Coexistence of Native-Like and Non-Native Misfolded Ferricytochrome C on the Surfac of Cardiolipin Containing Liposomes

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Coexistence of Native-like and Non-Native Partially Unfolded Ferricytochrome *c* on the Surface of Cardiolipin-Containing Liposomes

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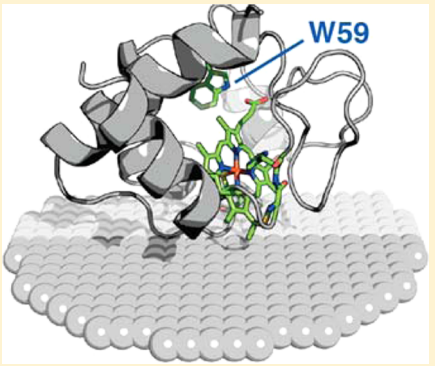
Supporting Information

ABSTRACT: Cytochrome *c*, in spite of adopting a rather rigid structure around its prosthetic heme group, is rather diverse with regard to its function and structural variability. On the surface of the inner membrane of mitochondria it serves as an electron transfer carrier. However, at conditions which have not yet been unambiguously identified, cytochrome *c* can adopt a variety of non-native conformations, some of which exhibit peroxidase activity. Cardiolipin-containing liposomes have served as ideal model system to investigate the various modes of interaction between cytochrome *c* and the inner mitochrondrial membrane. We probed the binding of horse heart ferricytochrome *c* to liposomes formed with 20% tetraoleoyl cardiolipin (TOCL) and 80% dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) as a function of lipid/protein ratio by fluorescence and visible circular dichroism spectroscopy. The obtained binding isotherms suggest that they reflect reversible binding processes, which excludes the possibility of significant protein insertion into the membrane. A global analysis of our data revealed the existence of two binding sites on the protein which causes rather different degrees of protein unfolding. We found that these two modes of interaction between protein and liposome led to conformational changes. While site 1 is relatively unaffected by NaCl, site 2 shows a more native-like state or a higher population of the native state in the presence of NaCl. At the highest utilized concentration of NaCl, there is only a 40% inhibition of the binding to site 2. We interpret our finding for this binding site as reflecting an equilibrium between electrostatically bound proteins with a high degree of unfolding and less unfolded proteins which bind either via H-bonding between lysine side chains and PO_2^- or hydrophobic interactions. With regard to site 2 binding, our results are reminiscent of the two-state equilibrium between a compact C and an extended E-state proposed by Pletneva and co-workers (Hanske et al. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 125−230). We conjecture that the nonelectrostatically bound proteins should have higher abilities to maintain the redox potential that is required for the function as an electron transfer protein.



INTRODUCTION

For a long period of time, cytochrome *c* has served as an ideal model system for studying the physicochemical aspects of electron transfer[1] and protein folding.[2−6] Over the last five to ten years, however, research on cytochrome *c* has shifted, driven by the necessity to understand this protein's role in triggering apoptosis.[7−9] The initial phase of the apoptotic process is already clearly established; in order to trigger apoptosis it is necessary for cytochrome *c* to initiate the aggregation of Apaf-1.[10] Very recent works by the Kagan group and others have shown that the protein is primed for this function by gaining peroxidase activity while still bound to the inner membrane surface of mitochrondria.[8,11−13] These findings and indications for conformational changes induced by cytochrome *c*−cytochrome *c* oxidase interactions[14−18] clearly indicate that it is necessary to identify and fully characterize the conformations that cytochrome *c* can adopt on the surface of anionic phospholipid-containing membranes, in structural and functional terms.

Liposomes and less-defined lipid dispersions containing anionic phospholipids have been used as model system for the inner mitochondrial membrane to which cytochrome *c* binds in vivo.[8,11,14,26,27,31−39,41−47] Nearly all studies conducted thus far show that cytochrome *c* undergoes conformational transitions upon binding to the surface of such liposomes as they do upon binding to the inner membrane.[8,11,31,32,34−39,41−47] Moreover, several lines of evidence suggest that cytochrome *c* binding can trigger the protein's insertion into the lipid membranes, if a major portion of the membrane surface is covered by proteins.[25,26,31] Even though cytochrome *c* binding to anionic lipids in general and to cardiolipin in particular has been studied with a plethora of experimental techniques, a clear and consistent picture has yet to emerge. Some of the most important results obtained thus far are briefly reviewed in the following to provide the reader with the context of our work.

The interaction between cytochrome *c* and the inner membrane involves cardiolipin (CL), a peculiar anionic lipid

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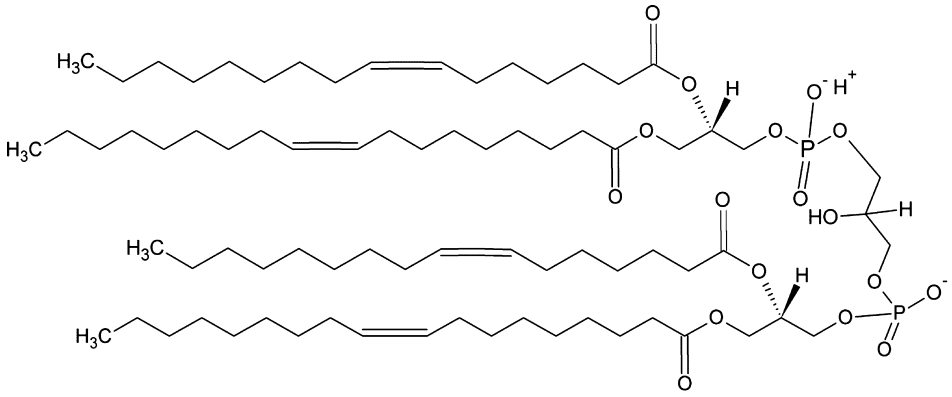

Figure 1. Structure of tetraoleoyl cardiolipin.

with four hydrophobic alkyl chains, as shown for tetraoleoyl cardiolipin in Figure 1. Kinnunen and co-workers identified two cytochrome *c* binding sites on cardiolipin-containing liposome surfaces termed A and C.[32] The binding of site A involving residues K72 and K73 is electrostatic in nature and the respective binding constant depends on the ionic strength of the solution. The binding via the C-site involving N52 has been suggested to occur mainly by means of hydrogen bonding. While the addition of NaCl inhibits binding to A, the C-site binding remains unaffected,[32−34] which seems to indicate that the binding to site A is reversible. However, the addition of liposomes to a specific mixture of cytochrome *c* and fluorescence-labeled CL-containing liposomes did not lead to any significant dissociation of the proteins from the surface.[32−34] Kinnunen and co-workers[34−36] proposed a stabilizing mechanism that they called the extended lipid anchorage. In their model, an alkyl chain of cardiolipin is inserted into the hydrophobic pocket of the protein. This hydrophobic channel is formed in close proximity to the helix containing the interacting amino acid residue N52. Kalanxhi and Wallace proposed a channel close to K72, K73, K86, and M80 as an alternative.[35] Sinibaldi et al. went even further by suggesting the penetration of two alkyl chains.[37] Irrespective of its location this hydrophobic interaction could indeed be expected to substantially impede the dissociation of cytochrome *c* from its binding sites. An alternative option for rationalizing the obtained stability of A-state binding is the protein's penetration into the membrane, the occurrence of which has indeed been proposed for liposomes with substantial protein coverage (low lipid/protein ratio).[31,38] Tuominen et al. showed that ATP, which binds to N91 binding site of cytochrome *c* with high affinity, changes the conformation of ferricytochrome *c* bound to site C but does not affect the structure of A-bound proteins.[39] Interestingly, the visible circular dichroism (CD) spectra of the B-band reported by Tuominen et al. suggest that the influence of A-site binding on the heme pocket structure of cytochrome *c* is rather modest. For the ferri-state it just causes a decrease of the very pronounced couplet by ca. 60%.[39] Since the change is symmetric, it is most likely reflecting a decrease of B-band splitting.[40] The binding to site C, however, which is dominant at acidic pH, has a dramatic influence on the CD spectrum, in that it changes the sign of the couplet. Thus, far, such a change has not been observed in solution nor has a physical explanation for this phenomenon yet been given. In addition, C-site binding was reported to become relevant also for liposomes with higher CL-fractions; this was attributed to a semiprotonated state of the phosphate head groups of cardiolipin caused by the decrease of the local pH at the lipid−water interface.[32] Nantes and co-workers investigated cytochrome *c* binding to mitochondrial mimetic vesicles with heart cardiolipin content and found a second ionic binding site of cytochrome *c* termed L that contains the residues K22, K27, H33, and K87, which also seems to be operative at more acidic pH.[41] Sinibaldi et al. probed the binding of cytochrome *c* to pure cardiolipin vesicles at neutral pH and observed two cooperative binding steps to what the authors termed high- and low-affinity binding sites.[42] They identified them with A and C, respectively, but the spectroscopic characteristics of the cytochromes bound to these sites are rather different from those reported by Tuominen et al.[39] The binding of both sites were shown to be disrupted by Cl^- ions, which is also inconsistent with the site C proposal. A more recent study from the same group revealed the involvement of K72 and K79 in both of their obtained binding processes.[43] Recently, Hong et al. performed fluorescence energy transfer and biolayer interferometer experiments from which they obtained evidence for an equilibrium between two conformations of cytochrome *c* bound to cardiolipin-containing liposomes (as opposed to two binding sites), one with a globular structure and the other one with more extended conformations.[44] Their data led the authors to propose that in both conformers the protein's binding to the liposome surface is electrostatic in nature. They claimed to have found no evidence for a site C or a more hydrophobic type of interaction. Bergstrom et al., who found that cytochrome *c* binding to cardiolipin-containing giant unilamellar vesicles (GUVs) causes the formation of pores big enough to penetrate into the interior of the liposomes.[31] Their observation can well be explained with the employed stoichiometric ratio of cardiolipin and cytochrome *c*, which ensures a rather large coverage of the liposome surface. A similar observation was earlier reported by Oellerich et al. as resulting from a high surface coverage of DOPG (dioleyl-phosphatidylglycerol) liposomes.[38] Very recently, Kawai et al. reported highly cooperative binding of cytochrome *c* to GUVs containing 49/30/20 mol % mixtures of POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine)/DOPE(1,2-dioleoyl-*sn*-glycero-3-phophoethanolamine/TOCL.[45] The experimental conditions were comparable with that generally used by Kinnunen and co-workers.[33,36] Fluorescence microscopy revealed the existence of rather large CL clusters.

