Multifaceted Effects of ATP on Cardiolipin-Bound Cytochrome *c*

Erik J. Snider, Julia Muenzner, Jason R. Toffey, Yuning Hong, and Ekaterina V. Pletneva*

†Department of Chemistry, Dartmouth College, Hanover, NH 03755

*Corresponding author: ekaterina.pletneva@dartmouth.edu

Submitted to *Biochemistry*

Supporting Information

Supporting Information Materials and Methods

Preparation of Labeled Variants, Experimental Setup and Analysis. Dns-labeled variants of horse heart cyt *c* were prepared and characterized as described.[1] Unfolding parameters from three different spectroscopic techniques for the new (Dns50, Dns99, and Dns104) as well as previously examined variants[1] are listed in Table S1. TOCL/DOPC liposomes (50 mol% CL) were prepared by extrusion[2] and used within 2-3 days of preparations. TR-FRET, ultracentrifugation, BLI experiments and their analyses were done as described.[1,2] Under our conditions here (TR-FRET and peroxidase assays) the average available surface area per protein was 96 nm^2, greater than the generous estimate of 74 nm^2 required for binding of one unfolded cyt *c*.

Cyt *c* mutants Glu92Cys and Lys99Cys were labeled with *N*,*N*'-dimethyl-*N*-(iodoacetyl)-*N*'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylenediamine and Alexa Fluor® 488 C_5 Maleimide (Life Technologies) using standard procedures.[1] The labeled variants were freshly purified by ion-exchange chromatography prior to FCS experiments. FCS measurements were done with a Nikon T*i* Eclipse inverted confocal microscope equipped with a PicoQuant FCS system as previously described.[2] The samples (50 nM labeled cyt *c*) were excited with a 485 nm pulsed laser diode (PicoQuant) and a Nikon Plan Apo IR 60× WI 1.27NA water objective was employed.

Analyses of FCS results were performed using SymPhoTime software (PicoQuant) followed by curve fitting in Sigma Plot 10.0 (Systat Software). Previously described models incorporating a diffusional and a conformational exchange terms (**D+EX**) yielded time constants τ_d for diffusion of liposomes carrying CL-bound cyt *c* and τ_{ex} for the protein conformational exchange.[2,3] Satisfactory fits of FCS curves for Alx99

also required inclusion of the triplet state component (τ_{tr}=3 μs; **D+EX+T**).[4] Measurements with cyt *c* and TOCL/DOPC liposomes (without added ATP or salt) yielded consistent values of the liposome diffusion time τ_d of ≈2 ms with both labeled variants (Figures S5 and S6). While the exchange component (τ_{ex}=0.2-0.3 ms) was clearly evident for Alx99, it was not resolvable for NBD92, reflecting constrained mobility of this hydrophobic label upon insertion into the lipid bilayer (Figure S5). Analysis of FCS curves for native Alx99 yielded τ_d=0.13-0.16 ms and τ_{ex}=20-30 μs, in accord with previous studies of dye-labeled cyt *c*.[3,4] Weak fluorescence of native NBD92 (Figure S5a) prevented its observation by FCS.

Effectors (NaCl, ATP, and other polyphosphates) were added to the buffer first to prevent liposome agglomeration that was noted when liposomes came in contact with high salt concentrations. Liposome solutions were next added to the dilute salt stocks, to a final total lipid concentration of 750 μM. Protein was added last, as one fifth of the total sample volume. Careful adherence to this experimental procedure ensured good reproducibility of results. Liposome-containing samples were incubated for at least 1 hour before data collection. The concentration of cyt *c* in all samples was 3 μM unless otherwise noted.

