Origin of the Conformational Heterogeneity of Cardiolipin-bound Cytochrome *c*

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SI Materials and Methods

Apocyt *c*. Ag_2SO_4 (80 mg) in 9 mL of water and 0.8 mL of acetic acid was added to cyt *c* (48 mg, Sigma Catalog No. C2506) dissolved in 1 mL of water. The solution was incubated in the dark for 4 hours at 44 ^oC and then dialyzed against 0.2 M acetic acid to remove the excess amount of Ag_2SO_4. The apoprotein was precipitated from the heme by addition of 10 volumes of cold (4 ^oC) acidic acetone (1 mL of 5 N H_2SO_4 and 100 mL of acetone). The pellet was collected by centrifugation and washed several times with acidic acetone, then resuspended in 2 mL of 0.05 M NH_4OAc solution (pH 5.0) containing 6 M GuHCl and 1 M dithiothreitol. The solution was let stand at 25 ^oC for 2 hours in the dark and was then centrifuged to remove a greenish yellow precipitate. The supernatant was dialyzed against 10 mM sodium phosphate buffer (pH 7.4) before application onto a HiTrap^{TM} SP HP column (GE Healthcare). The protein was eluted with a linear gradient of NaCl from 0 to 1.0 M in the same buffer.

Zncyt *c*. Cyt c (36 mg) was weighed and transferred to a 10 mL Teflon beaker. 4 mL of hydrogen fluoride pyridine (~70% HF, Sigma-Aldrich 184225) was added into the beaker and the solution was stirred in the dark for 3 min. HF was removed afterwards by evaporating under N_2 stream for 30 min. The remaining solution was applied to a desalting column using 50 mM NaOAc solution (pH 5.0) as an eluent. The colored portion was collected and dialyzed against 50 mM NaOAc (pH 5.0) and then 30 mM Tris-HCl (pH 7.0) buffer solutions. The dialyzed protein was diluted by 10 mL 6 M GuHCl solution in 100 mL Tris-HCl buffer (pH 7.0). A small amount of zinc acetate was dissolved in 2 mL 100 mL Tris-HCl buffer (pH 7.0) and transferred to the protein solution in 6 M GuHCl. The mixture was placed in a 50 ^oC water bath for 90 min in the dark. The reaction was monitored by UV-visible spectroscopy. Shift of the Soret band to 423 nm indicated the completion of the reaction. The solution was dialyzed against 10 mM Tris-HCl

buffer (pH 7.5) before application onto a HiTrap™ SP HP column (GE Healthcare). The protein was eluted with a linear gradient of NaCl from 0 to 0.75 M in the same buffer. Since Zncyt *c* is extremely light-sensitive, the derivative preparation, purification, and subsequent experiments were conducted in the dark with red light.

Preparation of Lipid Vesicles. Chloroform stocks of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,1',2,2'-tetraoleoyl-cardiolipin (TOCL) in a desired molar ratio, both from Avanti Polar Lipids, were dried under a stream of nitrogen gas and resuspended in a 25 mM HEPES buffer pH 7.4 to a final lipid concentration of 2.2 mM. The unilamellar vesicles were prepared by extrusion through a 0.1, 0.2, or 0.6 μm SPI-Pore membrane (11 cycles). Vesicle sizes were determined at room temperature by dynamic light scattering measurement with DynaPro NanoStar™ (Wyatt Technology Europe GmbH). Liposome solutions were stored at room temperature and used within 5 days. No evidence of vesicle aggregation was detected during this time period.

Protein-Liposome Binding Assays. Solutions containing different amounts of liposomes in a 25 mM HEPES buffer at pH 7.4 and identical concentrations of protein were prepared. Samples were mixed by pipetting and incubated 30 min prior to centrifugation. The samples were centrifuged for 60 min using a Beckman Airfuge tabletop ultracentrifuge equipped with a Beckman A-11 rotor. The centrifuge was run at 160,000× *g* using compressed nitrogen. After completion of each run, the supernatant was immediately removed and its absorption spectrum was measured. Bound (pelleted) protein was calculated as total minus free. No protein reduction was observed during the experiment. The protein concentrations were between 5.0 and 6.0 μM.

Model Compound and Quantum Yield Calculation. The model compound Bim-Cys was prepared from bimane iodoacetamide and *N*-acetyl cysteine as described.[1] The quantum yield Φ

of Bim-Cys was measured using quinine sulfate (Aldrich) in 0.1 N H_2SO_4 (Φ = 54.6%) as the reference *R* with the following equation:[1]

$$\Phi = \Phi_R \frac{A_R}{A} \frac{I}{I_R} \frac{n^2}{n_R^2} \tag{S1}$$

In Eq. S1, *A* is the absorbance at the excitation wavelength (386 nm for Bim), *I* is the integrated emission, and *n* is the solution refractive index. Solution refractive indexes were determined with an AO Scientific Instrument ABBE Mark II digital refractometer.

