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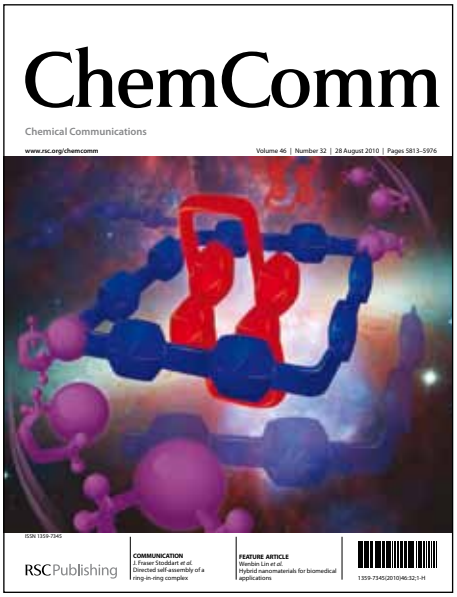

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

Salt as a Catalyst in the Mitochondria: Returning Cytochrome c to its Native State after it Misfolds on the Surface of Cardiolipin Containing Membranes

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Cytochrome c binding to cardiolipin receptors on the surface of TOCL/DOPC(20%:80%) liposomes induces a conformational change which is not reversible after the protein's dissociation at low ionic strength. Addition of 100 mM NaCl switches the protein back to its native state.

Cytochrome *c* is a multi-facet bio-molecule with a high degree of plasticity. It serves as an electron carrier between cytochrome c reductase and oxidase on the inner membrane surface of the mitochondria.[1] Moreover, it has been utilized as an ideal model system for fundamental protein folding studies.[2] Over the last five to ten years research on cytochrome *c* has shifted focus towards the protein's role in triggering mitochondrial apoptosis.[3] To this end, the protein has to dissociate from the inner membrane and subsequently penetrate the outer membrane to escape into the cytoplasm.[4] To accomplish this task the protein has to acquire peroxidase activity by which it oxidizes cardiolipin in the inner membrane.[4,5] This requires the protein to switch from its native fully folded to a partially unfolded conformation in which the functional heme group is more exposed to the protein environment.[6] It is unclear, however, to what extent these conformational changes are reversed upon the protein's dissociation from the inner membrane. This issue has to be clarified in order to understand how the protein can interact with the outer membrane and finally with the Apaf1 complex in the initiation of the apoptotic process.

Liposomes with anionic phospholipids have been frequently used as a model system for the inner mitochondrial membrane.[5,7–13] Cardiolipin is particularly effective for interacting with the protein via electrostatic interactions with its positively charged surface and some additional hydrophobic interactions.[14] Most of these interactions involve conformational transitions of the protein into partially unfolded conformations.[15] This communication shows that the reversibility of these transitions depend on the presence of anion binding to the protein in solution.

We first measured the circular dichroism (CD) spectrum of the Soret band region of ferricytochrome *c* as a function of lipid/protein ratio in the absence and presence of 100 mM (nearly physiological) concentration of NaCl. For the liposomes, we chose a 20%/80% mixture of TOCL(1,1'2,2'-tetraoleoyl cardiolipin) and DOPC(1,2-dioleoyl-sn-glycero-3-phosphocholine). This is close to physiological conditions with regard to the cardiolipin content of the liposome's lipid bilayer.[13] The pH of the sample was adjusted at 7.0 and stabilized with a 25 mM HEPES buffer. The observed spectra are shown in Figure 1 and 2, respectively.

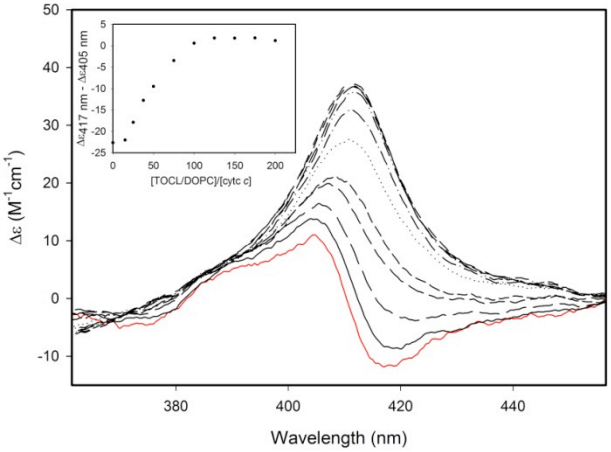

Fig.1 Soret band CD (top) measured as a function of the lipid/protein ratio of cytochrome c- liposome complexes. The red line represents cytochrome *c* in solution in the absence of lipids. Spectroscopic and sample parameters are in the Supporting Information. The positive couplet at 412 nm increases with liposome/protein ratio, which ranged from 15 to 200 in increments of 25. Inset: Difference between Δε-values measured at the position of the positive and negative component of the couplet in the native state's spectrum plotted as a function of the lipid/protein ratio.