Peroxidase Activity Assays. Formation of oxidized 2,2'azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS)[5] catalyzed by cyt *c* (3 μM) was monitored by absorption at 415 nm on an Agilent 8453 diode-array spectrophotometer. Samples (in a 25 mM HEPES buffer at pH 7.4; with TOCL/DOPC liposomes (50 mol% CL, 750 μM total lipid); with TOCL/DOPC liposomes and 10 mM ATP; with TOCL/DOPC liposomes and 86 mM salt) were incubated with ABTS (50 μM) for one hour before the

reaction was initiated by the addition of hydrogen peroxide (H_2O_2, 1 mM). Concentrations of H_2O_2 were verified spectroscopically (ε_{240}=43.6 $M^{-1}cm^{-1}$).[6] The signal was recorded immediately on addition of H_2O_2, and followed for three minutes. Under these conditions, peroxidase activity of native cyt *c* increased only slightly in the presence of ATP (2-fold increase in the initial rates), these changes cannot account for the higher activity of CL-bound cyt *c* with added ATP compared to that with salt. Control experiments with a lower concentration of H_2O_2 (100 μM) and a higher concentration of ABTS (150 μM) have revealed the same trends in the initial rates of ABTS oxidation (native < CL+salt < CL+ATP < CL, Figure S4a).

Formation of etoposide phenoxyl radicals[7] catalyzed by cyt *c* (3 μM) was monitored by EPR spectroscopy with a Bruker EMX 300 X-band EPR spectrometer. Samples (in a 25 mM HEPES buffer at pH 7.4; with TOCL/DOPC liposomes (50 mol% CL, 750 μM total lipid); with TOCL/DOPC liposomes and 10 mM ATP; with TOCL/DOPC liposomes and 86 mM salt) were incubated with etoposide (100 μM) for 1 hour before the reaction was initiated with the addition of H_2O_2 (100 μM). No reaction was noted in the absence of cyt *c*. Samples were transferred quickly to capillary tubes (1.5×90 mm) and the first spectrum was taken immediately after. Instrument parameters were: 3520 G center field; 100 kHz modulation frequency, and 10.11mW microwave power. The signal was recorded every two minutes for twenty minutes. After baseline correction, the first derivative output of the spectrometer was integrated to give the absorbance signal and then integrated again to give the area representative of the amount of etoposide phenoxyl radical.

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

Variant	CD[b]			Heme absorption			Dns fluorescence[c]		
	$[GuHCl]_{1/2}$, M	m_D, kJ mol^{-1}M^{-1}	$-\Delta G^o_f$, kJ mol^{-1}	$[GuHCl]_{1/2}$, M	m_D, kJ mol^{-1}M^{-1}	$-\Delta G^o_f$, kJ mol^{-1}	$[GuHCl]_{1/2}$, M	m_D, kJ mol^{-1}M^{-1}	$-\Delta G^o_f$, kJ mol^{-1}
Wild-type[d,e]	2.701 ± 0.059	11.5 ± 2.6	31.1 ± 7.7	2.595 ± 0.068	11.3 ± 2.7	29.3 ± 7.8	n.a.[f]	n.a.[f]	n.a.[f]
Dns50	2.582 ± 0.054	11.4 ± 2.2	29.5 ± 5.8	2.373 ± 0.088	9.8 ± 2.3	23.3 ± 5.4	2.642 ± 0.028	11.9 ± 1.3	31.5 ± 3.5
Dns66[e]	2.448 ± 0.057	13.8 ± 3.4	33.8 ± 9.1	2.448 ± 0.078	12.7 ± 3.7	31 ± 10	2.519 ± 0.050	13.4 ± 2.7	33.8 ± 7.5
Dns92[e]	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
Dns99	2.418 ± 0.025	13.5 ± 3.0	32.7 ± 7.2	2.377 ± 0.031	13.3 ± 1.6	31.7 ± 4.0	2.528 ± 0.025	13.4 ± 1.4	33.8 ± 3.5
Dns104	2.714 ± 0.185	7.5 ± 3.7	20.4 ± 10.1	n.d.[g]	n.d.[g]	n.d.[g]	2.742 ± 0.050	10.9 ± 2.1	29.9 ± 5.7

[a]At pH 7.4±0.1 and room temperature. The protein concentrations were between 7 and 11 μM. [b] Ellipticity was monitored at 222 nm. [c]Steady-state measurements of fluorescence intensities. [d]Obtained from equine heart (Sigma, C2506) and freshly purified by ion exchange chromatography. [e]From ref. *1*. [f]Not applicable. [g]The parameter was not determined.

