pH Induced Switch Between Different Modes of Cytochrome c Binding to Cardiolipin Containing Liposomes

Bridget Milorey[1], Reinhard Schweitzer-Stenner[1], Raghed Kurbaj[1], and Dmitry Malyshka[1i]*

[1]Department of Chemistry, Drexel University, 3141 Chestnut Street, Philadelphia, PA 19104, USA

Submitted: *ACS Omega*

Supporting Information

*Corresponding author: Reinhard Schweitzer-Stenner, Phone: 215-895-2268, Email: rschweitzer-stenner@drexel.edu

[i]Current address: College of Medicine, The Ohio State University, 370 W 9th Avenue, Columbus, OH 43210, USA

Figure S1: Soret band CD and UV-visible absorption spectra of ferricytochrome c measured at different cardiolipin (liposome) concentrations at pH 6.5 in the absence of NaCl. The arrows indicate the spectral response in the direction of increasing CL concentration. The protein concentration was 10 µM. The corresponding CL concentrations are 0, 100, 150, 200, 250, 300, 350, 400, 450, and 500 µM.

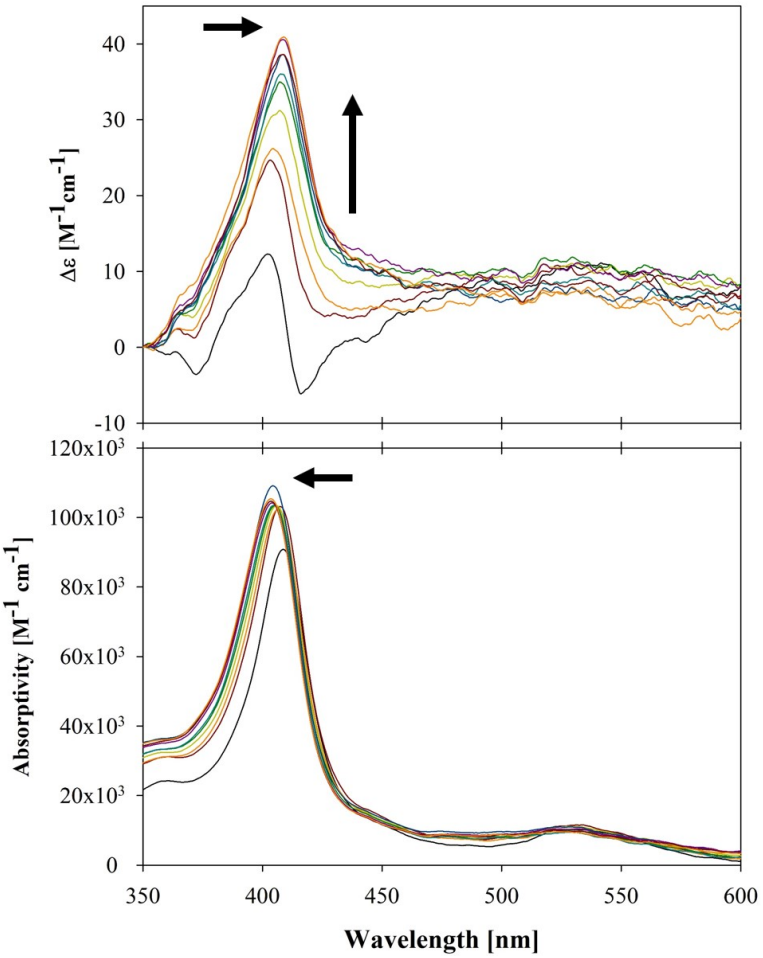


Figure S2: Non-coincidence between the wavenumber position of the positive CD Cotton band and the absorbance Soret band position (spectra shown in Figure S1) plotted as a function of the accessible cardiolipin concentration. The protein concentration was 10 μM.