Critical Distance R_0 Calculation. The critical distance, R_0, for Bim (***D***) and heme in cyt *c* (***A***) was calculated according to Eq. S2,[2]

$$R_0^6 = 8.785 \times 10^{-5} \left(\frac{\kappa^2 \Phi_D}{n^4} \right) \int F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda \tag{S2}$$

where κ^2, the value of the orientation parameter, was taken as 2/3 (validated with time-resolved fluorescence anisotropy measurements), Φ_D is the fluorescence quantum yield of the donor (18% for Bim), *n* is the refractive index of the solution, F_D is the normalized fluorescence spectrum of the donor, and *A* is the molar absorbance spectrum of the acceptor.

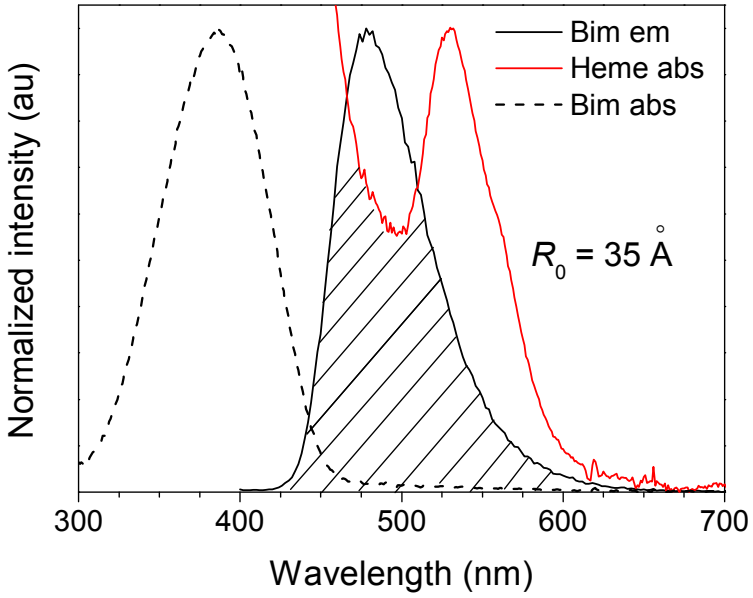

Figure S1. Spectral overlap of Bim-Cys emission and cyt *c* ferric heme absorption. The critical distance, R_0, was calculated to be 35 Å according to Eq. S2.

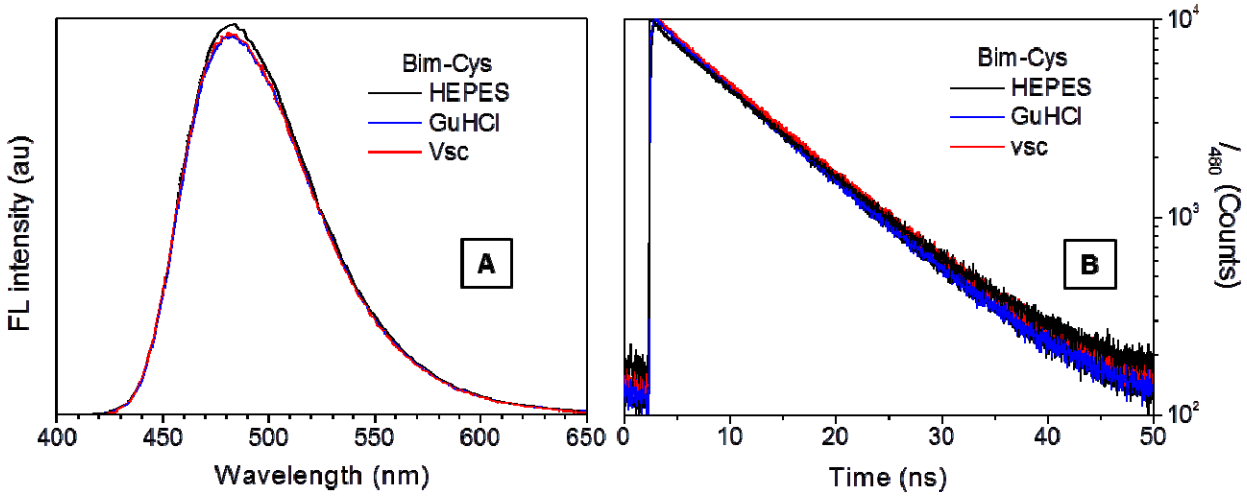

Figure S2. Steady-state fluorescence (λ_{ex} = 386 nm) and fluorescence decay curves of the model compound Bim-Cys in 6 M GuHCl at pH 7.4, and with TOCL/DOPC (50 mol % CL , 750 µM total lipid) vesicles in a 25 mM HEPES buffer at pH 7.4.