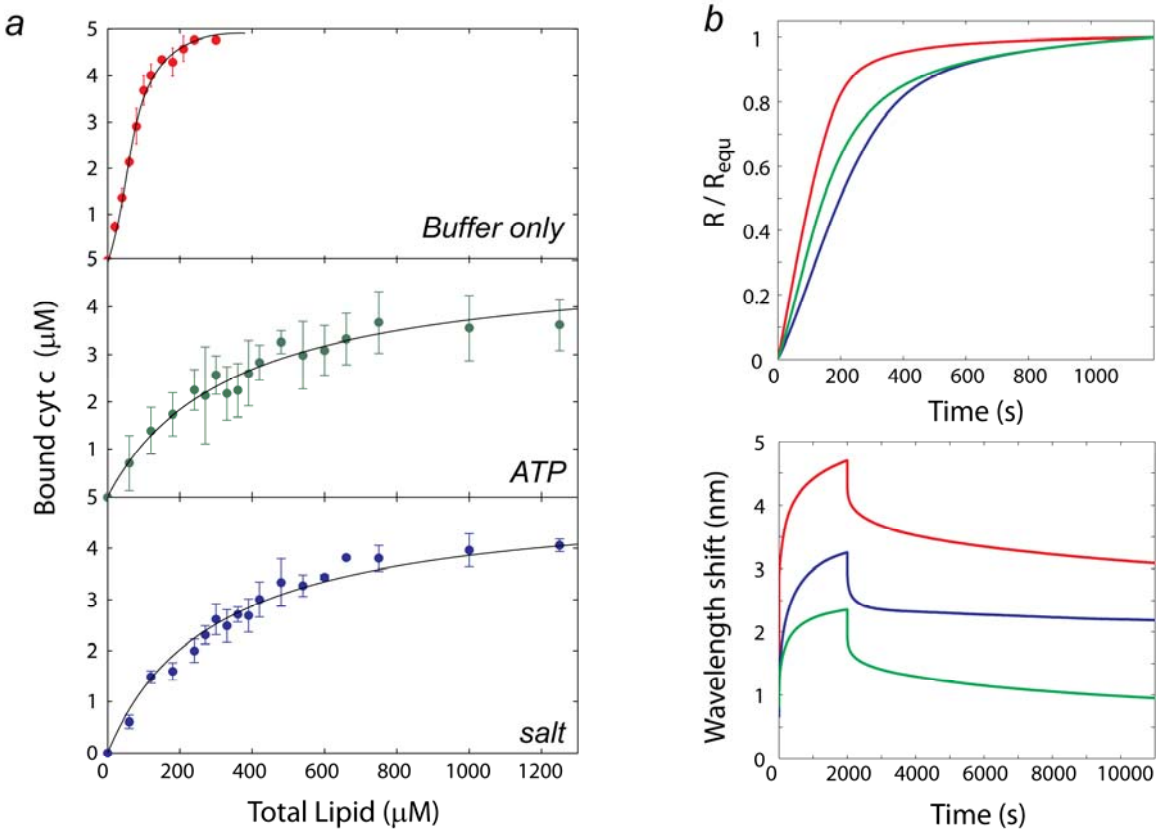

Figure S1. (*a*) Cyt *c* remaining in the supernatant after ultracentrifugation of cyt *c*-liposome solutions in a 25 mM HEPES buffer at pH 7.4 at various concentrations of total lipid (TOCL/DOPC liposomes, 50 mol% CL). The lines are not fits, shown to guide the eye only. (*b*) Biolayer interferometry experiments. Association (*top*) of TOCL/DOPC liposomes (50 mol% CL, 44 μM total lipid) with cyt *c* immobilized on a streptavidin biosensor tip in a 25 mM HEPES buffer alone and with added ATP and salt. Association (0 to 2000 s) and dissociation (2000 to 11000 s) of mobile cyt *c* (20 μM) to immobilized liposomes (*bottom*). The measurements were done as described (ref. *2*). The color scheme is the same as in the panel *a*.