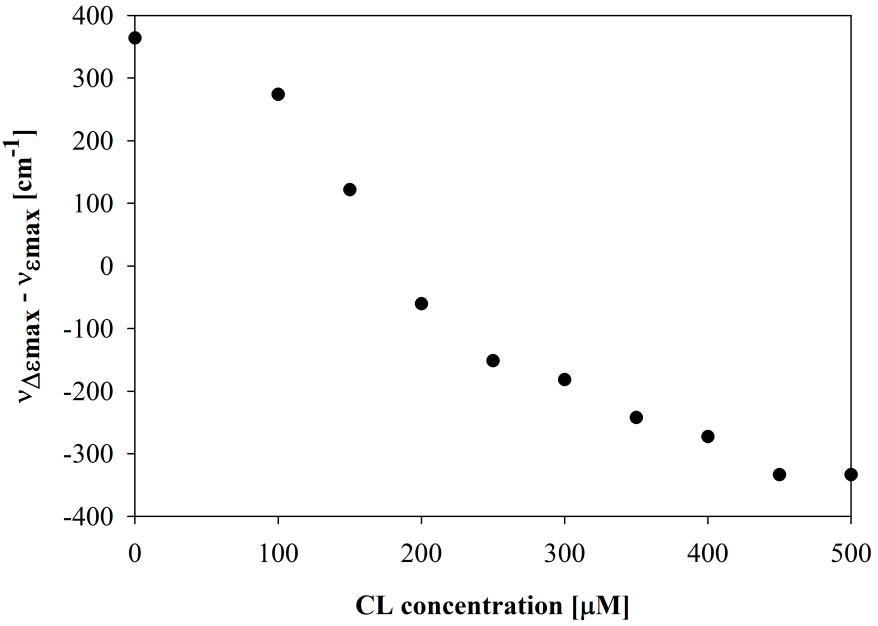
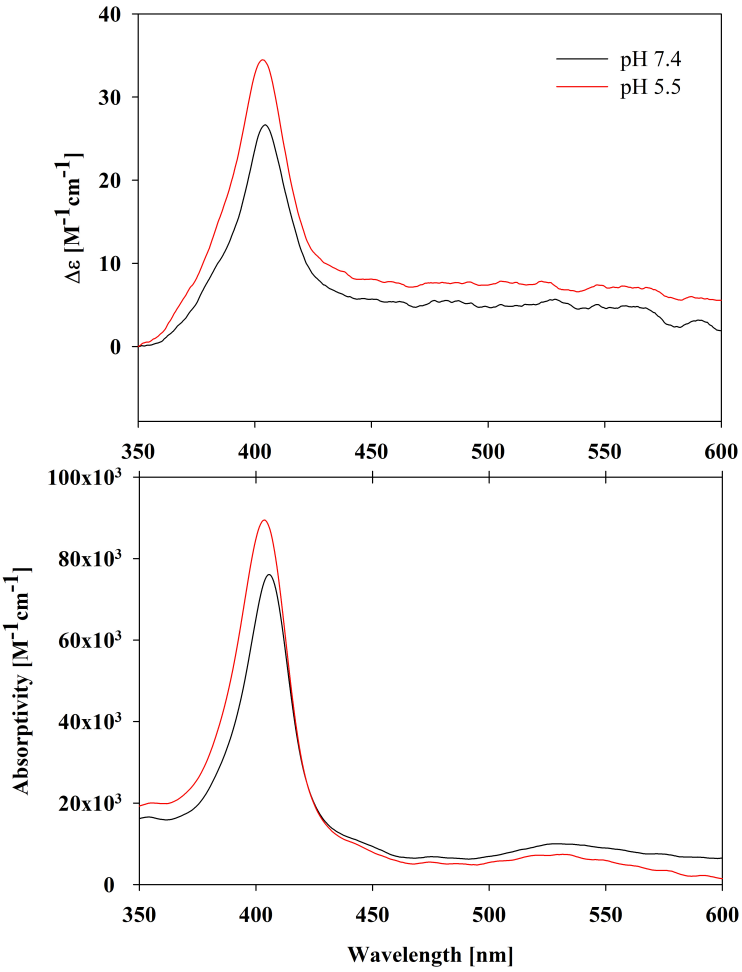


Figure S3: Soret band CD and UV-visible absorption spectra of ferricytochrome c in an 8 M urea solution measured at the indicated pH. The spectra taken at pH 7.4 reflect the hexacoordinate low spin state of the denatured protein where the heme iron is ligated with two histidines.