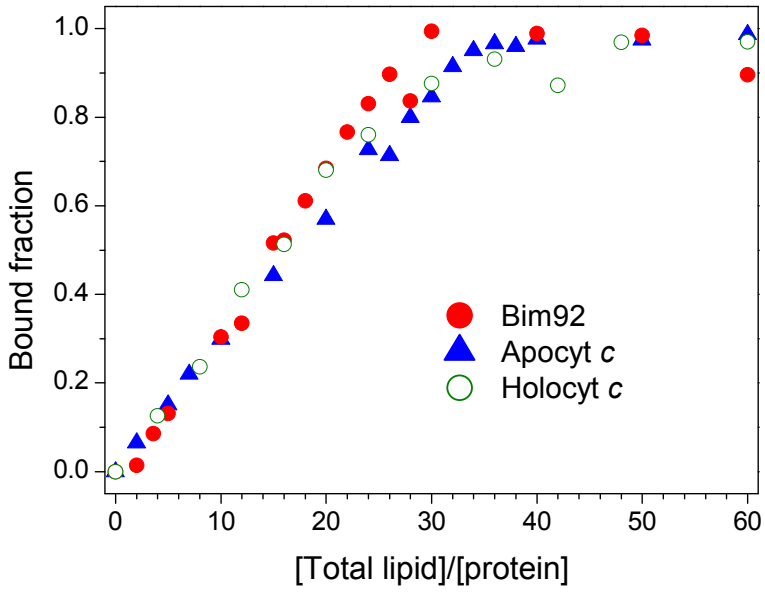

Figure S3. Fraction of cyt *c* remaining in the supernatant after ultracentrifugation of cyt *c*-vesicle solutions at various concentrations of total lipid. The protein concentrations were between 5.0 and 6.0 μM.

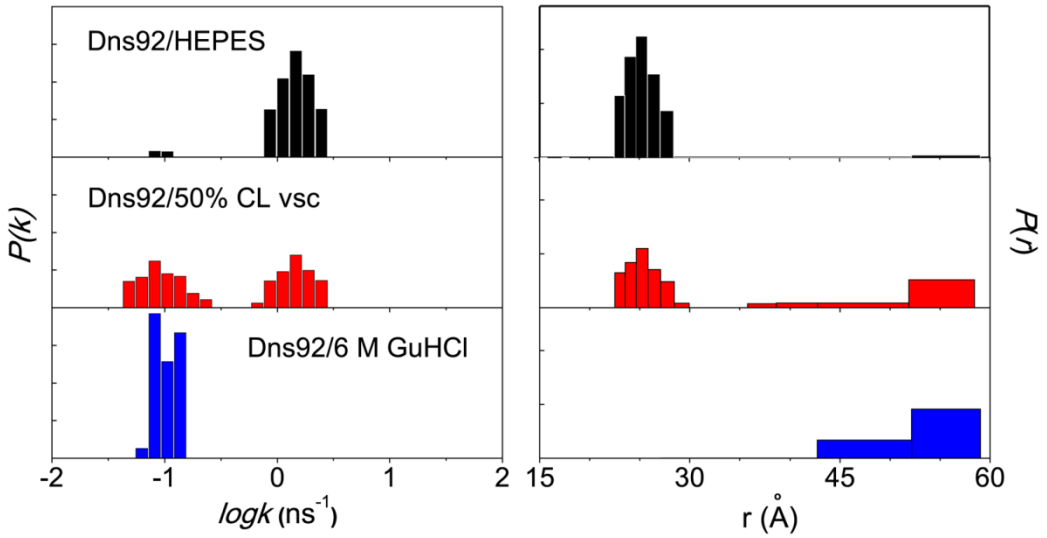

Figure S4. Distributions of rate constants $P(k)$ (*left*) and ***D–A*** distances $P(r)$ (*right*) for Dns92 cyt *c* in a 25 mM HEPES buffer at pH 7.4, with TOCL/DOPC (50 mol % CL, 750 μM total lipid) vesicles, and in 6 M GuHCl solution at pH 7.4. At distances longer than 59 Å, energy transfer rate constants and ***D-A*** distances cannot be determined reliably; the structures with $r \geq 59$ Å are represented by a single bar.