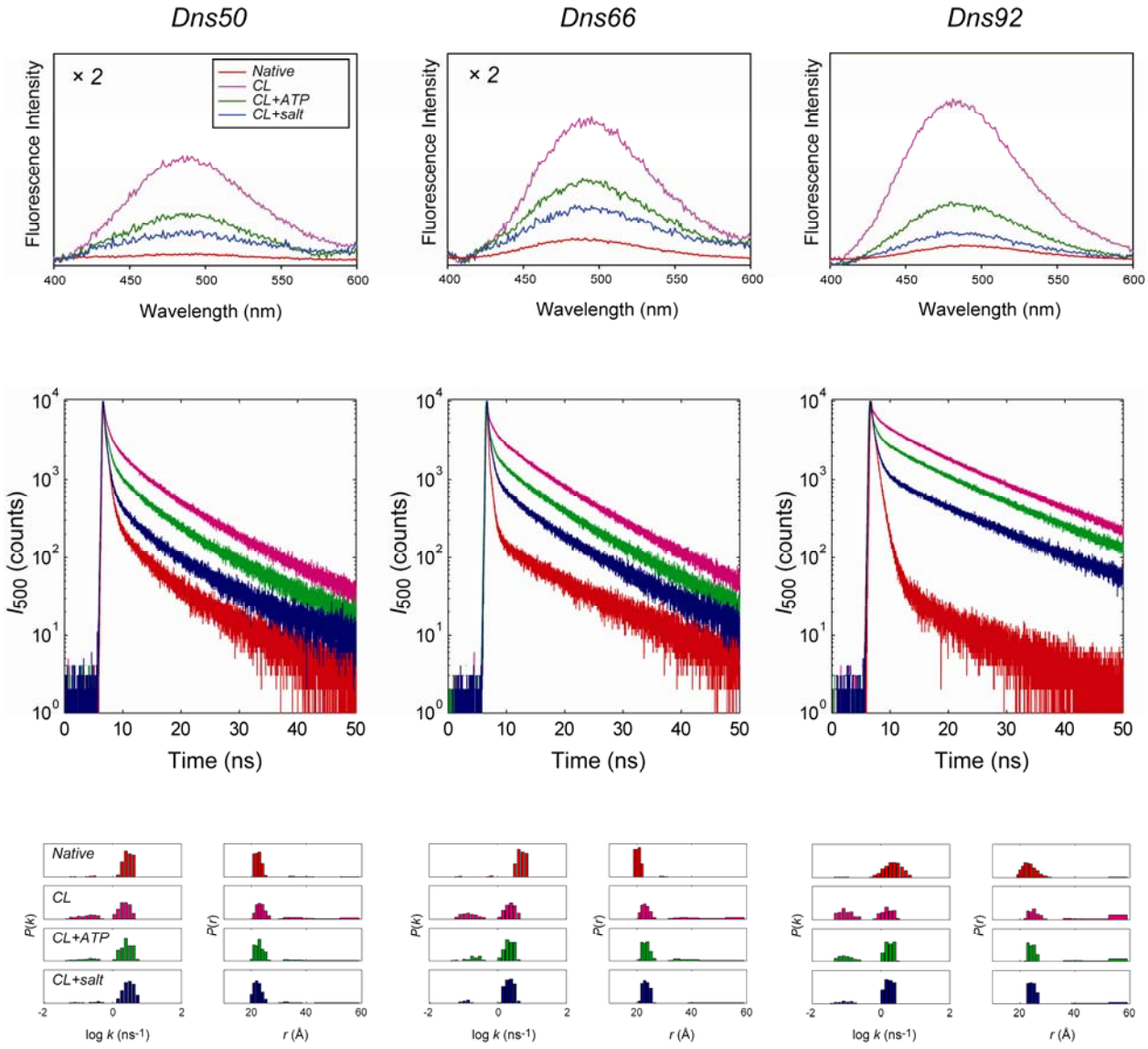

Figure S2. Fluorescence spectra (λ_{ex}=336 nm, *top*), TR-FRET kinetics (*middle*), and distributions of rate constants $P(k)$ and Dns-heme distances $P(r)$ (*bottom*) for five Dns variants in a 25 mM HEPES buffer at pH 7.4; with TOCL/DOPC liposomes (50 mol% CL, 750 μM total lipid); with TOCL/DOPC liposomes and 10 mM ATP; with TOCL/DOPC liposomes and 86 mM salt. At distances longer than 1.5×R_0=59 Å, energy-transfer rate constants and ***D-A*** distances cannot be determined reliably; the structures with r≥59 Å are represented by a single bar (*continued on the next page*).