Deriving spectroscopic response values from fluorescence, CD and absorption spectra

The spectral response plots in Figure 7 were produced as follows. After background subtraction, the F-band of the fluorescence spectra were decomposed into four Gaussian sub-bands using our program MULTIFIT. The same wavenumber positions and halfwidths were used for spectra taken in the absence and presence of NaCl. The total integrated intensity of four sub-bands was plotted as a function of the CL concentration. In addition to the total fluorescence intensity, we also measured the polarized fluorescence intensities I_{VV} and I_{VH} at 330 nm (v and h: vertically and horizontally polarized, the first subscript represents excitation, the second one the fluorescence). For the plot of CD-changes we departed from our earlier protocol[1] by now utilizing the change of the CD-Soret band:

$$\Delta\Delta\varepsilon = \Delta\varepsilon\left(\left[CL\right]\right) - \Delta\varepsilon\left(0\right) \qquad \text{(S1)}$$

where $\Delta\varepsilon(0)$ is the dichroism value of the protein in solution. Figure S4 depicts the corresponding difference spectra for different cardiolipin concentrations. For the display of the spectral response in Figure 7 we employed the difference value obtained for 413 nm ($\Delta\Delta\varepsilon_{413}$). Our switch from the earlier utilized response parameter (i.e. the difference between the values at the position of the positive and negative maximum of the native state couplet)* to that defined by eq. (1) was motivated by the greater sensitivity of the latter with regard to the spectral changes observed at pH 6.5.

Figure S4: Soret band CD difference spectra of ferricytochrome c calculated by subtracting the spectrum of the native protein from the spectrum measured at the indicated cardiolipin concentration. Difference spectra reflecting the spectral response in the absence as well as in the presence of 50 mM and 100 mM NaCl are shown in the left, middle and right figure, respectively.

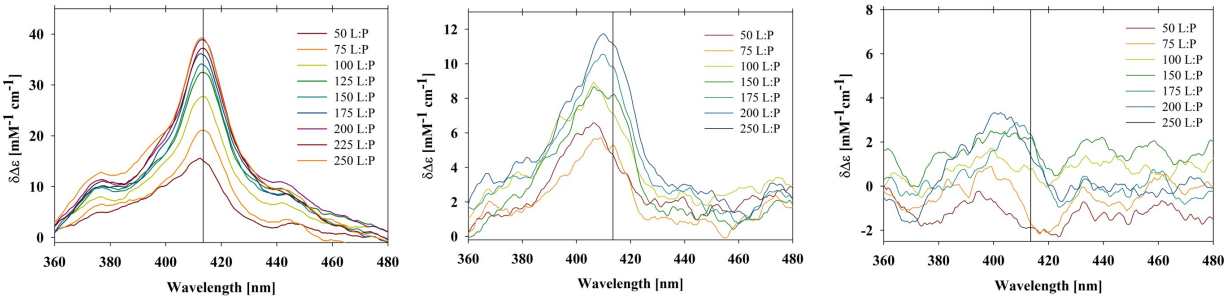
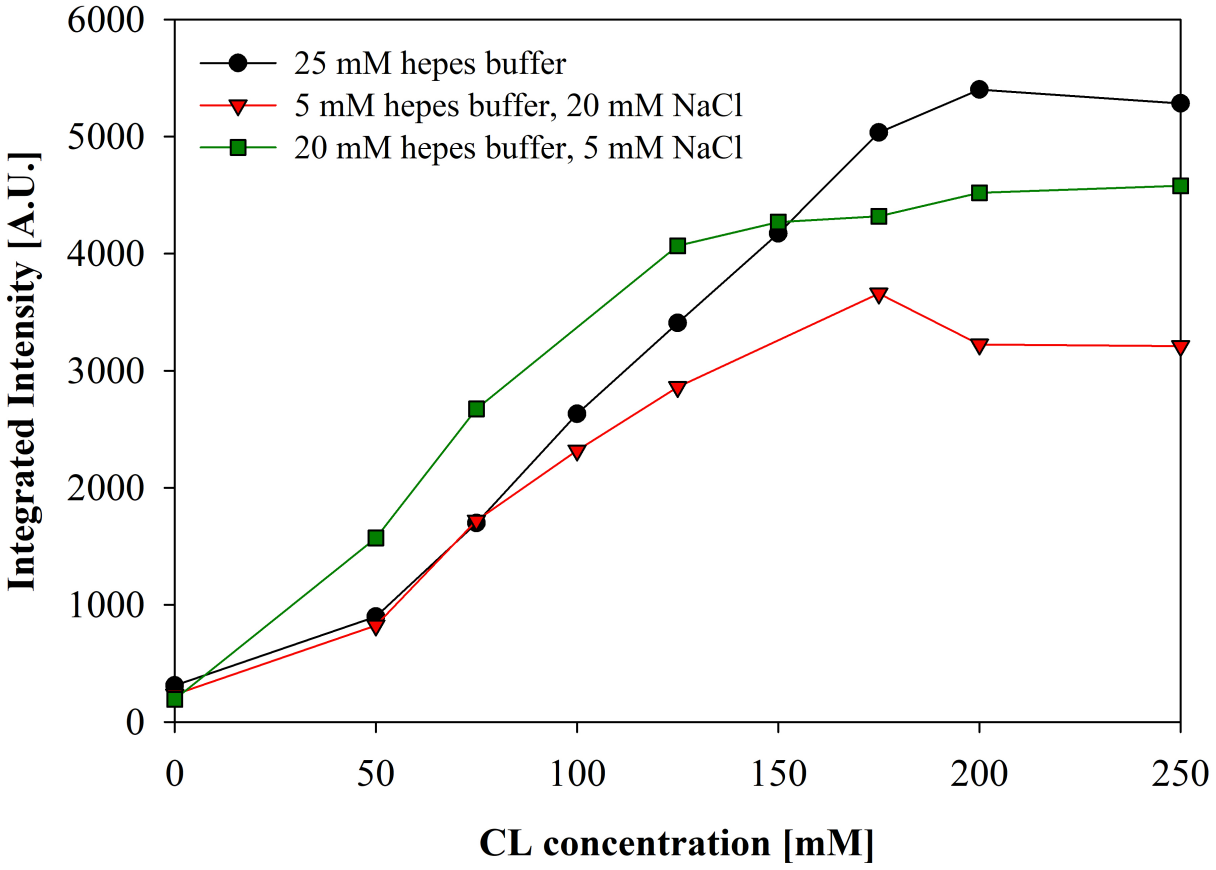