Obviously, in spite of the rather large body of experimental studies cited above, a unifying picture of how cytochrome *c* reacts with anionic lipid membranes has still to emerge. A thorough understanding of how the binding to lipid surfaces changes the structure (and function) of cytochrome *c* is of utmost physiological relevance. In the inner membrane space of intact

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mitochondria, the ionic strength is rather high and electrostatic interactions are diminished, so that hydrophobic forces could become relevant. Generally, this allows the protein to diffuse in three dimensions.[18]

The current study is aimed at determining and characterizing different modes of cytochrome *c*—anionic liposome interactions in the presence and absence of different concentrations of NaCl and low to moderate protein coverage of CL binding sites by combining different spectroscopic tools. For the liposome, we chose a 20%/80% mixture of TOCL(1,1′2,2′-tetraoleoyl cardiolipin) and DOPC(1,2-dioleoyl-*sn*-glycero-3-phosphocholine). This resembles physiological conditions with regard to the cardiolipin content of the liposome's lipid bilayer and the used maximal NaCl concentration. A dominance of a lamellar bilayer phase can be expected at these conditions.[46] Fluorescence binding data analyzed by Trusova et al. suggest some segregation of very low fractions of cardiolipin into lipid domains, which increases upon addition of NaCl.[46] We varied the lipid/protein (TOCL+DOPC/protein) ratio over a range from 0 to 200 (TOCL/protein: 0 to 40), which is comparable to what was used by Hanske et al.[47] but exceeds by far the range chosen by Sinibaldi et al.[42] The concentration of the protein allowed us to probe its binding to and interaction with liposomes by measuring its polarized and unpolarized fluorescence, visible circular dichroism (CD) and optical absorption spectrum as a function of the lipid-to-protein ratio of cytochrome *c*—liposome mixtures. These spectroscopies probe the tertiary structure in slightly different but somewhat overlapping regions of the protein. Visible absorption and CD spectra are particularly suitable for probing the ligation state of the heme and the strength of the internal electric field in the heme plane.[40] The fluorescence of the protein originates from the sole tryptophan (W59) of the protein. It is quenched in the folded state but increases substantially once the heme pocket opens during the unfolding of the protein. Steady-state fluorescence anisotropy derived from polarized fluorescence intensities inform about the rotational mobility and the excited state lifetime of the fluorophore.

Binding isotherms obtained from the obtained spectra were subjected to a self-consistent Langmuir-based thermodynamic analysis and reveal the existence of at least two, independent binding sites of the protein. While the binding via the site of highest affinity exhibits limited unfolding and a rather intact heme environment, the other binding process induces partial unfolding of the protein and exchange of the distal ligand. The addition of 150 mM NaCl only partially inhibits cytochrome *c* binding via site 2. The binding via site 1, however, remains unaffected. We interpret our results as indicating that site 1 binding is hydrophobic while the thermodynamic and spectroscopic parameters obtained for binding site 2 reflect the coexistence of predominantly hydrophobic and electrostatic binding.

MATERIALS AND METHODS

Preparation of Cytochrome *c* Solutions. Equine cytochrome *c* was purchased from Sigma-Aldrich Company (St Louis, MO) with no further purification and dissolved in 25 mM HEPES buffer. Potassium ferricyanide was added to the protein solution to oxidize any residual ferrous forms present in the sample. The protein solution was then titrated to pH 7.0 and passed through a Sephadex G-10 column (GE Healthcare) to remove any remaining oxidizing agents and impurities. The sample was then readjusted back to pH 7.0.

Preparation of Liposomes. TOCL (1,1′2,2′-Tetraoleoyl Cardiolipin) and DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) (Avanti Polar Lipids, Birmingham, AL) were dissolved in a 2:1 chloroform/methanol mixture. The protocol adopted for liposomes preparation resembles that of Hanske et al.[47] Solvent was removed by rotary evaporation at room temperature, and the remaining suspension was left to sit in a vacuum desiccator overnight. The lipid film was rehydrated with 25 mM HEPES buffer (pH 7.4) to obtain a concentration of 5 mM. The mixture was then left to sonify in an ice bath at 100 W for one to 2 h. The solution was centrifuged for 40 min at 12000 rpm to remove impurities. The supernatant was extracted and left to stabilize overnight. All liposome solutions were stored under nitrogen to prevent any oxidation.

Preparation of Cytochrome *c*-Liposome Binding Experiments. Liposomes were first diluted to the required concentration using 25 mM HEPES buffer (pH 7.4). The concentration of cytochrome *c* in all experiments was 5 μM. Experiments involving NaCl were prepared by adding aliquots of NaCl to the liposome—protein mixtures until the final salt concentration was achieved. Each liposome—protein mixture was made individually for the desired lipid/protein ratios.

Electronic Circular Dichroism Spectroscopy. Spectra were obtained using a Jasco J810 Spectrapolarimeter, which was purged and cooled with gaseous nitrogen. Aqueous cyt *c*-liposome solutions were measured with a 1 cm quartz cell purchased from International Crystal Laboratories (Gardfield, NJ). The spectra were measured in the range from 300 to 800 nm with a scanning speed of 500 nm/min, a data pitch of 0.05 nm, a bandwidth of 5 nm, and a response time of 0.5 s. Five spectra were accumulated per sample at 20 °C. The temperature was controlled with a Peltier solid-state heating and cooling module. All spectra were solvent-corrected using Jasco spectral analysis program.

Dynamic Light Scattering. Particle radii measurements were obtained using a Horiba Lb-500 Dynamic Light Scattering Particle Size Analyzer (Edison, NJ). A 10 mm path-length quartz cuvette was used to collect data at room temperature.

Fluorescence Spectroscopy. Fluorescence measurements were performed at room temperature using 10 mm path-length quartz cuvettes in a PerkinElmer LS55 Luminescence Spectrometer. Unpolarized excitation spectra were measured between 300 and 550 nm with 293 nm excitation wavelength and a scanning speed at 200 nm/min. Polarized fluorescence measurements were obtained at 340 nm with an integration time of 10 s. For both, the measurements of polarized and unpolarized spectra, an excitation slit width at 5.0 nm and an emission slit width at 2.5 nm were used. All spectra were baseline corrected using the program MULTIFIT.[48]

RESULTS

Size Distribution of Liposomes. A quantitative analysis of the fluorescence and CD data described below requires a homogeneous distribution of liposomes. We therefore used dynamic light scattering to probe the size distributions of liposome as a function of the lipid/protein ratio in the absence and presence of cytochrome *c*. As exemplified by the data shown in Figure S1 of the Supporting Information, the size distribution of liposomes was found to exhibit bimodal distributions with peaks at 1 and 0.1 μm for lipid/protein ratios below 25 in the presence of cytochrome *c*. In the absence of cytochrome *c*, however, the distribution peaks at 5 nm. On the contrary, single broad peaks positioned between 30 and 40 nm were obtained at

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lipid/protein ratios above 25, irrespective of the presence of cytochrome *c* (Figure S2 of the Supporting Information). Liposomes with diameters in the nanometer-range between 20 and 100 nm are generally classified as "smaller unilamellar vesicles" (SUV), those with diameters between 100 nm and a few micrometers are termed "large unilamellar vesicles" (LUV), and recently produced species with diameters of several micrometers are characterized "giant unilamellar vesicles" (GUV).[46,49−56] The sizes obtained for low lipid/protein ratios in the absence of cytochrome *c* rule out the formation of liposomes, they might instead indicate the presence of planar bilayers or inverted micelles.[57] In the case of the latter, the observed interaction with cytochrome *c* would be hydrophobic in nature. These data strongly suggest that cytochrome *c* binding to the rather limited surface of these bilayers/inverted micelles cause a complete coverage of their surface which eventually leads ever to the fusion of micelles into GUVs or to a clustering of micelles via protein−protein interactions.[38,58] Apparently, only binding data obtained with the rather homogeneous liposome distributions obtained at higher lipid/protein ratios are suitable for a more quantitative analysis which we are aiming at in this study.

Cytochrome *c* Binding to CL-Containing Liposomes. To identify coexisting modes of cytochrome *c* binding to CL-containing liposomes and to elucidate the concomitant conformational changes involving the tertiary structure of the protein, we measured the tryptophan (W59) fluorescence of ferricytochrome *c* as a function of the lipid-to-protein ratio. The occurrence of this fluorescence is indicative of (partial) protein unfolding upon its binding to the liposome surface. The fluorescence spectra in Figure 2 shows a very intense band

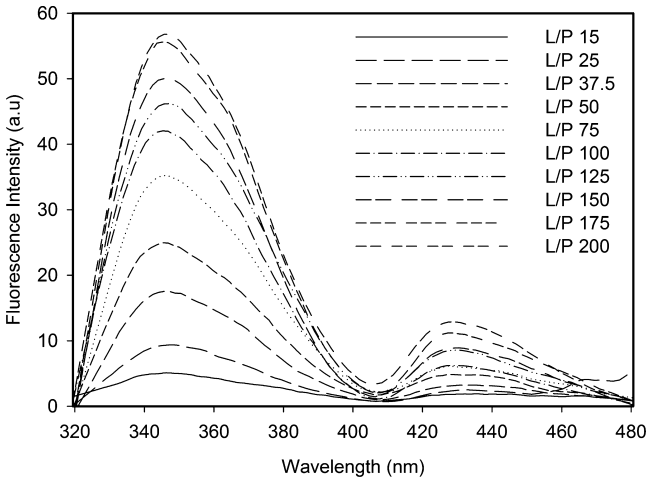

Figure 2. Fluorescence spectra of cytochrome *c*−liposome mixtures at varying lipid concentrations.

with varying peak positions between 330 and 350 nm and a much weaker band peaking in the region between 420 and 460 nm. We assign the former to fluorescence (F-band) and the latter to rather intense phosphorescence emission (P-band). The occurrence of the latter is somewhat surprising since we did not employ any measures to deoxygenate the sample, normally a prerequisite for observing phosphorescence at room temperature.[59] We suspect that our observation might be in part due to a photolytical removal by the intense xenon lamp irradiation of our fluorescence spectrometer.[60] Recent experiments on ferricytochrome *c* in solution carried out in our laboratory revealed a strong pH-dependence of the P-band which appears uncorrelated with the occurrence of the F band.[61] While the F-band luminescence was obtained for acidic and alkaline states of the protein, the P-band appeared solely in the fluorescence spectra of the alkaline states IV and V. The fluorescence data in Figure 2 reveal the expected increase in tryptophan fluorescence intensity with increasing lipid concentration, indicating (a) an increasing number of liposome-bound cytochromes and (b) a partial unfolding of the liposome-bound proteins which involves changes of their tertiary structure. The occurrence of fluorescence is indicative of at least partial unfolding that leads to an increase of the distance between the heme and W59 and thus to a reduction of fluorescence quenching.[62] The peak position of the F-band slightly blueshifts from 345 to 342 nm at a lipid/protein ratio of 75. The P-band exhibits a similar behavior, which shows that F- and P-band emissions are both related to W59 excitation.