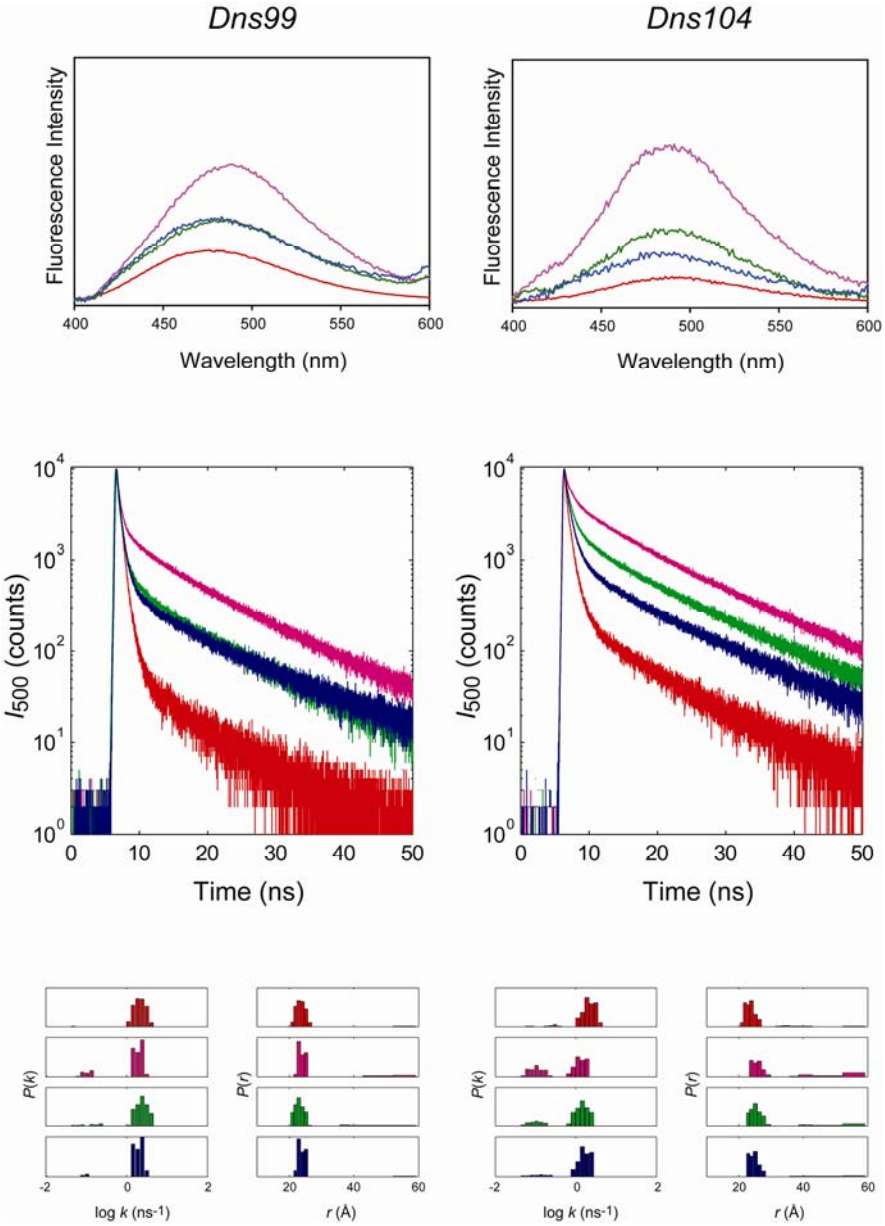

Figure S2 (CONTINUED). Fluorescence spectra (λ_{ex}=336 nm, *top*), TR-FRET kinetics (*middle*), and distributions of rate constants $P(k)$ and Dns-heme distances $P(r)$ (*bottom*) for five Dns variants in a 25 mM HEPES buffer at pH 7.4; with TOCL/DOPC liposomes (50 mol% CL, 750 μM total lipid); with TOCL/DOPC liposomes and 10 mM ATP; with TOCL/DOPC liposomes and 86 mM salt. At distances longer than 1.5×R_0=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.