Figure S5: W59 Fluorescence response to the addition of cardiolipin measured in the presence of different buffer and NaCl concentration. The data indicated that the buffer has some influence on both cytochrome c binding to cardiolipin containing liposomes as well on the equilibrium between nf^+ and f^+. For the dose response data in Figure 7 of the manuscript we used a buffer concentration of 25 mM. Replacing it partially by NaCl reduces the fluorescence amplitude (amount of f^+). Interestingly, it also shifts the response curves to lower CL concentration, which indicates that the buffer has some inhibitory influence either on the initial binding or on the surface concentration of bound cytochromes at which the transitions from nf^+ to f^+ occurs.



Revisiting spectroscopic response data measured at pH 7.4

We refitted the spectroscopic response data for ferricytochrome *c* binding to 20% TOCL/80%DOPC liposomes at pH 7.4. In our earlier study the simulation of the dose response curves had been performed manually and free parameters were optimized in a way that minimized the reduced χ^2-value to a value below 2.[1] In the current study, we employed model 1 in a more rigorous way. The spectroscopic response data measured without NaCl were subjected to a non-linear least square fitting (cf. Material and Methods). With the exception of K_0 we allowed all parameters listed in Table S1 to vary. For K_0 we used the value reported in the earlier study.[1] Next, we simulated the spectroscopic response data for the three employed NaCl concentrations (50, 100 and 150 mM). Differences between predicted and experimentally obtained responses were than minimized by solely varying K_0 and K_{Na} (cf. eqs. 7a and b in section 4). As shown in Figure S5, this procedure yielded a quite satisfactory reproduction of the entire set of experimental data. Only the polarized fluorescence measured with NaCl is slightly underestimated at high CL-concentration. For the sake of self-consistency, we did not try to eliminate these minor discrepancies by varying additional parameters.