We also measured the steady-state anisotropy:

$$r_S = \frac{I_{II} - I_\perp}{I_{II} + 2I_\perp} \quad (1)$$

of the W59 fluorescence as a function of the above lipid/protein ratios. I_{II} and $I_\perp$ are the intensities of the fluorescence polarized parallel and perpendicular to the polarization of the exciting light. The relationship between r_S and the rotational correlation time ϕ is generally described by the Perrin equation:

$$r_S = \frac{r_0}{1 + \frac{\tau_w}{\phi}} \quad (2)$$

where r_0 is the anisotropy in the absence of rotational diffusion and τ_w denotes the W59 fluorescence lifetime. Generally, r_S values reflect the rotational relaxation time of the utilized fluorophore, which is a direct measure of the microviscosity of the chromophore's environment.[63] Figure 3 depicts the obtained

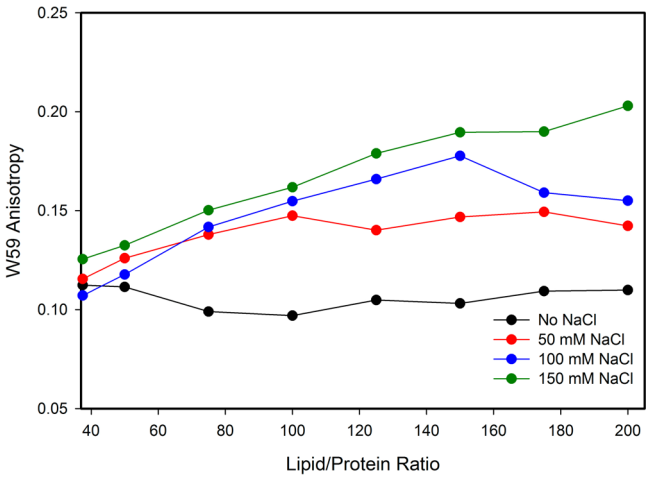

Figure 3. Fluorescence anisotropy of W59 in ferricytochrome *c* as a function of lipid/protein ratio at the indicated NaCl concentrations.

r_S-values at 340 nm. All the values lie between 0.1 and 0.13, thus indicating a rather mobile W59 residue irrespective of the lipid/protein ratio. Variation of r_S with increasing lipid/protein ratio are within the limit of uncertainty. The r_S-value for the unbound folded cytochrome *c* is difficult to determine experimentally, because the unquenched fluorescence intensity of W59 is very low. However, one can safely assume that r_S is not much lower than the r_0 value of 0.4 (as obtained if the angle between

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absorption and emission moment is zero) based on the following argument. The lifetime of the quenched W59 fluorescence in the folded state of the protein can be expected to be an order of magnitude shorter (~0.2 ns) than that of the respective unquenched fluorescence in holocytochrome *c*.[64] Since the rotational correlation time ϕ is approximately 5.1 ns,[65] eq 2 yields $r_S \approx r_0$, provided that the rotation of W59 residues is severely restricted, which can be expected to be the case in folded cytochrome *c* where it is hydrogen-bonded to one of the heme propionic acid peripheral substituents.[66] The lower r_S values observed in the presence of liposomes reflect the increased lifetime of the W59 fluorescence in membrane-bound proteins as well as the increased side chain mobility of the residue.[67] The observed reduction of r_S indicates a (maximal) τ/ϕ ratio of ≈2.3.

The fluorescence data might not reveal a complete picture since they do not reflect binding processes that induce only minor structural changes of the protein that does not involve substantial motions of W59 relative to the heme plane. We therefore measured the CD spectrum of the Soret band region of ferricytochrome *c* as a function of lipid/protein ratio. Soret CD spectra have been shown to be very sensitive indicators of any tertiary structure changes that involve heme–protein interactions.[40,68−73] These CD and the corresponding absorption spectra of the liposome–protein mixture are shown in Figure 4. The CD data were taken from a recent publication.[74] For the native, unbound protein (seen in red), the CD spectrum shows the well-known couplet, which is indicative of band splitting due to the presence of a strong internal electric field and the occurrence of asymmetric vibronic perturbation due to heme–protein interactions.[71,75] Upon the addition of liposomes, this couplet gradually disappears and is replaced by a positive Cotton band, which is indicative of a more open heme crevice with the M80 containing Ω-loop further away from the heme group.[76] The magnitude of the Cotton band is reminiscent of the Soret band CD signal, which Hagarman et al.[75] observed for the non-native state V of ferricytochrome *c* and Soffer et al.[77] for a misfolded state of the protein that they could stabilize even at native conditions after exposing the protein to alkaline conditions at pH 11.5. A similar change of the visible CD spectrum was reported by Sinibaldi et al. for ferricytochrome *c* binding to liposomes with 100% cardiolipin.[42]

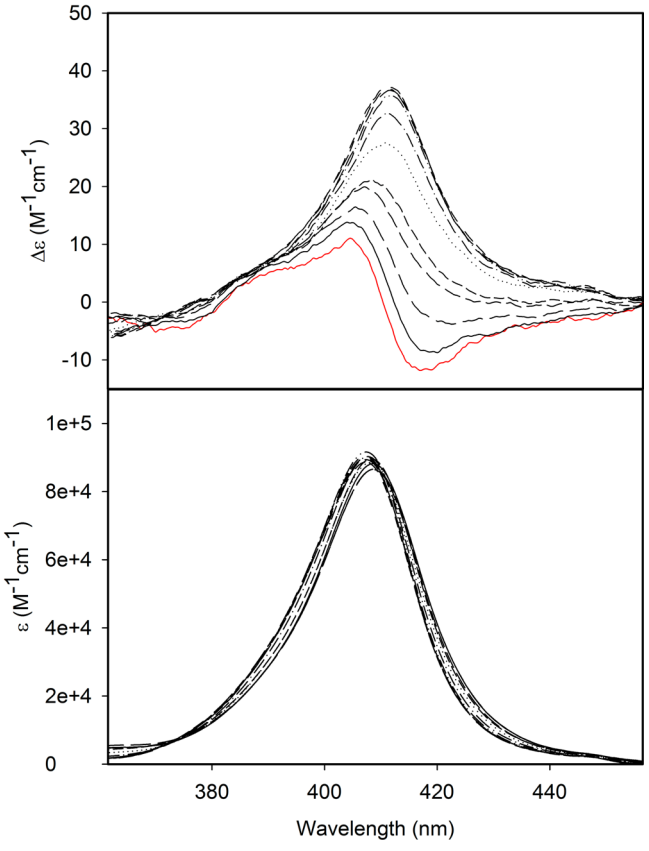

Figure 4. Soret band CD (top) and absorption (bottom) spectra of cytochrome *c*–liposomes mixtures. The CD intensity increases with increasing lipid/protein ratio.

Interactions between the protein and liposomes are also reflected by changes of the noncoincidence between the peak positions of the positive Soret CD cotton band and of the corresponding absorption band profile. Figure 5 displays the wavelength difference between positive cotton and absorption band as a function of the lipid/protein ratio. The data revealed a shift of the positive CD band from the high-energy side of the Soret band to a red-shifted position on the low-energy side with increasing lipid/protein ratio, which very much seems to coincide with the fluorescence titration. This makes the CD/absorption spectra of the partially unfolded state(s) distinct from what we earlier observed for partially unfolded ferricytchrome *c* in solution, for which the above noncoincidence is generally small or even undetectable.[75,77]

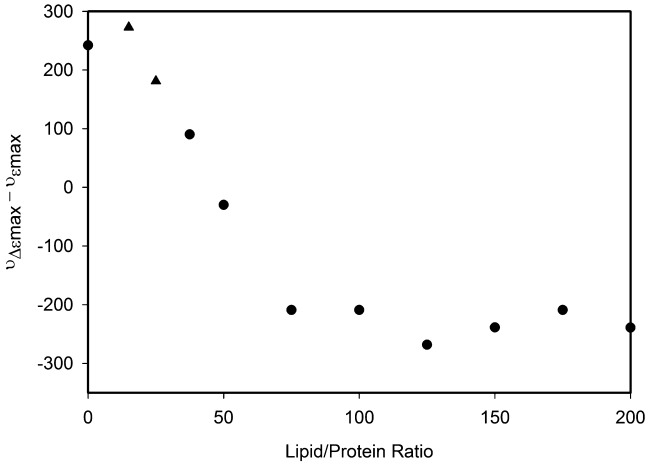

Figure 5. Change in wavelength position of the CD band and absorbance band of the Soret region.