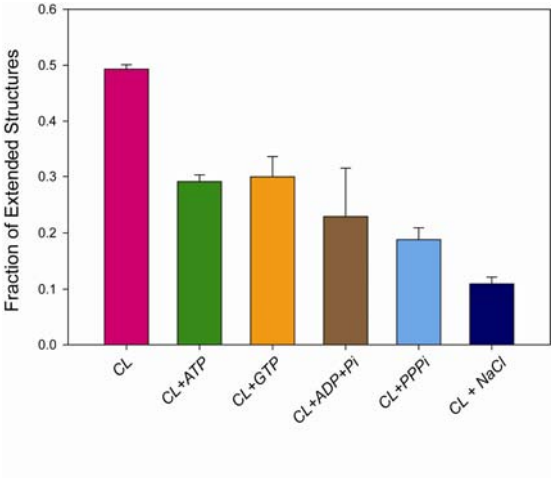

Figure S3. Fraction of extended structures from TR-FRET of Dns92 (3 μM) in a 25 mM HEPES buffer at pH 7.4 with TOCL/DOPC liposomes (50 mol% CL, 750 μM total lipid, *magenta*) and with TOCL/DOPC liposomes and added effectors at identical ionic strength.

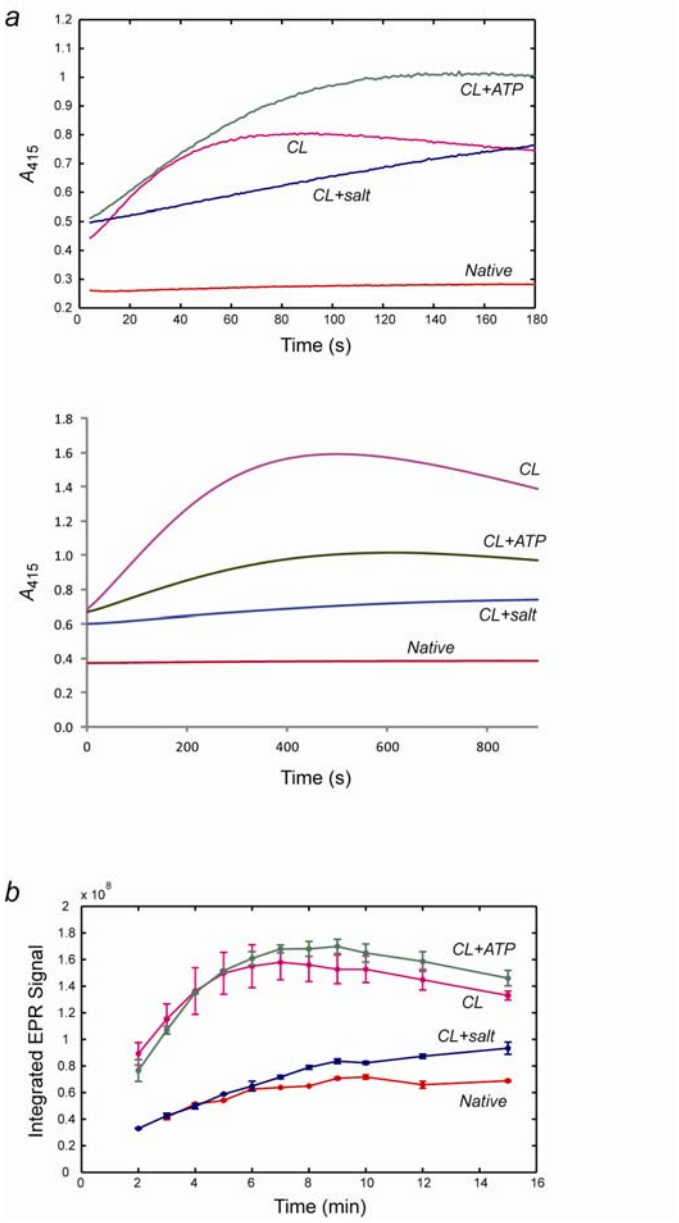

Figure S4. Peroxidase activity of cyt *c* (3 μM) in a 25 mM HEPES buffer at pH 7.4; with TOCL/DOPC liposomes (50 mol% CL, 750 μM total lipid); with TOCL/DOPC liposomes and 10 mM ATP; with TOCL/DOPC liposomes and 86 mM salt monitored by (*a*) ABTS oxidation ([ABTS]=50 μM and [H_2O_2]=1 mM, *top*) and at a lower H_2O_2 concentration (*control*) ([ABTS]=150 μM and [H_2O_2]=100 μM, *bottom*) and (*b*) etoposide phenoxyl radical formation ([etoposide]=100 μM and [H_2O_2]=100 μM) assays. Initial rates of product formation from multiple experiments are presented in Figure 2.