Fitting parameters	Obtained values
$K_0[M^{-1}]$	$6 \cdot 10^{2} \pm \cdot 10^{2}$[a]
$K_{c,high}$	2.71±0.32[b]
$\Delta\Delta\varepsilon_{nf}[M^{-1}cm^{-1}]$	-17±19[b]
$\Delta\Delta\varepsilon_{f}[M^{-1}cm^{-1}]$	17±25[b]
$f_{nf}[M^{-1}cm^{-2}]$	$2.2 \cdot 10^{5}$[b]
$I_{F,f}$	$3.06 \cdot 10^{3}$±0.43[b]
$I_{VV,f}$	70±13[b]
$I_{VH,f}$	31±13[b]
K_{mod}	0.028±0.004[b]
n	1.58±0.08[b]
$K_{Na}[M^{-1}]$	60±10[a]

Table S1: Fitting parameters obtained from the re-analysis of spectroscopic response data obtained at pH 7.4[1] ([a]Errors obtained manually, [b] errors of fitting parameters),

Parameters	[NaCl]=0	[NaCl]=50 mM	[NaCl]=100 mM	[NaCl]=150 mM
$K_{c,high}$	2.71	0.67	0.38	0.27
$\Delta\Delta\varepsilon_{nf}[M^{-1}cm^{-1}]$	-17.37	-22.0	-18	-18

Table S2: Variation of indicating fitting parameters as a function of NaCl concentration

Figure S6: Spectral response data of visible circular dichroism (upper panel left), the integrated intensity of a W59 fluorescence sub-band (middle panel, left) and the integrated intensities of CT 1(lower panel, right), polarized fluorescence I_{vv} (middle panel, right) and I_{vh} (lower panel, right) measured as a function of cardiolipin concentration concentration at pH 7.4 in the absence and the presence of NaCl. The right figure in the upper panel displays the mole fractions of *nf* (solid lines) and *f* (dashed lines) conformations as a function of cardiolipin concentration for the utilized NaCl concentrations: 0 mM (black), 50 mM (red), 100 mM (blue) and 150 mM (purple). The corresponding response data sets are displayed as follows: 0 mM (brown circles), 50 mM (green squares), 100 mM (top down triangles and 150 mM (orange squares). The CT band data were obtained with no NaCl (open circles) and 100 mM NaCl (filled circles). The solid lines result from fits and simulations described in the manuscript. Note that corresponding data points in the different figures were obtained for the same cardiolipin to protein ratio. The experimental data were reported earlier.[1]