Like the couplet observed for the native state, this pronounced noncoincidence is indicative of the splitting of the B-band due to electronic and vibronic perturbations.[69] In the protein, this perturbation is caused by a rather strong internal electrical field. Hence, our data suggest that this electric field is changing upon the protein's binding to the liposome surface. One possible reason could be that an electric field, created by the liposomal surface charges, overlaps with the electric field of the protein in the heme cavity. An increase of the total electric field strength would cause a blueshift of the B-band.[78−80] Such a shift was indeed observed, but it is traditionally assigned to a change of the axial ligand from M80 to a histidine.[38] The fact that the positive CD-band "walks" from the high to the low energy side of the Soret absorption band suggests a reorientation of the electric field in the heme plane that involves a move of the electric field vector across a line connecting two of the C_m atoms.[69] If the

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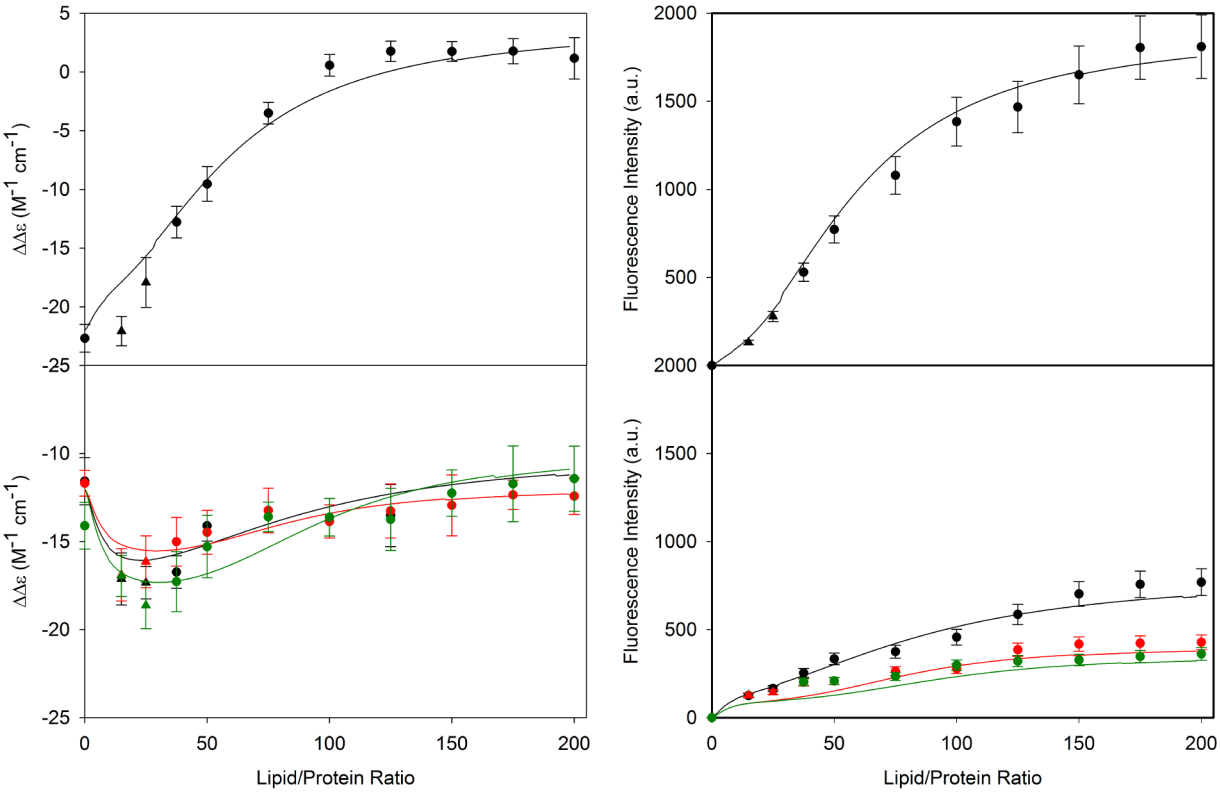

Figure 6. Effect of lipid concentration on the $\Delta\Delta\varepsilon$ of couplet peak of the ECD spectra (left columns) and integrated fluorescence intensity obtained at 340 nm (right columns) in the absence (top row) and presence (bottom row) of varying NaCl concentrations. The solid curve results from the fit discussed in Theoretical Analysis of Fluorescence and CD Titration. Our NaCl concentrations were 50 (black), 100 (red), and 150 (green) mM. The two omitted lipid/protein ratios are symbolized by triangles.

vector coincides with the diagonal line of the heme, the Stark splitting is zero. For the cytochrome *c*-liposome mixture, that seems to happen at a lipid/protein ratio of 75. For high lipid/protein ratios, the large noncoincidence between absorption and CD-band suggests that only the low-energy component of the B-band transitions carries substantial rotational strength, a phenomenon earlier observed for deoxymyoglobin.[70]

In order to exhibit the dose response of fluorescence and circular dichroism in quantitative terms, we proceeded as follows. For the former, we used our program MULTIFIT to decompose the fluorescence F-band into three Gaussian sub-bands for which the wavenumber positions and the bandwidths were kept constant. The integrated intensities of the most intense sub-band F2 are plotted in the right panel of Figure 6 as a function of the lipid/protein ratio of the liposome−protein mixture. The CD spectra were further analyzed by plotting the lipid/protein ratio dependence of the difference $\Delta\Delta\varepsilon = \Delta\varepsilon_{417} - \Delta\varepsilon_{405}$. This procedure eliminates the influence of baseline shifts to which individual spectra are subjected to. The respective titration curves are also shown in the left panels of Figure 6. The dichroism data of the liposome−protein mixture show a rather unusual behavior in that they go through a minimum at low lipid/protein ratios before continuously increasing to the saturation level. Taken together, CD and fluorescence data suggests the involvement of at least two modes of liposome−protein interactions in the absence of salt.

To further elucidate the binding interactions occurring at low lipid/protein ratios, we directly recorded the polarized fluorescence I_{II} and $I_{\perp}$ which are generally used for the determination of the steady-state anisotropy as a function of the lipid/protein ratio. Contrary to the integrated fluorescence intensities displayed in Figure 6, the plotted I_{II} and $I_{\perp}$ values in Figure 7 are apparent intensities measured for an emission wavelength of 340 nm. The data for both parallel (black) and perpendicular (red) polarized fluorescence show a gradual increase with increasing liposome concentrations.

It should be noted that all titration curves in Figures 5, 6, and 7 also contain the data points (triangles) that were obtained with the lipid/protein ratios (i.e., 15 and 25) for which our light-scattering data suggest protein-induced fusion of planar bilayers/micelles into GUVs.

Probing Na^+-Induced Dissociation and Cl^--Induced Refolding of Cytochrome *c*. In the presence of NaCl, Na^+ ions can compete with the positively charges patches on the surface of cytochrome *c* for anionic phospholipid binding sites.[46] Though never thoroughly considered, one should also expect Cl^- to contribute to this inhibition by interacting with the positively charged patches of the protein. In order to explore how these ions affect the binding of cytochrome *c* as well as the reversibility of the induced structural changes, we measured the W59 fluorescence, the polarized 340 nm fluorescence, and the visible CD spectra of the liposome−protein mixture as a function of the lipid/protein ratio after adding 50, 100, and 150 mM of NaCl to the protein−liposome mixtures. The fluorescence and CD spectra are shown in Figures S3 and S4 of the Supporting Information (CD spectra measured with 100 mM NaCl were earlier reported by Pandiscia and Schweitzer-Stenner).[74] Figures 6 and 7 display the binding isotherms obtained from these spectra. At 50 mM NaCl, the fluorescence of the liposome−protein mixture is reduced by about half compared to the fluorescence of the bound protein on the liposome surface. Upon the addition of 100 and 150 mM NaCl, the fluorescence intensity

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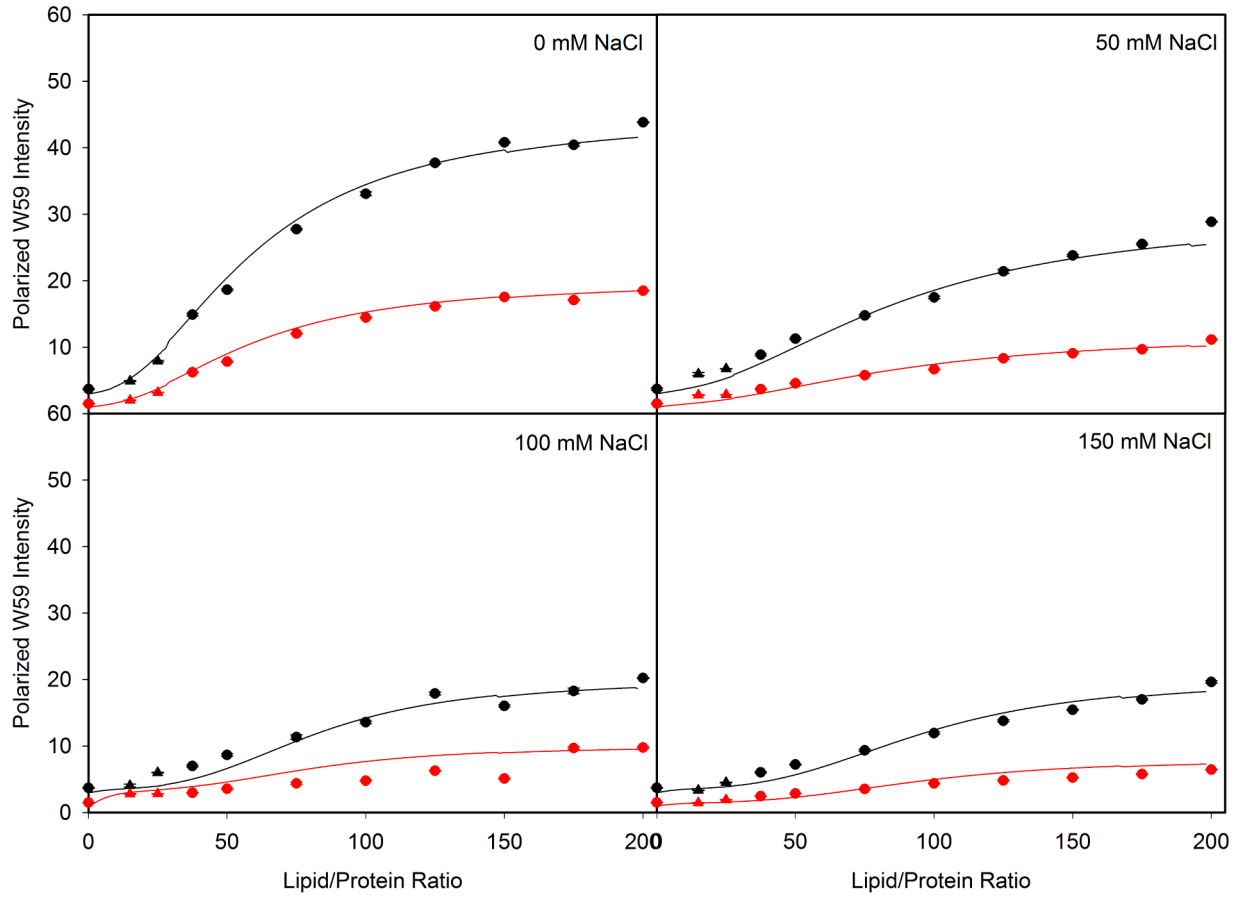

Figure 7. Parallel (black) and perpendicular (red) polarized fluorescence spectra in the absence and presence of varying NaCl concentrations.

is again cut in half compared with 50 mM. Differences between spectra taken with 100 and 150 mM NaCl are minimal, indicating that saturation has been reached. In order to check whether the fluorescence quenching in the presence of NaCl is solely due to the protein's structural conversion and is not caused by direct quenching processes, we measured the fluorescence of ferricytochrome *c* in the presence of urea with and without 100 mM NaCl. The two spectra were found to be practically identical (Serpas, Pandiscia, and Schweitzer-Stenner, unpublished).