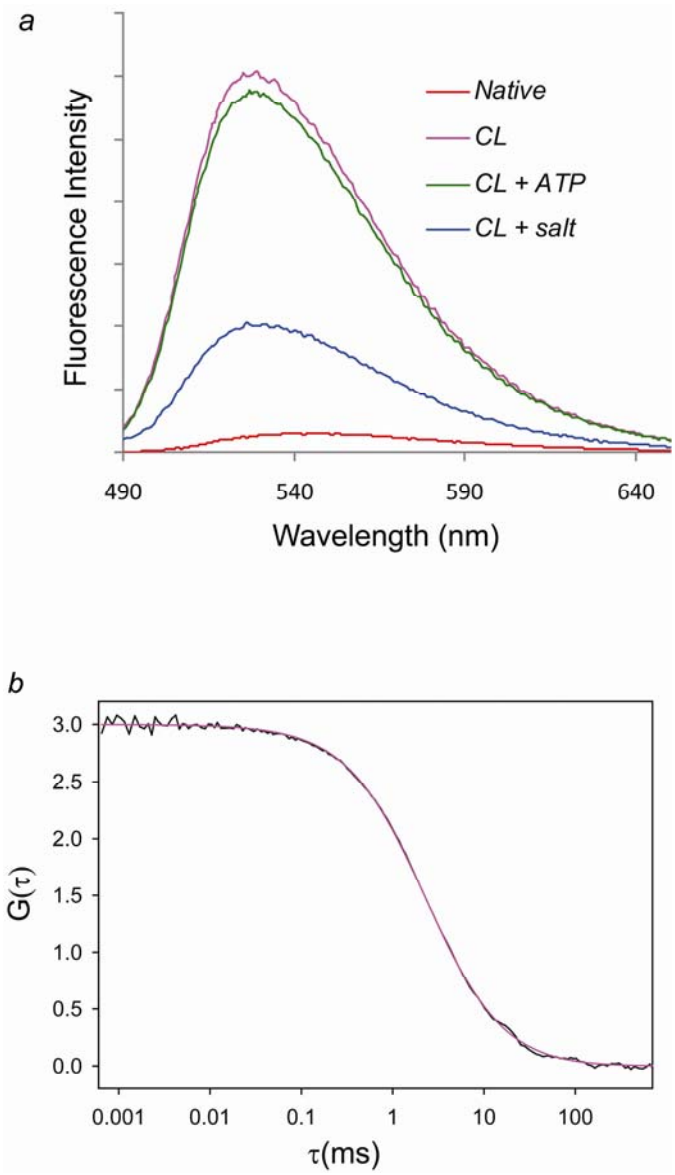

Figure S5. (*a*) Fluorescence spectra of NBD92 cyt *c* (3 μM) in a 25 mM HEPES buffer at pH 7.4; with TOCL/DOPC liposomes (50 mol% CL, 750 μM total lipid); with TOCL/DOPC liposomes and 10 mM ATP; with TOCL/DOPC liposomes and 86 mM salt. (*b*) A representative FCS curve for NBD92 (50 nM) in a 25 mM HEPES buffer at pH 7.4 with TOCL/DOPC liposomes (50 mol% CL, 750 μM total lipid). Data fit (*magenta*) to a diffusional-term only model (**D**) yielded τ_d=2.3 ms.