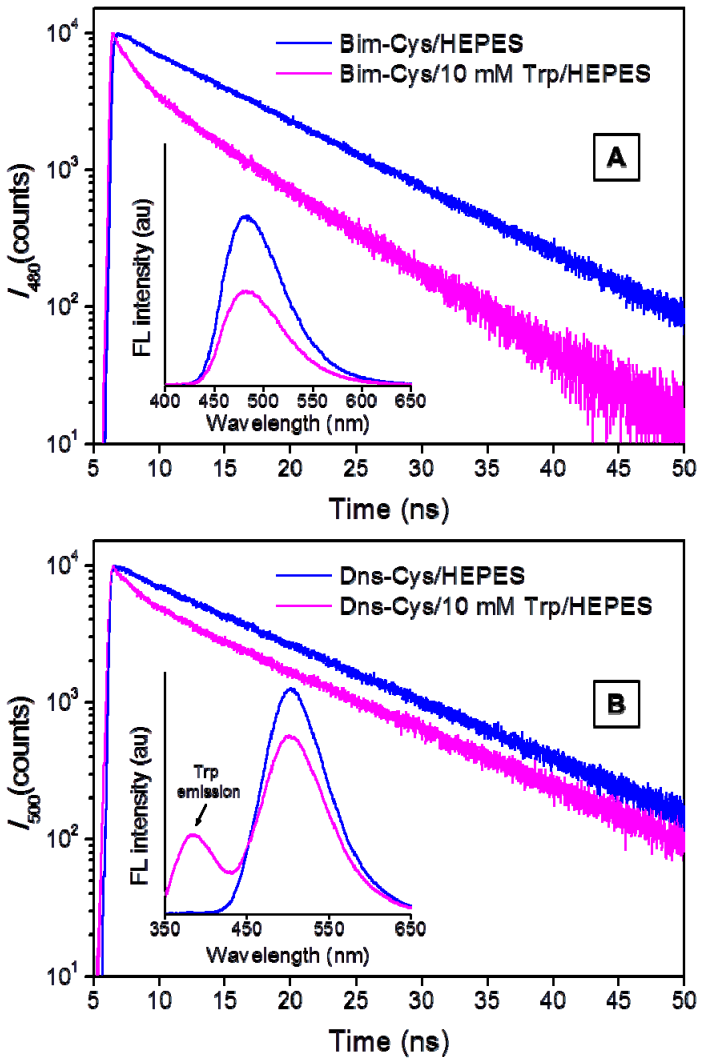

Figure S5. Fluorescence decay curves and steady-state spectra of the model compounds (A) Bim-Cys and (B) Dns-Cys both with and without 10 mM L-tryptophan (Trp) in a 25 mM HEPES buffer at pH 7.4.

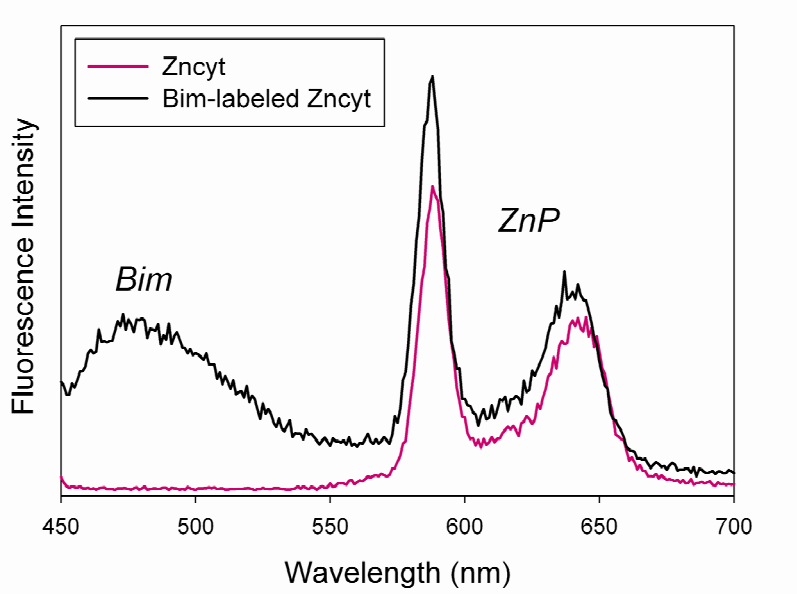

Figure S6. Fluorescence spectra (λ_{ex}=386 nm) of Zncyt and Zncyt labeled with the bimane group at residue 104 in a 100 mM sodium phosphate buffer pH 7.4. [Zncyt] = 1 μM.