We also determined the steady-state fluorescence anisotropy for the above NaCl concentrations. The corresponding data are displayed in Figure 7. As one would expect based on the results from above fluorescence titrations, the addition of NaCl also decreases the polarized fluorescence. Most interestingly, however, the steady-state anisotropy not only increases with increasing salt concentration for lipid/protein ratios above 50 but also in the presence of salt the anisotropy now becomes more dependent on the lipid/protein ratio. As indicated above, we can rule out any direct fluorescence quenching by NaCl as the cause of these changes. A more detailed analysis of the data will be provided below.

THEORETICAL ANALYSIS OF FLUORESCENCE AND CD TITRATION

We utilized a comprehensive theoretical model outlined below to arrive at a consistent simulation of all reported titration curves with a consistent set of fitting parameters. Because a homogeneous population of liposomes (i.e., SUVs) was only ensured for lipid/protein ratios above 37.5, the data points taken with lower lipid/protein ratios (15 and 25) were not used for the fitting procedure. A comparison of the CD and the fluorescence titrations clearly reveal differences with respect to slope and saturation behavior. While the fluorescence is monotonously increasing, the dichroism difference values either stay nearly constant at the initial phase (in the absence of salt) or they first decrease (becoming more negative) and start to increase only slightly once the lipid/protein ratio increases above 25. This led us to assume that a minimal model should comprise at least two binding sites with rather different affinities. The lipid/protein ratio dependence on the steady-state anisotropy in the presence of salt (Figure 3) seems to indicate the occurrence of even 3 binding processes. Assuming two binding sites, the analysis was based on the following reaction scheme:

$$\begin{array}{ccc} cyt_{C1} - CL & \Leftrightarrow & cyt_{C2} - CL \\ \Updownarrow K_1 & & \Updownarrow K_2 \\ CL & & CL \\ + & & + \\ cyt_n & & cyt_n \end{array}$$

where the subscripts *n* indicates the native, full, folded state and C1 and C2 conformations of membrane-bound cytochrome *c*. This model assumes that the protein undergoes a conformational change upon binding and that after dissociation from CL on the liposome surface, the protein does switch back to its fully folded state, contrary to an earlier expressed assumption.[74] Moreover, we do not assume the occurrence of any irreversible binding, since this would give rise to a linear increase of the binding

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isotherm at low lipid concentration until the saturation of all binding sites is achieved. None of the observed isotherms meet this requirement. Treating cytochrome *c*−CL interactions as an equilibrium reaction is in accordance with approaches from other laboratories.[42,45,47]

Conformational changes generally give rise to spectroscopic changes, which we utilize in this paper. A Langmuir-type binding isotherm containing spectroscopic response function, which accounts for the CD ($\Delta\Delta\varepsilon$) as well as the fluorescence data (f) measured in our study, can be written as follows:

$$s(\mu) = s_0 + \sum_{j=1}^{2} [s_j \cdot (K_j \cdot \mu)^{n_j}] \quad (3)$$

where s_0 is the spectroscopic value of the fully folded protein, $s_j = s_1, s_2$ denote the respective spectroscopic value for the partially unfolded protein produced by the binding of the site j to CL-receptors on the liposome surface. K_j (j = 1,2) is the affinity constant for the jth binding process involving the protein and CL receptors on the liposome surface, μ is the lipid/protein ratio of lipids that are not in contact with the protein, and n_j are the corresponding Hill coefficients. The latter heuristically reflect the fact that the binding of proteins to membrane surfaces induce changes of the latter that can affect subsequent binding processes.[26] The grand partition sum, G is written as

$$G = 1 + \sum_{j=1}^{2} [(K_j \mu)^{n_j}] \quad (4)$$

The theory described thus far accounts for cytochrome *c*−liposome interactions in the absence of salt. If Na^+ and Cl^- ions are present, they can react with the CL head groups and the protein, respectively. Both reactions can inhibit cytochrome *c* binding, which heuristically can be accounted for by a formalism which describes the reduction of the equilibrium constants K_j' in a dose response manner:

$$K_j' = K_j \cdot (r_j - 1) f_{1,j} + 1 \quad (5)$$

Here, r_j is the amount by which K_j is reduced upon saturation with ions. $f_{1,j}$ is a binding function that describes inhibitory ion binding with respect to the jth binding site. This function was accounted for by a simple Langmuir isotherm:

$$f_{1,j} = \frac{(K_{\mathrm{Na},j}[\mathrm{NaCl}])^m}{1 + (K_{\mathrm{Na},j}[\mathrm{NaCl}])^m} \quad (6)$$

where $K_{\mathrm{Na},j}$ is the effective equilibrium binding constant of Na^+ with regard to the jth binding site. Different values for $K_{\mathrm{Na},j}$ are indicative of different efficiencies with regard to the inhibition of the binding of sites 1 and 2 to CL on the liposome surface.

The 16 sets of titration data displayed in Figures 6 and 7 were simultaneously fit with a common set of thermodynamic and spectroscopic constants. The effective number of free parameters was 16, which has to be compared with 128 data points. The values for s_0 are determined experimentally for cytochrome *c* in solution. The concentration of free lipids which enters all the above equations has been calculated from the total lipid concentrations by combining the law of mass conservation with simple, earlier, introduced numerical methods.[81]

As revealed by the solid lines in Figures 6 and 7, the resulting fits are very satisfactory, in spite of the imposed restrictions. The respective thermodynamic parameters are listed in Table 1, the spectroscopic parameters can be found in Table S1 of the Supporting Information. The reduced χ_r^2 value is 1.12, which indicates a good and statistical significant fit. We use the parameter dependences of χ_r^2 and visual inspections of the fits after the variation of fitting parameters to estimate their statistical uncertainties. Details of this calculation are described in the Supporting Information. The obtained error values are listed in Table 1 and Table S1 of the Supporting Information. Apparently, most of the thermodynamic parameters exhibit rather modest statistical errors.

Table 1. Fitting Parameters Obtained from Our Comprehensive Theoretical Model Outlined in This Paper

	no NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl
K_1[a]	0.175	0.175	0.175	0.175
K_2[a]	0.045	0.037	0.030	0.027
K_1[b] (M^{-1})	$3.5 \times 10^4 \pm 1.5 \times 10^4$	$3.5 \times 10^4 \pm 1.5 \times 10^4$	$3.5 \times 10^4 \pm 1.5 \times 10^4$	$3.5 \times 10^4 \pm 1.5 \times 10^4$
K_2[b] (M^{-1})	$9.0 \times 10^3 \pm 1.0 \times 10^3$	$7.4 \times 10^3 \pm 1.0 \times 10^3$	$6.0 \times 10^3 \pm 1.0 \times 10^3$	$5.4 \times 10^3 \pm 1.0 \times 10^3$
K_1[c] (M^{-1})	1.75×10^5	1.75×10^5	1.75×10^5	1.75×10^5
K_2[c] (M^{-1})	4.5×10^4	3.7×10^4	3.010^4	2.7×10^4
n_1	1.45 ± 0.05	1.5 ± 0.05	1.5 ± 0.05	1.5 ± 0.05
n_2	3.5 ± 0.5	3.5 ± 0.5	4.5 ± 0.5	4.5 ± 0.5

[a]With regard to the lipid/protein ratio. [b]With regard to the lipid concentration in the outer layer of the liposome bilayer. [c]With regard to the cardiolipin concentration in the outer layer of the liposome bilayer.

While the obtained reduced χ^2 value suggests good and statistically reliable fitting of the data, a closer inspection, particularly of the fits to the fluorescence data, suggests some minor, though systematic, deviations between fit and experimental data. The former slightly overestimates the latter at intermediate and slightly underestimates them at higher lipid/protein ratios which one might interpret as reflecting of a third binding process. As indicated above, the steady increase of the steady-state fluorescence anisotropy with the lipid/protein ratio in the presence of salt points in the same direction. However, a global fit of an extended, three binding site model, while reducing the discrepancies, brought about a reduced χ^2 value substantially below 1. Such fits are generally considered statistically unreliable. The respective K_2 and K_3 values emerging from this fit were only modestly different with overlapping statistical errors. The corresponding spectroscopic parameters exhibited rather large correlations. We therefore conclude that the available data do not allow any discrimination between possibly existing binding sites 2 and 3.

While it is obvious that the fluorescence and dichroism data observed in the presence of NaCl require at least a model considering two binding sites, this is less obvious for the data recorded for [NaCl] = 0. We therefore checked whether these data could be fitted with a single step binding model. The result was very clear. We could fit either the fluorescence or the CD data with such a model but not both data sets together. This observation underscores the value of using different spectroscopic methods to assess the binding of the protein to the liposome surface.