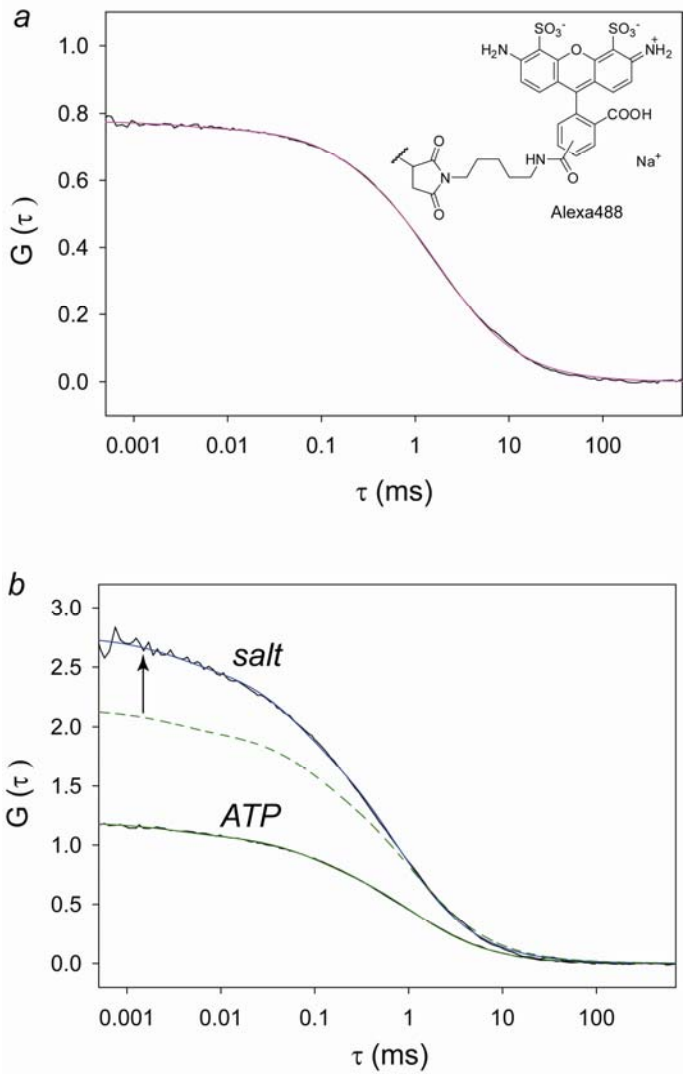

Figure S6. Representative FCS curves for Alx99 (50 nM) in a 25 mM HEPES buffer at pH 7.4 (*a*) with TOCL/DOPC liposomes (50 mol% CL, 750 μM total lipid), *inset* – structure of Alexa488 label; (*b*) with TOCL/DOPC liposomes and added 10 mM ATP or 86 mM salt. Data fit (*magenta,* **D+EX+T**) yielded τ_d=1.8 ms and τ_{ex}=0.22 ms. The higher signal-to-noise (compared to NBD92, Figure 3) FCS curves for Alx99 show only minor differences at shorter times for the protein ensembles with added ATP and salt, consistent with a small increase in the amplitude of the conformational exchange component for CL-bound cyt *c* in the presence of salt. The *dashed green* curve shows the scaled fit of the FCS trace with added ATP to illustrate these differences (*arrow*) between the curves.

References

(1) 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.

(2) Hong, Y., Muenzner, J., Grimm, S. K., Pletneva, E. V. *J. Am. Chem. Soc.* **2012**, *134*, 18713–18723.

(3) Haldar, S., Mitra, S., Chattopadhyay, K. *J. Biol. Chem.* **2010**, *285*, 25314-25323.

(4) Werner, J. H., Joggerst, R., Dyer, R. B., Goodwin, P. M. *Proc. Natl. Acad. of Sci. USA* **2006**, *103*, 11130-11135.

(5) Hirota, S., Hattori, Y., Nagao, S., Taketa, M., Komori, H., Kamikubo, H., Wang, Z., Takahashi, I., Negi, S., Sugiura, Y., Kataoka, M., Higuchi, Y. *Proc. Natl. Acad. of Sci. USA* **2010**, *107*, 12854–12859.

(6) Arnao, M. B., Acosta, M., del Río, J. a., Varón, R., García-Cánovas, F. *Biochim. Biophys. Acta Bioenerg.* **1990**, *1041*, 43-7.

(7) Tyurina, Y. Y., Kini, V., Tyurin, V. A., Vlasova, I. I., Jiang, J., Kapralov, A. A., Belikova, N. A., Yalowich, J. C., Kurnikov, I. V., Kagan, V. E. *Mol. Pharmacol.* **2006**, *70*, 706-17.