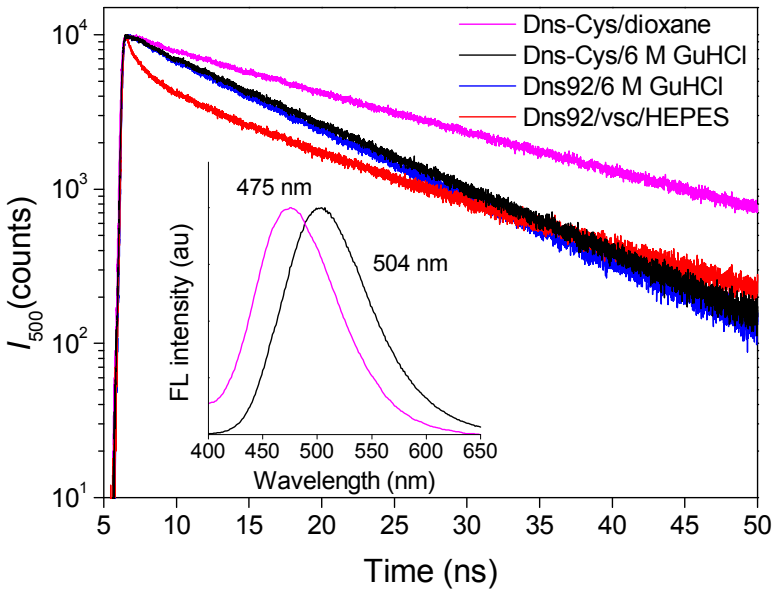

Figure S7. Fluorescence decay curves of the model compound Dns-Cys in dioxane and 6 M GuHCl solution as well as Dns92 in a 25 mM HEPES buffer at pH 7.4 with TOCL/DOPC (50 mol % CL, 750 μM total lipid) liposomes and in 6 M GuHCl solution at pH 7.4. The decay curves of Dns-Cys can be fitted to a single exponential function with lifetimes (τ) of 16.16 ± 0.04 and 9.87 ± 0.02 ns in dioxane and in GuHCl, respectively. The decay curves of Dns92 are fitted by a double exponential function, with longer-lived lifetimes (τ_{long}) of 12.45 ± 0.09 and 9.48 ± 0.02 ns with liposomes and in GuHCl solution respectively. *Inset*: Normalized fluorescence spectra of Dns-Cys (λ_{ex} = 336 nm) in dioxane and 6 M GuHCl solution. The numbers represent the emission maxima of the spectra.