In order to facilitate the comparison with other binding studies in the Discussion, Table 1 lists K_j values in three different units: with regard to the lipid-to-protein ratio (no unit), in units of molar with regard to the total bulk concentration of lipids concentration in the outer layer of the liposomes, and in units of molar with regard to the effective bulk concentration of the

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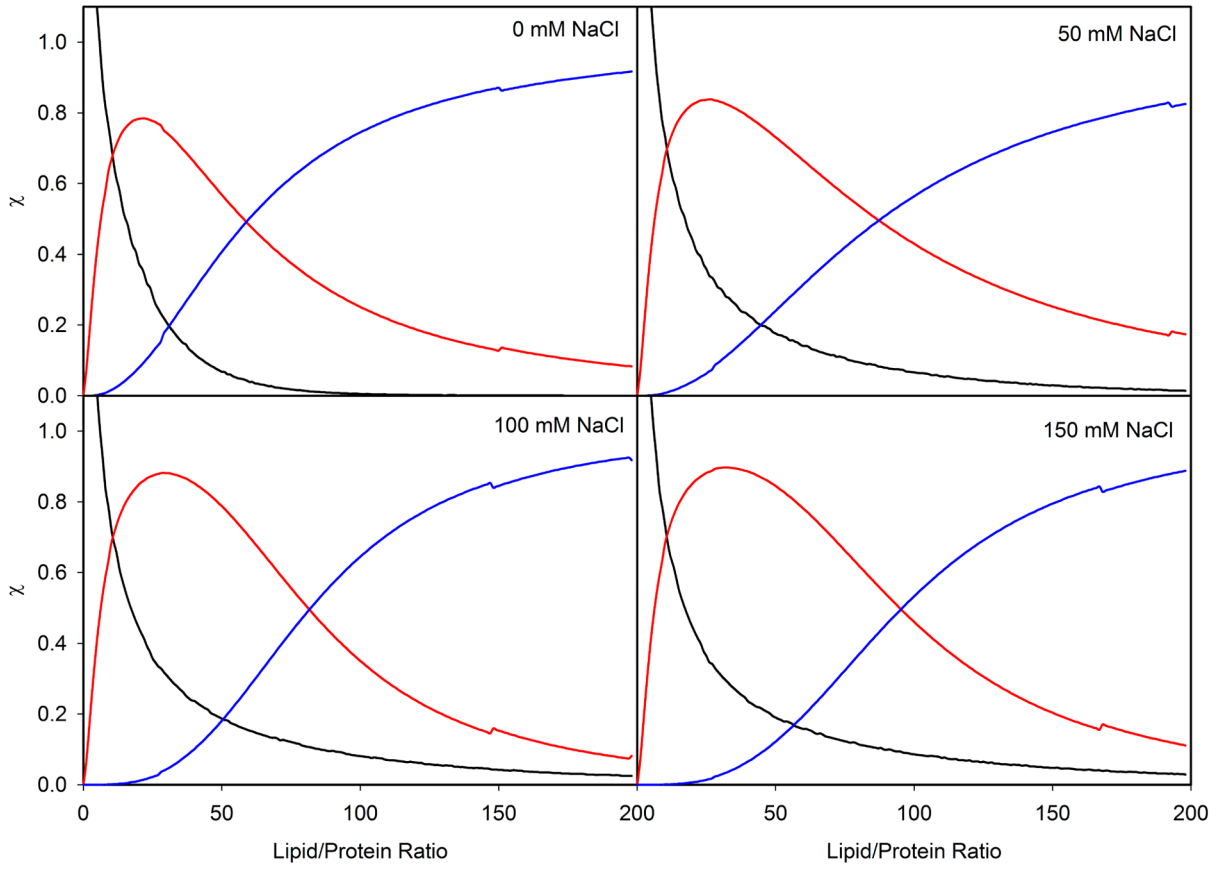

Figure 8. Mole fractions of cytochrome-liposome mixtures in the absence and presence of varying NaCl concentrations. Unbound proteins are shown in black, proteins bound via site 1 are in red, and via site 2 in blue.

corresponding CL lipids. With respect to the latter, we assumed an equal distribution of CL in the outer and inner layer of the SUVs. This might be an oversimplification. Gallet et al. showed that the inner membrane of yeast mitochondria generally has more CL in the outer membrane, which, however, can be reversed by the oxidative phosphorylation production of ATP.[82] In our case, it is more likely that CL is enriched in the outer membrane since this minimizes the electrostatic repulsion between their phosphate head groups. The respective equilibrium constants expressed with regard to the estimated CL concentrations should therefore be considered as an upper limit.

With the obtained equilibrium constants, we calculated the mole fractions of the involved species as a function of the lipid/protein ratio. The results are displayed in Figure 8. In the absence of NaCl, the liposome−protein mixture is dominated by proteins bound via site 1 at low lipid/protein ratios, via site 2 at intermediate-to-high lipid/protein ratios, as one would expect. Upon the addition of salt, the site 2 fraction is slightly shifted to higher lipid/protein ratios. The data could be fitted by assuming that NaCl has no detectable influence on site 1 binding.

In the absence of NaCl, the binding via site 1 induces some fluorescence, but does not change $\Delta\Delta\varepsilon$ at all, thus causing the different initial slopes of the respective titration curves in Figure 6. This result suggests that the protein mostly maintains its native structure. On the contrary, binding via site 2 induces substantial fluorescence and CD changes, the latter adopting positive values. This suggests that the latter process involves substantial changes of the tertiary structure.

To fit the CD titrations obtained in the presence of salt, we had to allow a rather strong influence of NaCl on the spectroscopic parameter s_j. This is in accordance with earlier findings of Pandiscia and Schweitzer-Stenner, who discovered (Figure S5 of the Supporting Information) that the addition of NaCl re-establishes the Soret band couplet of the unbound protein. The latter is affected by Cl^- binding as well. As a consequence, the s_0 value representing $\Delta\Delta\varepsilon$ had to be set to -12 $mol^{-1}cm^{-1}$. This finding is reminiscent of what Shah and Schweitzer-Stenner reported for the influence of phosphate ions on the visible CD spectrum of ferricytochrome c in solution.[71] Second, the $\Delta\Delta\varepsilon$ values (Table S1 of the Supporting Information) associated with site 2 binding return to negative values, thus reflecting the couplet shape of the respective CD spectra (ref 74 and Figure S4 of the Supporting Information). For site 1, $s_1 = \Delta\Delta\varepsilon$ is not much affected by the presence of salt. The salt-induced changes of the CD-spectra are accompanied by only a partial reduction of the intrinsic fluorescence (66% and 18% of the original fluorescence for sites 1 and 2, respectively, at 150 mM NaCl, c.f. Table S1 of the Supporting Information), which indicates that the adopted conformation is not that of the native protein.

The degree of direct binding inhibition by both, sodium and chloride ions, can be estimated by comparing the obtained apparent binding constants for K_2 (Table 1). This yields 18%, 37%, and 40% inhibition for 50, 100, and 150 mM NaCl, respectively. These values reflect the above indicated saturation of NaCl interactions with cytochrome c−liposome complexes above 100 mM. On the basis of the electrostatic theory of Heimburg and Marsh, one estimates a corresponding Debye length of 7.81 Å for 150 mM NaCl, which corresponds to 50% of the protein's radius.[26] We used eq 6 to estimate an effective

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equilibrium constant for sodium-CL interactions and obtained a value of $K_{Na,2} = 0.015\ mM^{-1}$.

Hence, our data strongly suggest that the addition of salt only partially inhibits cytochrome *c* binding to the CL receptors on the liposome surface. The spectral changes (CD and fluorescence) are to a substantial degree caused by salt induced structural changes of the protein itself, which move the protein to a structure with a more native-like heme environment.

As indicated above, we did not consider the fluorescence and CD data measured at low lipid/protein ratios when we fitted the above model to our data. However, as shown in Figures 6 and 7, the respective data points do not substantially depart from the fits, suggesting that the protein binding to the small micelles formed at these conditions resemble mostly the site 1 binding to the SUVs formed at higher lipid/protein ratios. That seems to suggest that at least this binding step is not very dependent on the form and or size of the bilayer.

DISCUSSION

Summary of Results. In this study, we measured the total integrated W59 fluorescence, the polarized fluorescence at 340 nm, and the visible Soret band CD of ferricytochrome *c* as a function of the concentration of lipids added to the protein solution. The polarized fluorescence values were used to calculate the steady-state anisotropy. The chosen mixture of 20% CL and 80% DOPC form liposomes with the ionized phosphate groups of CL functioning as a quasi-receptor for cytochrome *c* binding. A consistent analysis of both the CD and the fluorescence data required the consideration of at least two independent protein binding sites with different binding affinities and spectroscopic properties. Binding via the high affinity site 1 causes rather limited structural changes of the protein in samples, whereas binding via site 2 at higher lipid/protein ratios induces W59 fluorescence and the conversion of the Soret band couplet into a positive Cotton band. W59 belongs to one of the two yellow foldon regions of the protein, whose stability ranks in the middle of the foldon hierarchy.[2] The high intrinsic fluorescence of site 2 bound cytochrome *c* is likely to be diagnostic of a rather extended protein structure. The addition of NaCl has no detectable inhibitory effect on binding via site 1 but inhibits the binding via site 2 by ca. 40% at the presence 150 mM NaCl. Moreover, it has a direct influence on the structure of bound proteins, as judged by rather significant changes which involve the intrinsic dichroism and, to a lesser extent, the intrinsic fluorescence values of the liposome-bound proteins.[74] Such changes should not be neglected in the analysis of binding studies that utilize fluorescence and dichroism spectra as a probe of protein binding to liposomes.

The barely detectable variation of the steady-state fluorescence anisotropy obtained for [NaCl] = 0 indicates a synchronized increase of fluorescence lifetime due to reduced energy transfer to the heme and the W59 rotational mobility. The increase of r_S with an increase of NaCl concentration and the concomitant stronger dependence on the lipid/protein ratio can have two sources; namely, higher fluorescence quenching (reduced lifetime) and a more compact structure (reduced mobility). We will return to this issue below when we discuss our results in more detail.

Comparison with Binding Data Reported in the Literature. Our results must also be discussed in the context of several related binding studies reported in the literature. The binding experiments of Kinnunen and associates have dominated the discussion on cytochrome *c*−liposome interactions due to their identification of two different protein binding sites A and C.[32] These authors studied cytochrome *c* binding by measuring the quenching of the fluorescence of a membrane incorporated pyrene lipid due to fluorescence resonance energy transfer to the heme group of the bound cytochrome *c*. This method has the advantage of probing the binding of cytochrome *c* directly. We wondered whether the first binding process inferred from our data corresponds to the site A binding reported by Kinnunen and co-workers. This conjecture is corroborated by the corresponding visible CD spectra reported by Kinnunen and co-workers, which shows an only slightly reduced B-state couplet. This observation is at least in qualitative agreement with our findings with regard to the CD spectrum of the protein bound by site 1. However, identifying site 1 with A encounters a major obstacle owing to the fact that a closer inspection of the binding data reported by Rytömaa and Kinnunen suggest (a) an even higher binding affinity and (b) a fractional occupation of CL-receptors on the liposome surface. The binding isotherm observed for cytochrome *c*−17.5%CL/82.25%PC (phosphatidylcholine) mixtures (Figure 1 in ref 32) suggests saturation around 0.6 μM, while the total lipid concentration used in this experiment was 55 μM. This corresponds to a minimal concentration of ca. 4.2 μM for CL in the outer layer of the liposomes' bilayers (assuming again a 50%/50% distribution with regard to inner and outer layer). We estimated the binding constant and the fraction of maximal available CL-receptors in terms of a simple one-step binding model, thereby explicitly considering the differences between free and total protein concentration. We thus observed that the binding constant should lie between 5×10^6 and 1×10^7 M^{-1} with only 5% of the CLs available for binding. That is substantially higher than our site 1 affinity of 1.75×10^5 M^{-1}. One might argue that an equilibrium thermodynamic analysis is unsuitable for the data of Rytömaa and Kinnunen because they showed that the binding to the A-site is not reversible. However, if this was the case, one would expect a nearly linear slope of their fluorescence titration until full saturation is reached. The published data do not show this behavior. Consequently, Kawai et al. treated their own A-site binding data with a thermodynamic equilibrium model.[45] Moreover, the partial susceptibility of the proposed A-site binding to Na^+ indicates that at least a fraction of the binding is in fact reversible. Altogether, it seems that our site 1 should not be identified with the A-site proposed by Rytömaa and Kinnunen.[32] The microscopic data of Kawai et al. suggest strong CL-demixing to occur among the lipids of the GUVs that they used for their experiments. If contrary to the claim of Trusova et al.,[46] such a demixing also occurs in the bilayer of the liposomes generally used by Kinnunen and associates, the fractional binding indicated by their isotherms might become understandable within the framework of the theory of Heimburg and Marsh.[26]