The CD spectrum of the unbound native protein displays a couplet, which is indicative of B-band splitting, in part due to the presence of a strong internal electric field.[16] In the absence of NaCl and upon the addition of liposomes, the couplet gradually disappears with increasing lipid/protein ratios and is replaced by a positive Cotton band, which reflects a more open heme crevice with the M80 containing Ω-loop further away from the heme group (Figure 1).[17] This parallels the observation Sinibaldi et al. reported for cytochrome binding to liposomes with 100% TOCL.[18] The magnitude of the Cotton band is reminiscent of the Soret band CD signal, which Hagarman et al.[19] observed for the non-native state V of ferricytochrome and Soffer et al.[20] 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. The inset of Figure 1 shows the difference between the Δε values measured at the position of the positive and negative component of the couplet in the native state's spectrum as a function of lipid/protein ratio. These data are indicators of the protein's binding to the liposome surface. We also measured the corresponding UVCD spectra, which are shown in Figure S1 of the Supporting Information. The spectra clearly indicate that the secondary structure of the protein remains mainly unaffected by the conformational transition of the protein.

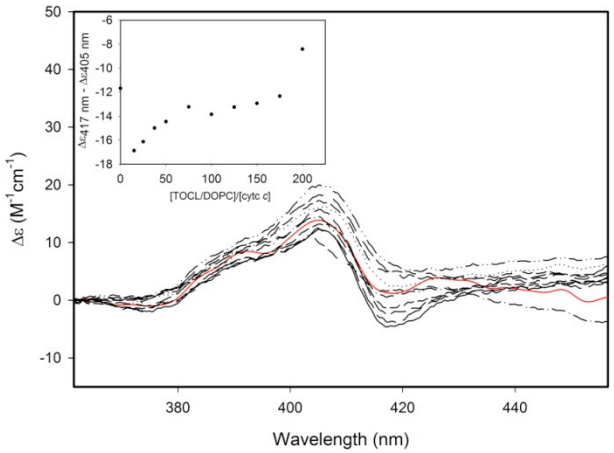

Fig. 2 Soret band CD (top) measured as a function of the lipid/protein ratio (inset) of cytochrome *c*-liposome complexes in the presence of 100 mM NaCl. The red line represents cytochrome *c* in solution in the presence of 100 mM NaCl. Spectroscopic and sample parameters are in the Supporting Information. Lipid/protein ratios ranged from 15 to 200 in increments of 25. Inset: Difference between Δε-values measured at the position of the positive and negative component of the couplet in the native state's spectrum plotted as a function of the lipid/protein ratio.

Figure 2 reveals that the addition of 100 mM NaCl nearly recovers the couplet of the fully folded protein. The binding isotherm (Figure 2 inset), indicates a significantly reduced binding of the protein to the liposomes, suggesting that the binding mechanism is at least partially electrostatic in nature, as reported earlier.[21,8] The dominance of the couplet structure in all spectra further suggest that the unbound proteins are all in the native state, indicating reversibility for the protein's conformational change. The corresponding UVCD spectra in Figure S2 are again suggesting that the secondary structure remains mostly unaffected. It should be noted that the presence of salt reduces the couplet of the unbound, native protein, which reflects a direct influence of anion binding to positively charged patches on the protein's surface on the electrostatic potential of the protein in the heme pocket.[22]

The results reported thus far resemble finding from earlier studies. Here, we go a step further by checking the reversibility of the conformational changes of cytochrome c induced by its bin ding to CL. To this end we subjected the protein-liposome solutions to ultracentrifugation, again in the absence and presence of 100 mM NaCl. This allowed us to probe the unbound fraction of the protein. Surprisingly, in the absence of salt, the CD spectra still display positive Cotton bands for all lipid to protein ratios (Figure 3), which indicates that the protein has maintained the non-native, partially unfolded state after dissociation from the surface. This observation resembles the recent discovery of a misfolded state of ferricytochrome c that remains stable at physiological conditions.[20]