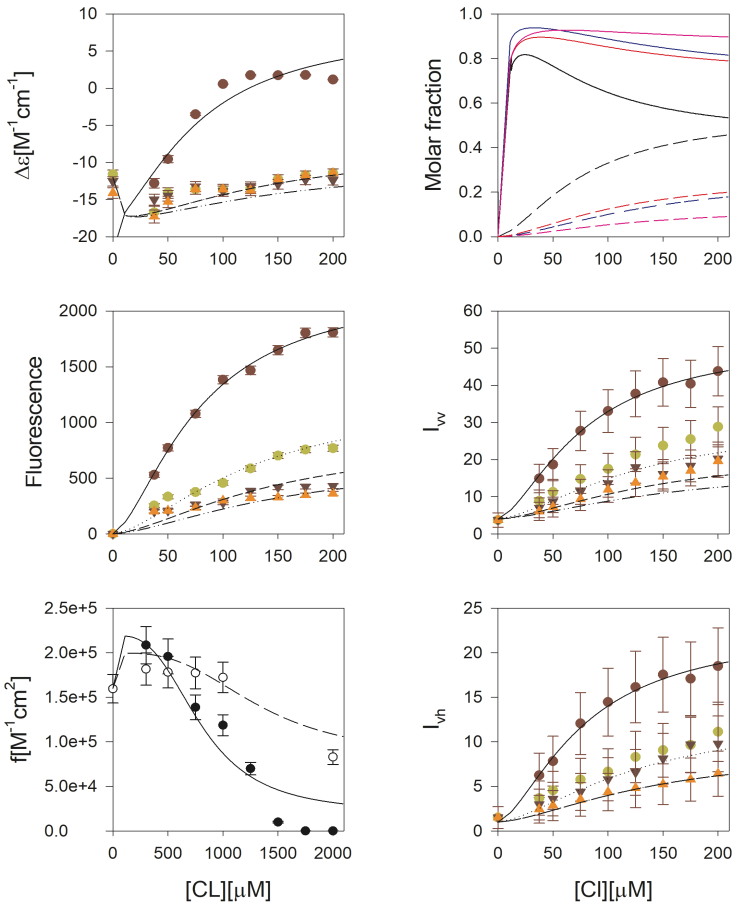
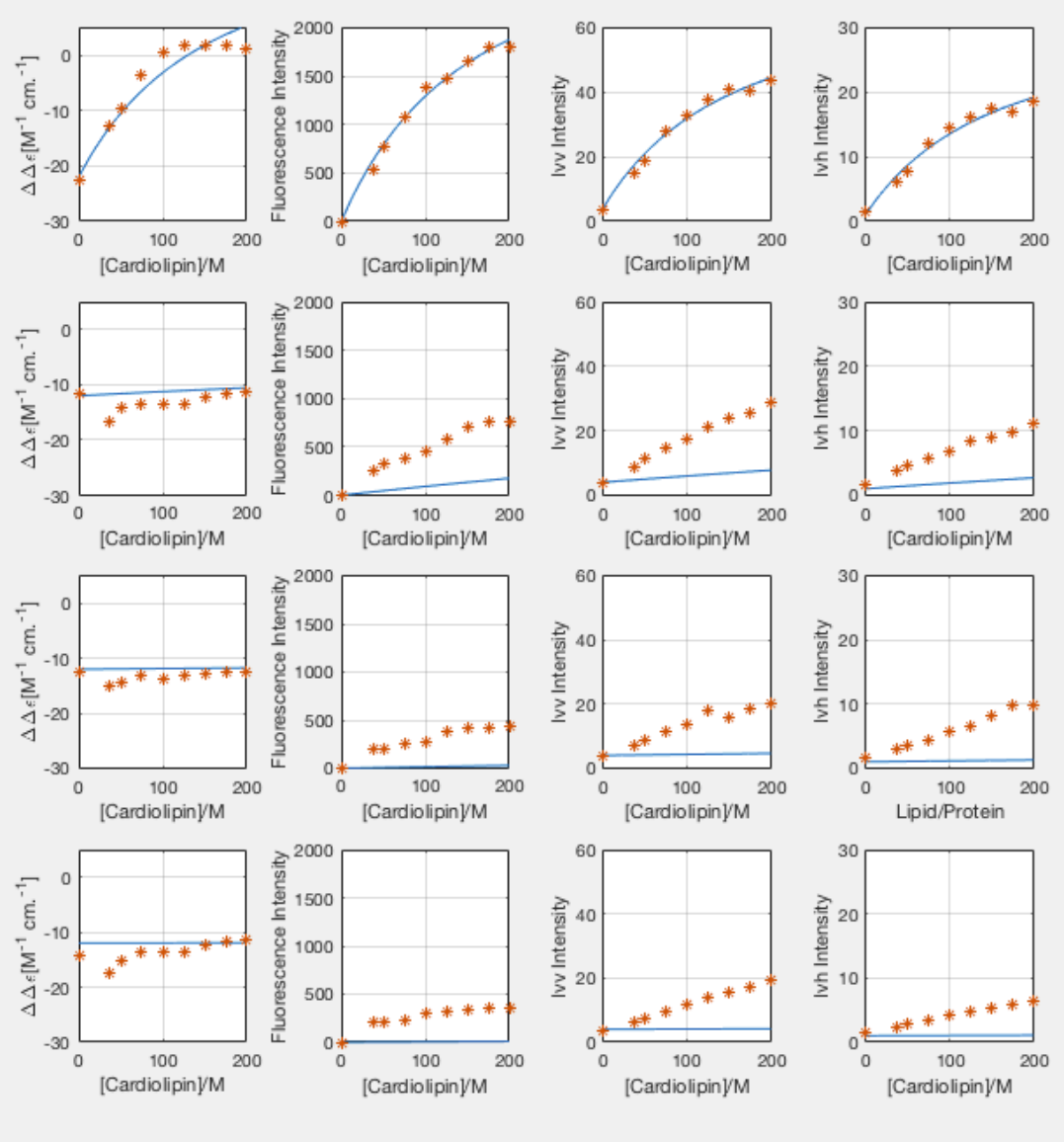