The existence of a so-called C-site binding, which is not electrostatic in nature was inferred from the observation of an increased quenching of pyrene fluorescence at acidic pH in the presence of 50 mM NaCl and a less effective inhibition of pyrene quenching for liposomes with a higher CL content (50% and more).[32] C-site binding as introduced by Rytömaa and Kinnunen[32] involves hydrogen bonding between a protonated phosphate group as donor and acceptor side chains situated on the protein surface. These authors proposed such a semi-protonated state of the CL phosphate groups as being caused by the accumulation of hydronium ions in the liposomes' double layer. This conjecture, however, is at variance with recent findings of Malyshka et al., who reported that between pH values

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of 5−12, there is no protonation of the lipid's phosphate head groups.[83]

More recently, Sinibaldi et al., by probing cytochrome *c* binding to liposomes with 100% CL, observed two independent binding sites with binding affinities of 4.9×10^4 and 2.4×10^4 M^{-1} with regard to the molar lipid concentration.[37] If one considers the fact that only ca. 50% of their CLs are in the outer membrane, the real binding constants are 9.8×10^4 and 4.8×10^4 M^{-1}. The value for site 1 lies slightly below the values which we obtained for site 1 (1.75×10^5), whereas the corresponding site 2 values are practically identical (4.5×10^4 M^{-1}, in our case). Altogether, their equilibrium constants are very close to ours, in spite of the difference with regard to the CL content of the liposomes. However, several differences are noteworthy. First, our data do not indicate any substantial structural changes induced by site 1 binding, whereas the dichroism values of Sinibaldi et al. suggest partial unfolding. Second, the data of Sinibaldi et al. seem to indicate NaCl-induced inhibition for both binding sites (58% for site 1 and 86% for site 2). Third, their binding model assumes that the two binding processes are independent (i.e., they reflect different binding sites on the liposomes rather than on the protein). We wonder how such heterogeneity can occur on the surface of liposomes with 100% CL. Our model assumes the binding processes to reflect two different protein binding sites. We also performed simulations with a model for which we assumed different binding sites on the membrane, consistent with the binding model of Sinibaldi et al.[42] This approach failed in yielding a satisfactory fit to our experimental data. This led us to conclude that our dependent site model (i.e., the binding to site 1 requires the absent of site 2 binding and vice versa) is more appropriate.

We now consider the results of some experiments on cytochrome *c*−CL interactions carried out by Pletneva and co-workers. Hanske et al.[47] substituted the heme iron by Zn and measured the Zn-porphyrin fluorescence anisotropy as a function of lipid concentration for a fixed concentration of cytochrome *c*. The thus obtained binding curve is monophasic and the corresponding binding constant is 1.5×10^4 M^{-1}. Since they used 50%/50% mixtures of TOCL and DOPC, the corresponding binding constant for CL-binding is actually 6.0×10^4 M^{-1} (again, we consider the fact that only half of the lipids are in the outer membrane of the liposomes[84]). This lies close to our K_2 value. The main part of the work reported by these authors involved measuring and analyzing the fluorescence energy transfer between four dansyl labels of the protein and the heme. Distance distributions emerging from these data strongly suggest the coexistence of two protein conformations, a compact, molten globule-like conformation C and a more extended structure E in which the contacts between N- and C-helix are broken. Data reported by Hong et al. suggest that the E-state becomes less populated at very low lipid/protein ratios.[44] At high lipid/protein ratio (750 in the study of Hong et al., if one considers only the outer layer), the population of the E-state is significant (60%) if the CL content lies above 30%. This value should be lower for our liposome−protein mixtures because of the lower CL-content (~15% for a lipid/protein ratio of 750).

Finally, we like to discuss the very thorough work of Oellerich et al., who studies the binding of ferricytochrome *c* to LUVs formed with diolenoyl-phosphatidylglycerol (DOPG) at very low to intermediate lipid/protein ratios.[38] The authors analyzed populations and characterized different protein conformations with various optical spectroscopies, resonance Raman spectroscopy among them. They found that in the intermediate lipid/protein regime (20−100), binding of the protein to the LUVs induced conformational transitions into a mixture of non-native hexacoordinated low spin and high spin proteins. The binding in this region is electrostatic in nature. At very low lipid/protein ratios, however, the protein binds more tightly due to partial penetration into the LUV's bilayer. The coverage of the LUV surface is very high. Oellerich et al. estimated a very high binding constant for this process (10^{10} M^{-1}), which exceeds even the high affinity of Kinnunen's A-site binding. One might consider the possibility that the lower surface coverage in our study just decreases the effective binding constant (compared with binding scenario investigated by Oellerich et al.) in that it allows for a lesser penetration into the outer layer of the liposome. With regard to the intermediate lipid/protein region, our optical spectra do not indicate any admixture of hexacoordinated high spin proteins. The Soret band positions rather indicate a non-native hexacoordinated state for site 2 binding. As in our study, Oellerich et al. observed a phase separation of liposome aggregates at low lipid/protein ratios, which they also assigned to the nearly complete coverage of the liposome surfaces by the protein.

Interpretation of Binding Data of This Study. A rather obvious way of assigning the different binding sites inferred from our data could utilize the A, C, and L-sites of the protein identified by Kinnunen, Nantes, and their respective co-workers.[32,33,41] For reasons given above, we rule out site A. Site C, if it exists, and site L seem to be unlikely candidates since they require a more acidic bulk than that provided in our experiments. On the other side our data reveal a coexistence between electrostatic and nonelectrostatic (hydrogen bonding, van der Waals interactions) binding sites, which is very much reminiscent of the A/C-state model.

We wonder whether a classification of cytochrome *c*−liposome interactions in terms of these two sites is way too simplistic. Figure 9 displays the distribution of positively charged lysine residue patches in horse heart cytochrome *c*, which are asymmetrically distributed over the protein. Any of these patches could be potentially involved in the protein's binding to the CL-phosphate groups on the liposome surface. In this context, it is remunerative to remember the much earlier work of Margoliash and co-workers, who investigated in detail the binding of anions to the positively charged lysine patches. They identified two phosphate binding sites termed phosphate sites I (K87) and II (K25, H26, and K27) where the latter exhibits a lower binding affinity than the former (by an order to magnitude).[85,86] These two sites are rarely invoked in the current debate of cytochrome *c*−CL interactions.

Kostrzewa and co-workers performed spin-label ESR on yeast cytochrome *c* bound to negatively charged DOPG membranes and also found that lysine residues 25, 72, 86, and 87 make up the active site of the protein−membrane interaction.[87] Furthermore, they concluded that spin-labeled lysine residues did not show significant immobilization upon binding to lipid membranes when compared to cytochrome *c* in bulk solution, corroborating the work of Oellerich and co-workers that cytochrome *c* does not penetrate into the membrane surface at low liposome occupation.[38]

We do not disregard the possibility that our theoretical binding model might be too simple for two reasons. First, it is thinkable that instead of two binding processes, our data are in fact diagnostic of a distribution of conformations, binding sites, and binding affinities. If one assumes that cytochrome *c* binding induces a partial demixing of CL and DOPC,[88] the protein could

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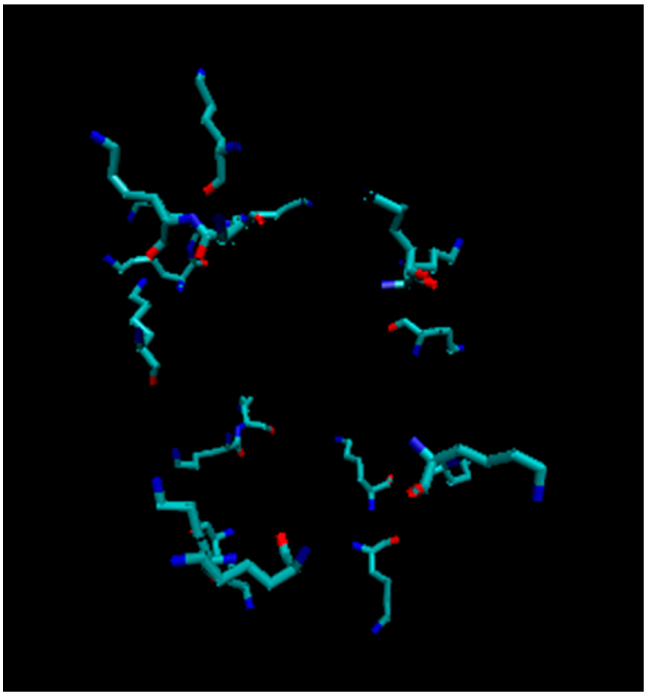

Figure 9. Positively charged lysine side residue in the structure of ferricytochrome *c* (pdb 2FRC). The figure was created with VLC software.

also bind at one particular area on a positive patch, move slightly and by that adjustment, a different area of said patch could then bind to the membrane surface. Thus, alternating between different binding locations on the same positive patch, depending on the orientation of the protein. However, even in the case that such scenarios apply, our spectroscopic and thermodynamic parameters can be considered as effective values which allow us to distinguish two different types of binding, which themselves might still be associated with heterogeneous ensembles of protein−liposome mixtures. Second, in the case of the formation of CL patches, the rather elementary Langmuir-based binding model might lead to an overestimation of binding sites.[88] However, with regard to our data, this would mostly affect the saturation level (i.e., the parameters s_j) and would lead only to a minor underestimation of the binding affinity. Moreover, even if demixing is operative, the situation on the surface of our liposomes should still be more reminiscent of an independent receptor scenario than the 100% dioleoylphosphatidyllglycerol liposomes considered by Heimburg and Marsh.[88]