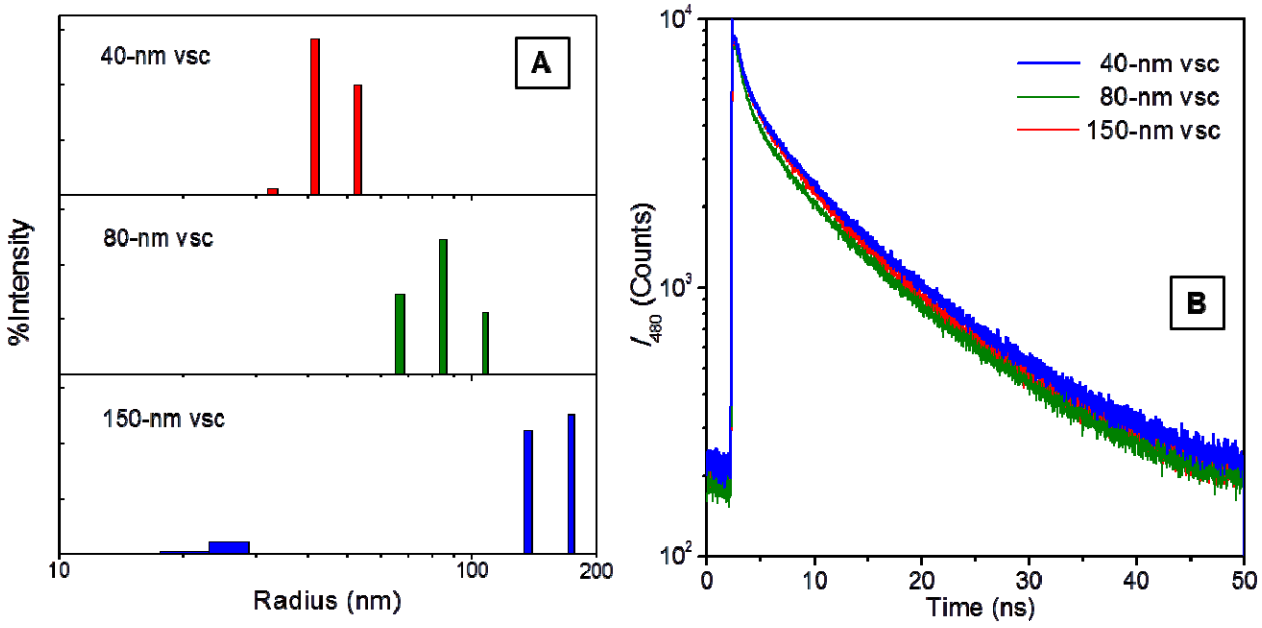

Figure S8. (A) Size distribution of TOCL/DOPC (50 mol % CL, 750 μM total lipid) liposomes and (B) TR-FRET decay curves of liposome-bound Bim92 with different radii in a 25 mM HEPES buffer at pH 7.4.

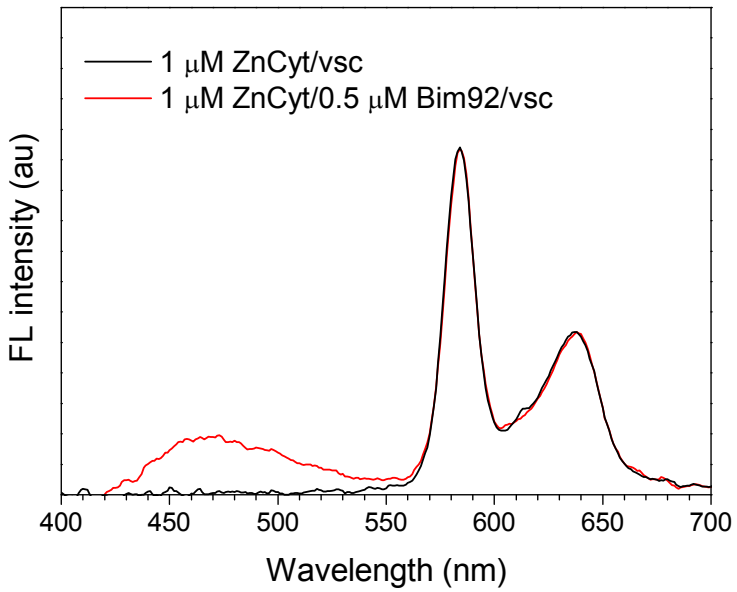

Figure S9. Fluorescence spectra (λ_{ex} = 386 nm) of ZnCyt with and without added Bim92 in the presence of TOCL/DOPC liposomes (50 mol % CL, 750 μM total lipid).