Figure S7: Spectral response of circular dichroism (left column), W69 fluorescence (2nd column from left), I_{vv} and I_{VH} (third and fourth column from left) to ferricytochrome c binding to 20% TOCL/80% DOPC liposomes (SUVs) at pH 7.4 without (upper panel) and different NaCl concentration (upper panel: 0M; 2nd panel: 50 mM, 3rd panel: 100 mM, 4th panel: 150 mM. The data were taken from Pandiscia and Schweitzer-Stenner.[1] The solid lines represent a simulation based on a fitting to the data obtained without NaCl. Details are described in the main text.



Error analysis.

We used the covariance option of our non-linear fitting program to obtain the covariance matrices for the free parameter of fits to the considered data sets. The standard deviation inferred from the square roots of their diagonal elements are listed in Tables 1 (for pH 6.5) and S1 (for pH 7.4). While the errors are rather small for n, K_{mod} and $I_{F,f/f'}$ they are very large for spectral response parameters of polarized fluorescence and particularly large for the respective circular dichroism values. The reason for these uncertainties can be inferred from the covariance matrices which reveal very large correlation effects between corresponding parameters of the nf and f^+ state. This is indicative of very shallow minima of the χ^2-function in the two-dimensional space defined by such correlated parameter pairs. We argue that the real statistical errors are likely to be much smaller since we could use all these spectroscopic parameters to actually correctly predict the spectroscopic responses obtained at various NaCl concentrations. It is therefore likely that an incorporation of all data sets in the least square fitting would have resulted in smaller statistical errors. The error for $K_{c,high}$ is moderate for the pH 7.4 fitting, but rather large for the L-site data set. The reason for this is obvious. If $K_{c,high}$ is substantially larger than 1 large changes of its value leads only to minor variations of the nf/nf^+ and f/f^+ mole fractions. It should be mentioned that the listed uncertainties of K_0 and K_{Na} were obtained manually by varying these values in simulations of spectral response curves for the investigated NaCl concentrations.

Figure S8: Effective binding constant $K_{eff}=K(1+K_c)$ plotted as a function of cardiolipin concentration for ferricytochrome c binding to 20%TOCL/80%DOPC liposomes at pH 6.5 (black) and 7.4 (red) in the absence (solid lines) and presence of 100 mM NaCl (dashed lines). The affinity parameter K accounts for van der Waals type contributions (both pH) and electrostatic interactions (pH 6.5) as described in the main text.