Our spectroscopic data of the complex formed by site 1 are somewhat reminiscent of the state III* that ferricytochrome *c* adopts at mildly alkaline conditions (pH 9) and low ionic strength in solution. In this state, the protein exhibits a quasi native-like B-band couplet, a reduced 695 nm band and weak W59 fluorescence.[89] This all indicates that the M80 ligand is weakened but still bound to the heme iron.[61] Site 1 is nearly unaffected by the presence of NaCl, which seems to rule out any electrostatic interaction. The lack of influence of NaCl also excludes protein penetration into the outer liposome layer as the primary cause for this binding.[31,38,90] Furthermore, our protein concentrations are not large enough to induce lateral pressure on the membrane surface to cause penetration.[26] The same line of argumentation applies in part to site 2 binding, owing to the partial inhibition of binding by sodium ions. To explain this coexistence between electrostatic and nonelectrostatic binding, one might consider the possibility of a consecutive action of electrostatic and noncovalent interactions, somewhat reminiscent of a combined A/C-site binding. Such an effect has indeed been observed by Sanghera and Pinheiro for the binding of the Syrian hamster prion protein SHaPrP(90−231) to anionic lipid membranes. The authors found compelling experimental evidence for a two-step binding process that comprises initial electrostatic lipid−protein interactions, which form advantageous contacts that are not governed by electrostatic interactions.[91] This could involve the generally invoked hydrophobic contacts, but we wonder whether hydrogen bonding between ammonium groups and the PO_2^- head groups of cardiolipin should be regarded as a yet not considered option. In principle, there is no necessity for CL-protein hydrogen bonding to require the phosphate group to function as a donor. Recent NMR studies by Kooijman et al. show that such hydrogen bonding can indeed occur between with phosphatidic acid binding domains of lipid suspensions,[92] ionized phosphate groups should have similar capabilities. Site 2 binding can therefore be understood as involving the equilibrium between two CL-bound states of the membrane, one being electrostatically bound, whereas the other one is stabilized by hydrophobic interactions and/or by H-bonding. The two bound states are in equilibrium with unbound protein in solution owing to the fact that we obtained the same effect of NaCl binding on the CD spectra of cytochrome *c*-liposome mixtures, irrespective of whether salt was added prior or after mixing proteins and liposomes (data not shown). This could not have happened if the nonelectrostatic binding state could only be established via prior electrostatic binding. We therefore propose the following reaction scheme for binding site 2:

$$cyt_{C2}^{ES} - CL \leftrightarrow cyt_{C2}^{H} - CL$$
$$\searrow \qquad \swarrow$$
$$CL$$
$$+$$
$$cyt_n$$

where the superscripts ES and H denote electrostatic and hydrogen bonding/hydrophobic binding. To some extent, it resembles the A/C-site model of the Kinnunen group[32] but with much lower affinities. K_2 must therefore be treated as an apparent binding constant, which reflects the average of binding to the two protein isomers on the liposome surface. If binding to the ES state has a slightly higher affinity, the elimination of this step by NaCl binding would decrease the apparent binding constant as observed. Moreover, the spectral changes obtained upon the addition of NaCl suggest that the ES state is more native-like and less unfolded than the H state. The former exhibits less W59 fluorescence than the latter and a more native-like Soret band CD signal.

Our model is in qualitative agreement with the observed increase of the steady-state anisotropy with increasing ionic strength (Figure 3), if one assumes that proteins in the ES state exhibit lesser W59 quenching and thus a much lower anisotropy than the H state of the protein. The values obtained in the absence of salt are thus average values of these two conformers. The addition of salt mostly eliminates the ES state, thus increasing the apparent steady-state anisotropy.

The idea of the existence of two coexisting states of cytochrome *c* on the surface of CL-containing liposomes have

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been put forward before by Pletneva and co-workers, as briefly mentioned above.[44] Their E-state is more unfolded than their more compact C-state; it is therefore reasonable to assume that the former exhibits much more W59 fluorescence than the C-state. With respect to cytochrome *c* in solution, the compact state A populated at pH 2 in the presence of NaCl and the fully denatured state produced by an access of urea might serve as models for C and E, respectively. The integrated fluorescence intensities of the respective F_2-subband are 200 and 2500 (data not shown), respectively. From these numbers, it follows that the s_{20} values of 1900 (no salt) and 350 ([NaCl] = 150 mM) would indicate 74% and 6.5% of E-state population, respectively. This simple calculation certainly overestimates the E-fraction, but the result is consistent with the observation that the E-state population is substantially reduced by the addition of salt.[44] Altogether, there is a striking similarity between the ES/H conformers inferred from our data and the C/E coexistence deduced from the more site-specific studies of Pletneva and co-workers.

A more recent study by Muenzner et al. explored the kinetics of cytochrome *c* binding to liposomes with 50% TOCL/50% DOPC at very high lipid concentrations, which corresponds to a lipid/protein ratio of 125, if one considers only the outer membrane.[93] One has to exercise some caution when comparing their data to ours, owing to the different lipid mixture of their and our liposomes. However, even if we use our results only as a qualitative guideline, it is reasonable to suggest that site 2 binding should be dominant at their experimental conditions. The authors found that the binding process can be subdivided into four kinetically distinguishable processes (i.e. the very fast binding process), a rearrangement affecting segments of the protein, partial insertion into the membrane, and eventually the formation of the extended structure of state E. The latter step was found to occur only at high lipid/protein ratios. A partial, though limited, insertion into the membrane could explain the observed blue shift of the W59 fluorescence F band in the site 2 binding region.

Finally, we like to mention that our thermodynamic analysis also required the use of Hill coefficients for each binding process, in agreement with Sinibaldi et al.[42] and Kawai et al.[45] This is not surprising because one can expect that the membrane surface itself changes upon binding to the protein. Heimburg et al. showed that binding of cytochrome *c* to negatively charged phospholipids induced bilayer curvature of the lipids, which in turn could increase the probability for additional binding of proteins.[26,90] The Hill coefficient could also reflect the average number of CL-lipids constituting the binding site for a single protein.[88] On the basis of their microscopic data, Kawai et al. suggested a protein induced reorientation of the lipids with the CL-rich domains as reason for the observed positive cooperativity.

Structure−Function Relation. Is there anything we can learn about the real biological behavior of cytochrome *c* from our data? A high affinity, native-like state of cytochrome *c* is needed to properly orchestrate electron transfer in the IMM. We propose that our site 1 and possibly also the H-conformer of site 2 binding could fall into this category, since the presence of native M80 ligand would allow for the protein to carry out its main function of electron shuttling, while this can be excluded for conformations adopted via site 2 binding. The presence of a physiological concentration of salt in the inner membrane space would stabilize the two more native conformers further.

How can cytochrome *c* gain peroxidase activity? To answer this question, one must first recall the relationship between cytochrome *c* binding, protein structure, and peroxidase activity. The absorption spectra of site 2 proteins peak at 407 nm, which rules out any substantial population of a pentacoordinated state but is more likely to reflect either histidine[94] or lysine ligation. Peroxidase activity, however, is thought to be associated with a pentacoordinated iron in the resting state.[95,96] Hence, further structural changes are necessary for the protein to acquire this enzymatic capability. However, if histidine is the sixth ligand in the site 2 mixtures, it might not be too difficult to replace it in the course of a reaction with H_2O_2. This notion particularly applies if a substantial fraction of proteins bound via site 2 is actually in the E-state, in which substrates should have an easy access to the active site of the protein.[93] The work of Muenzner et al. indicates indeed a predominant peroxidase activity of the E-state.[93]

SUMMARY

It was the purpose of this study to obtain a thorough thermodynamic and spectroscopic characterization of cytochrome *c* binding to cardiolipin-containing liposomes at conditions which, in terms of cardiolipin fraction and salt concentration, bear some similarity to what the protein encounters on the inner membrane of the mitochondria. We identified two different binding processes with different affinities, which we relate to different binding sites on the protein. We denoted them as sites 1 and 2 binding processes in the order of decreasing binding affinity. Site 2 binding leads to substantial structural changes which have been obtained in studies from other laboratories.[39,42,47] Site 1 binding is peculiar in that it does not involve major changes of the protein's tertiary structure; it is reminiscent of the intermediate state III* of ferricytochrome *c* which Verbaro et al. recently discovered at pH 9 and low ionic strength.[89] Site 1 and to a lesser extent site 2 binding were found to be unresponsive to the addition of salt, which suggest hydrophobic interaction or hydrogen bonding as the underlying process. None of our binding sites nor the binding processes investigated by Santucci, Pletneva, and their respective co-workers should be identified with site A and C binding proposed by Rytömaa and Kinnunen,[32] which involve a very high-affinity binding to a partial fraction of cardiolipins at rather high lipid/protein ratios. The spectral changes and the partially reduced binding associated with binding step 2 are interpreted as reflecting an equilibrium between two protein conformers of bound cytochrome *c*. One interacts electrostatically with CL, and it is therefore susceptible to the addition of salt. This conformation is rather unfolded and gives rise to most of the W59 fluorescence observed at high lipid/protein ratios. The other conformer binds to CL either via Lys−PO_2−H-bonding or hydrophobic interactions, it is less unfolded, and becomes predominant at high lipid/protein ratios in the presence of salt. Both conformers are in equilibrium with proteins in the solvent. At very low lipid/protein ratios, the concentrations of lipids are too low to form SUVs, and instead micelles are formed. Upon cytochrome *c* binding, these micelles fuse into GUVs. Interestingly, the binding to the micelles resemble site binding to SUVs, suggesting that this binding step is rather independent of the vesicle size.

Our binding studies as well as the conformational studies of the Pletneva group were carried out under conditions at which only a small fraction of the liposome surface was occupied by proteins. This minimizes lateral pressure and avoids a deeper insertion of proteins into the lipid bilayer. Thus, our studies

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should be distinguished from work carried out with much lower lipid/protein ratios, which led to the observation of irreversible membrane insertion.[34,37,45]

■ ASSOCIATED CONTENT

Supporting Information

Additional fitting parameters and the mole fractions of the involved species of the protein−liposome mixtures; dynamic light scattering data and fluorescence spectra; soret band CD spectra; and occupation of lipids/liposome as a function of lipid/protein ratio. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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