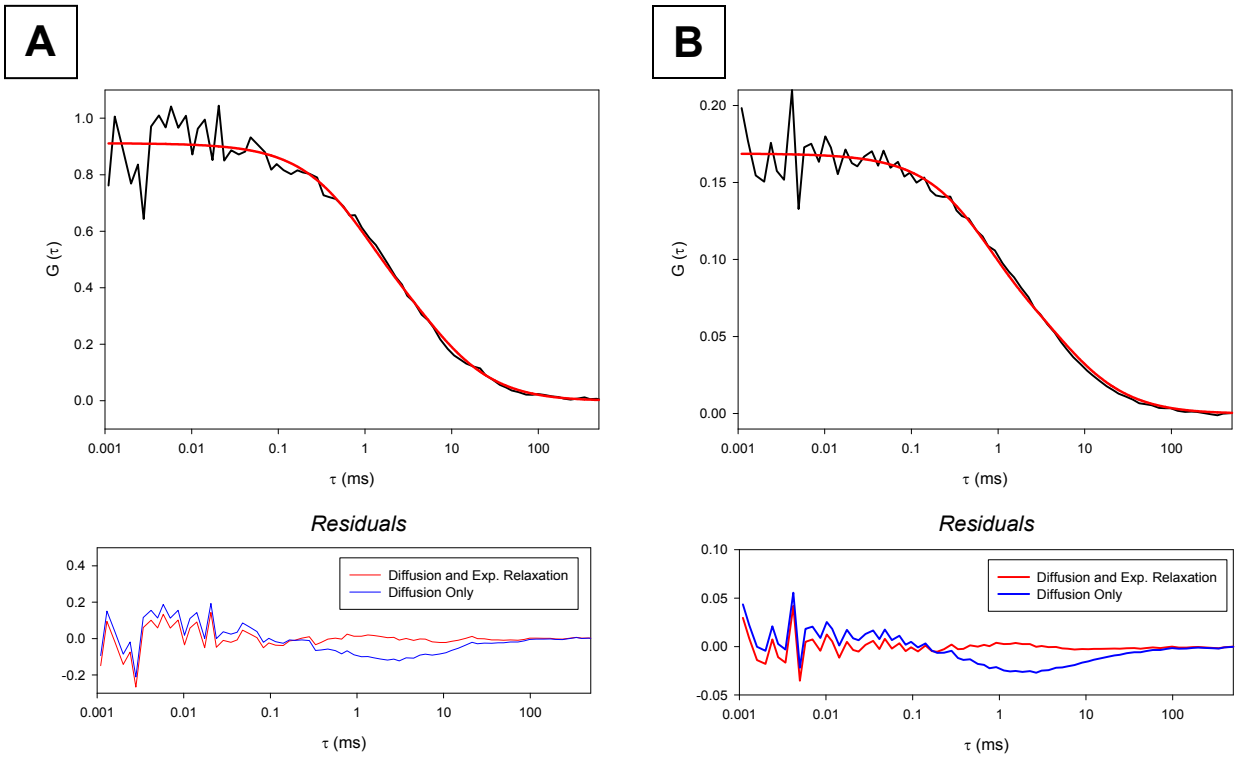

Figure S10. Autocorrelation curves of Bim92 cyt *c* in a 25 mM HEPES buffer at pH 7.4 with TOCL/DOPC vesicles (50 mol % CL). (A) 10 nM protein and 750 µM total lipid and (B) 50 nM protein and 75 µM total lipid. Lower panels show fit residuals of the corresponding autocorrelation curves with (*red*) and without (*blue*) an exponential relaxation term.

