For demonstration, Table S3 shows the expected values for the energy transfer rate constant, \(k_T\), of the four complexes discussed in the main text for a reactivity distance of 15.3 Å, which is the shortest reactivity distance determined by BD simulations for the [Fe\(^{2+}\)Mb:Fe\(^{3+}\)cyt \(b_3\)] complex at pH 7.\(^\text{12}\)

**Table S3.** \(k_T\) at 15.3 Å (s\(^{-1}\))

|                | MgMb     | ZnMb    |
|----------------|----------|---------|
| \(\text{Fe}^{3+}\text{cyt }b_3\) | 7.4\times10^8 | 1.3\times10^{10} |
| \(\text{Fe}^{2+}\text{cyt }b_3\) | 4.1\times10^8   | 8.0\times10^9   |

The RET expected rate constants are at least an order of magnitude greater for the [ZnMb:cyt \(b_3\)] complexes than the [MgMb:cyt \(b_3\)] complexes while the observed quenching rate constants, \(^1k_f\)'s, are approximately the same for these complexes, suggesting that if RET was a significant component of the quenching, one would note at least an order of magnitude greater \(^1k_f\)'s for the [ZnMb:cyt \(b_3\)] complexes than for the [MgMb:cyt \(b_3\)] complexes. Further, no direct evidence of RET is available because Fe\(^{3+}/\text{Fe}^{2+}\)cyt \(b_3\) does not fluoresce. Previous reports have indicated that ET should be considered as the primary process for complexes in which the acceptor does not fluoresce and the distance between the donor and the acceptor is toward the lower limit of reactivity distances typically studied in RET.\(^\text{13}\) In fact, plotting the calculated rate constants for the two possible quenching processes – ET and RET – in Figure S3 for the
[\text{MgMb:Fe}^{3+}\text{cyt } b_5] \text{ complex (indicated by } k_q \text{ regardless of the process) as a function of reactivity distance displays that RET is expected to dominate at longer distances (> 20 Å) while ET is expected to dominate at shorter distances. The calculated ET rate constants (} k_{ET} \text{) come from a previous report.}^{12} \text{ Most significantly, for the 15-20 Å reactivity center distances (predicted by BD}^{12} \text{ and highlighted as the region between the two vertical dashed lines in Figure S3) of these complexes, electron transfer is favored to RET.}

\textbf{Figure S3.} \text{The predicted } k_{ET} \text{ and } k_T \text{ as a function of reactivity center distances for the } [\text{MgMb:Fe}^{3+}\text{cyt } b_5] \text{ complex. The vertical dashed lines signify the range of reactivity center distances (15-20 Å) predicted by BD simulations for the analogous complex } [\text{Fe}^{2+}\text{Mb:Fe}^{3+}\text{cyt } b_3].
5. Marcus Plot for $[^1\text{ZnMb}:\text{cyt b}_5]$ Complexes

The driving forces for the oxidative ($-\Delta G^0 = 1.1$ eV) and reductive ($-\Delta G^0 = 0.9$ eV) $[^1\text{ZnMb}$ ET charge-separation processes differ by $\delta(-\Delta G^0) \approx 0.2$ eV (Scheme S1). Figure S4 highlights the surprising circumstance that the two driving forces are symmetrically placed around the maximum of the parabola at $-\Delta G^0 = \lambda$ where ET is activationless. As a result, the $FC$ terms for the two processes are fortuitously the same explaining why the oxidative and reductive quenching singlet ET reactions have similar $^{1}k_f$'s (Table 3). Additionally, the respective oxidative and reductive ET charge separation processes for the $[^1\text{ZnMb}:\text{cyt b}_5]$ complexes have driving forces that are within $\delta(-\Delta G^0) \sim 0.1$ eV (Scheme S1) of those for the $[^1\text{MgMb}:\text{cyt b}_5]$ complexes (Scheme 1; Table 1), accounting for the M-independence of ET rate constants. In short, the apparently surprising similarities in the $^{1}k_f$ for the oxidative and reductive quenching of $[^1\text{ZnMb}$ and $[^1\text{MgMb}$ are understandable on simple energetic grounds.

Figure S4. $FC$ term as a function of driving forces ($-\Delta G^0$) and $\lambda = 1.0$ eV for ET in the $[^1\text{ZnMb}$ complexes with cyt $b_5$. Oxidative quenching or ET from $[^1\text{ZnMb}$ to Fe$^{3+}$ cyt $b_5$ is in red and reductive quenching or ET from Fe$^{2+}$ cyt $b_5$ to $[^1\text{ZnMb}$ is in blue.
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