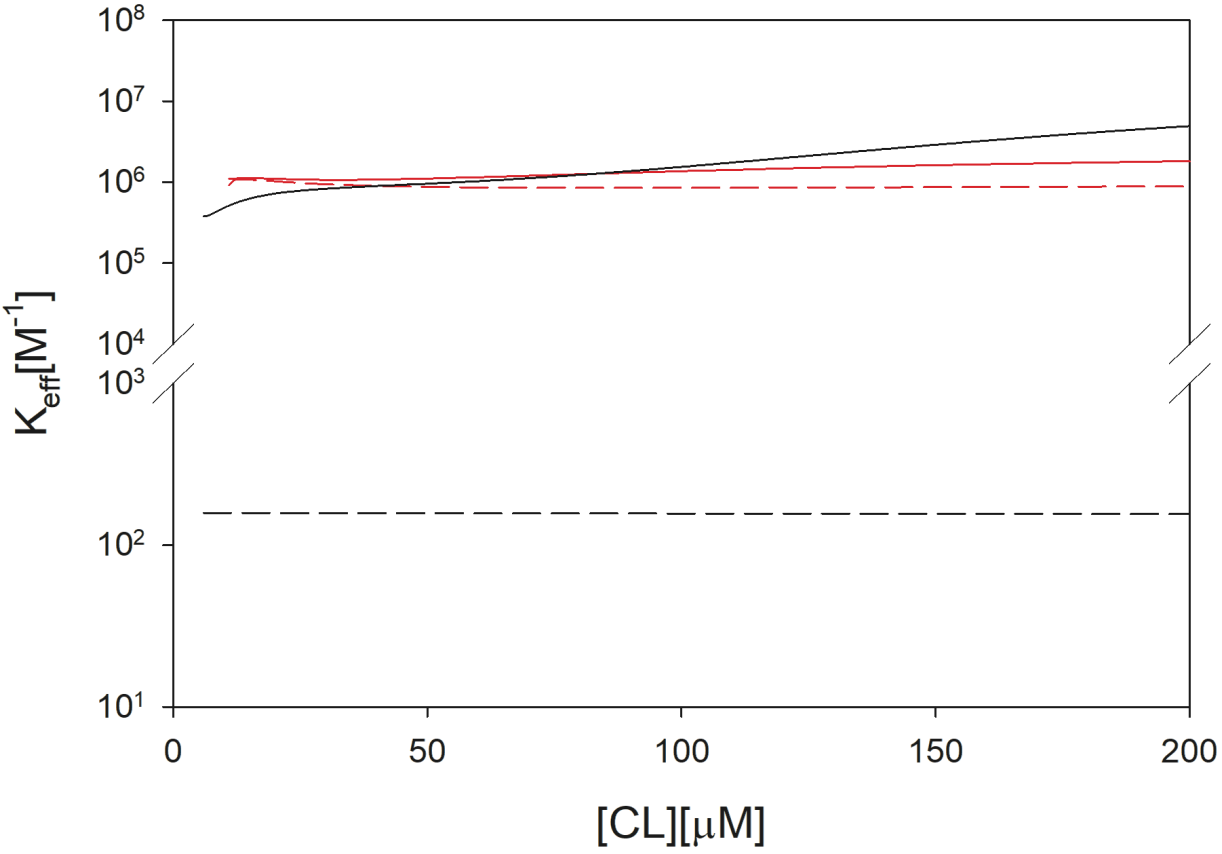
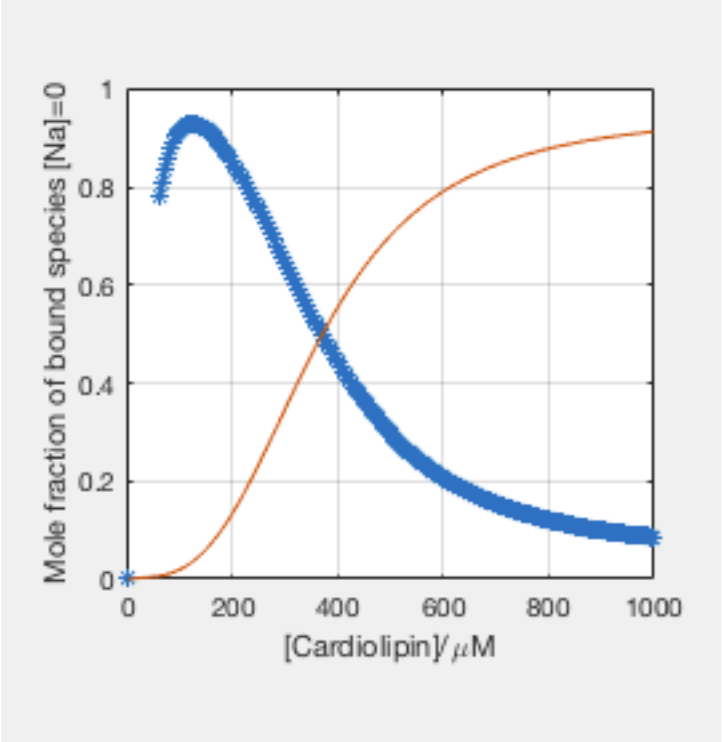


Figure S9: Mole fraction of the nf^+ (blue) and f^+ (red) population of ferricytochrome as a function of cardiolipin concentration in the absence of NaCl as calculated with the parameters used to fit the response data in Figure 7.



References

1. Pandiscia, L. A.; Schweitzer-Stenner, R. Coexistence of Native-Like and Non-Native Cytochrome c on Anionic Liposomes with Different Cardiolipin Content. *J. Phys. Chem. B* 2015, *119*, 12846–12859.