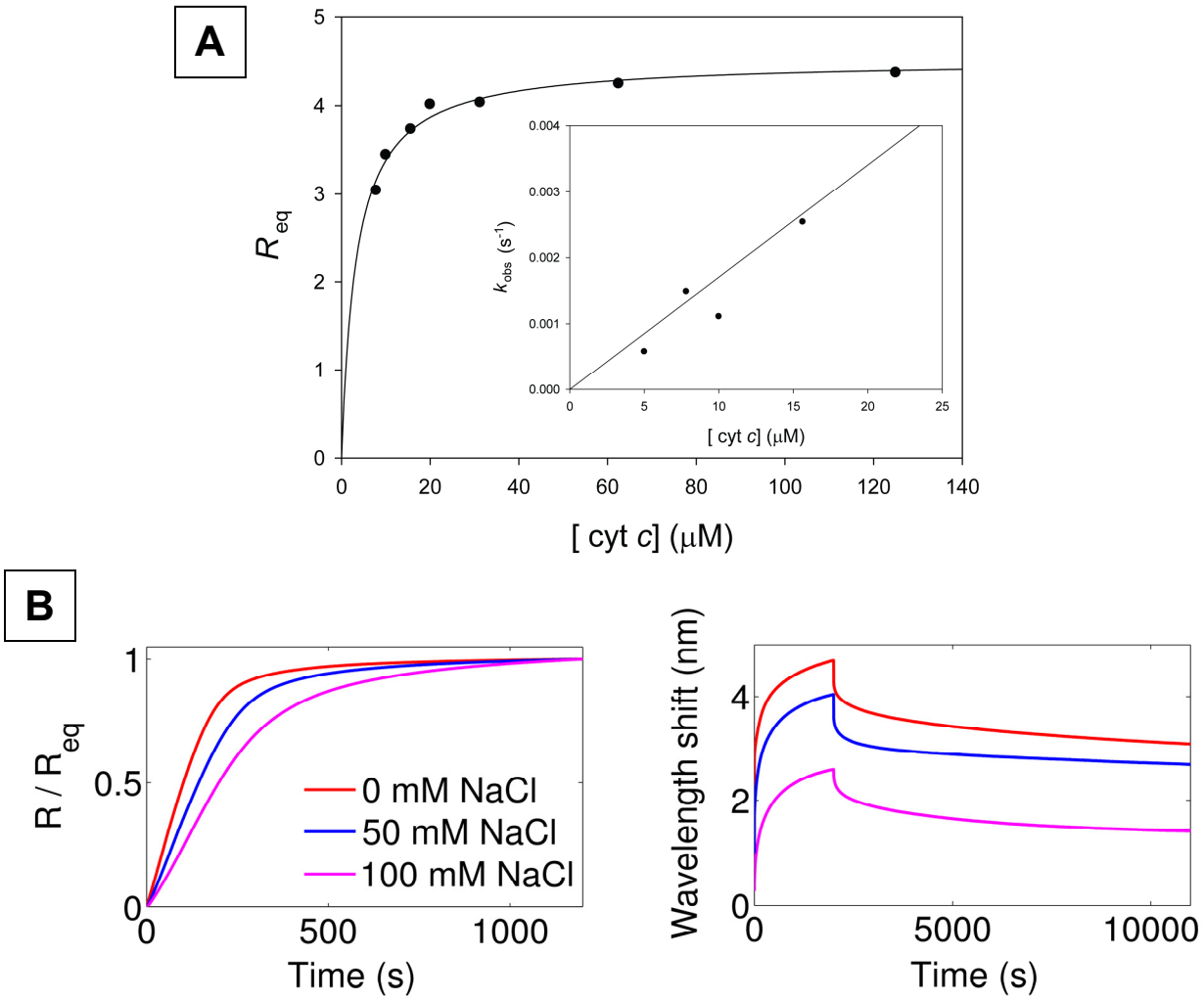

Figure S11. (A) Dependence of the equilibrium wavelength shift R_{eq} on the concentration of mobile cyt *c*. Vesicles (50 mol% CL) were captured by binding to cyt *c* immobilized on a streptavidin biosensor tip and interactions between mobile cyt *c* and captured vesicles (Figure 7, step 2) in a 25 mM HEPES buffer at pH 7.4 were examined. Fit of the R_{eq} dependence to Eq. 7 yielded the apparent K_a value of $(3.0\pm0.3)\times10^5$ M^{-1}. (*Inset*) Linear regression of the k_{obs} values at lower concentrations of mobile cyt *c* (Figure 7, step 2) yielded the apparent k_{on} value of 171±7 $M^{-1}s^{-1}$. The rise curves at higher concentrations of cyt *c* (>20 μM) could not be fit well to a monoexponential function. (B) (*left*) Association of vesicles (50 mol% CL, 44 μM) with immobilized cyt *c* (Figure 7, step 1) at indicated concentrations of NaCl. All traces were normalized to their respective R_{eq} values for ease of comparison. (*right*) Association (0 to 2000 s) and dissociation (2000 to 11000 s) of mobile cyt *c* (20 μM) to immobilized vesicles (Figure 7, steps 2 and 3).

Table S1. Thermodynamic Parameters for the Unfolding Transitions of Wild-type (wt) and Dye-labeled Variants of Horse Heart Cytochrome *c*[a]

	CD[b]			Heme Absorption			Dye Fluorescence[c]		
Variant	$[GuHCl]_{1/2}$	m_D	$-\Delta G^0_f$	$[GuHCl]_{1/2}$	m_D	$-\Delta G^0_f$	$[GuHCl]_{1/2}$	m_D	$-\Delta G^0_f$
	M	kJ mol^{-1} M^{-1}	kJ mol^{-1}	M	kJ mol^{-1} M^{-1}	kJ mol^{-1}	M	kJ mol^{-1} M^{-1}	kJ mol^{-1}
wt[d]	2.701 ± 0.059	11.5 ± 2.6	31.1 ± 7.1	2.595 ± 0.068	11.3 ± 2.7	29.3 ± 7.1	n.a.[e]	n.a.[e]	n.a.[e]
Bim92	2.588 ± 0.144	9.3 ± 3.9	23.9 ± 11.5	2.518 ± 0.103	12.7 ± 5.2	32.2 ± 14.4	2.668 ± 0.058	14.7 ± 4.7	39 ± 13
Dns92[d]	2.427 ± 0.028	14.1 ± 1.6	34.2 ± 3.9	2.438 ± 0.029	15.4 ± 2.1	37.5 ± 5.1	2.449 ± 0.056	19.5 ± 5.9	48 ± 14

[a] At pH 7.4 ± 0.1 and room temperature. The protein concentrations were between 5 to 8 μM. [b] Monitored is ellipticity at 222 nm. [c] Steady-state measurement of fluorescence intensities. [d] Data from ref. 3 and shown for comparison.[3]

References

(1) Pound, G. J., Pletnev, A. A., Fang, X., Pletneva, E. V. *Chem. Commun.* **2011**, *47*, 5714-5716.
(2) Lakowicz, J. R. "Principles of Fluorescence Spectroscopy"; 2nd ed.; Kluwer Academic/Plenum Publishing: New York, 1999.
(3) Hanske, J., Toffey, J. R., Morenz, A. M., Bonilla, A. J., Schiavoni, K. H., Pletneva, E. V. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 125-130.