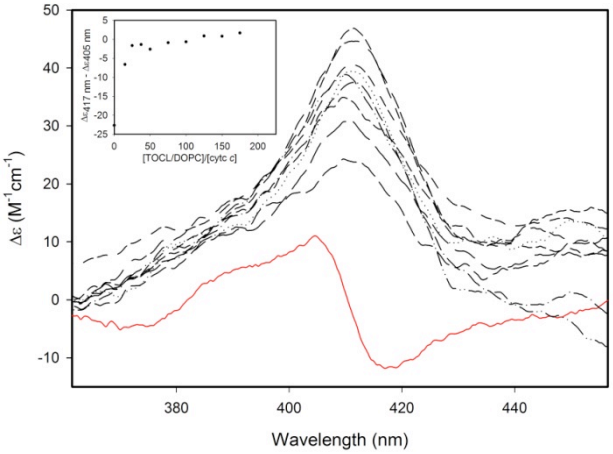

Fig. 3 Soret band CD (top) measured as a function of the lipid/protein ratio (inset) of the protein supernatant. The red line represents cytochrome *c* in the absence of liposomes. Spectroscopic and sample parameters are in the Supporting Information. The positive couplet at 412 nm increases with liposome/protein ratio. Lipid/protein ratios ranged from 15 to 200 in increments of 25. Inset: Difference between Δε-values measured at the position of the positive and negative component of the couplet in the native state's spectrum plotted as a function of the lipid/protein ratio.

The addition of NaCl prior to the centrifugation processes yields a rather different picture. As shown in Figure 4, all of the recorded spectra resemble or are very similar to the couplet observed for the native protein in the presence of 100 mM NaCl. The Δε values depicted in the inset show only minor changes at high lipid/protein ratios which might indicate an incomplete conversion to the native state for the dissociated proteins. This observation suggests that Cl^- ions not only inhibit cytochrome c binding to the cardiolipin receptors on the liposome surface but also insure that the induced conformational changes are reversible. This is a very astonishing result and points to an important regulatory role of these ions in the inner membrane space of mitochondria.

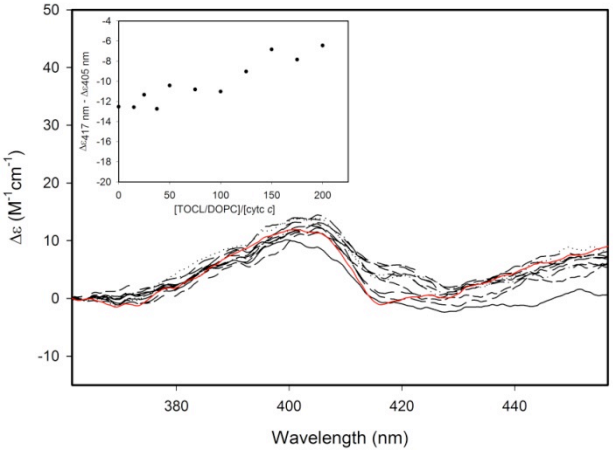

Fig. 4 Soret band CD (top) measured as a function of the lipid/protein ratio (inset) of the protein supernatant in the presence of 100 mM NaCl. Spectroscopic and sample parameters are in the Supporting Information Lipid/protein ratios ranged from 15 to 200 in increments of 25. Inset: Difference between Δε-values measured at the position of the positive and negative component of the couplet in the native state's spectrum.

In order to ensure that the supernatant contains only proteins unbound to liposomes, we employed dynamic light scattering (DLS) experiments to check the size of the particles in our samples. Our 20%/80% liposome mixtures of CL/PC gave a median particle size of 38.4 ± 7.5 nm, cytochrome *c* in the presence of salt alone yielded a particle size of 4.0 ± 0.5 nm, while the supernatant samples had a particle size ranging from 4.8 ± 0.9 to 6.4 ± 0.9 nm, confirming that the protein in the supernatant had dissociated from the surface.

We wondered whether the salt-induced reversibility of the conformational transition is liposome mediated, i.e. indirect modifications of protein – cardiolipin interactions by the binding of Na^+-ions to negatively charged groups on the liposome surface[23] or directly induced by the interaction between Cl^- ions to positively charges patches of the protein in solution. To distinguish between these two modes of interaction, we first centrifuged liposome-cytochrome c samples in the absence of salt and subsequently added 100 mM NaCl. We then measured the respective CD spectra and found them practically identical with those in Figure 4 (cf. Figure S3). Hence, Cl^- ions function as an allosteric effector that restores the protein's native state.

Conclusions

Our results are surprising in many respects. Firstly, our data regarding cytochrome c – liposome interactions in the absence of salt indicate that at least a substantial fraction of the observed binding is reversible (a quantification has to await further studies), while the induced conformational transition is not. This is thus the second time that we observed an apparently frustrated state of ferricytochrome being stable at neutral pH.[20] Secondly, the fact that this misfolded conformation switches back in the presence of NaCl is even more surprising. This suggests that the former is stabilized by repulsive interactions between positively charged surface groups, which may prevent the N- and C-helix of the protein from forming the necessary non-covalent bonds to establish the native state. A similar though somewhat less dramatic effect has been observed at acidic pH where Cl^- binding can switch a mostly unfolded ferricytochrome c into the much more folded molten globule state A.[24] Our results are reminiscent of what Ingwall et al. reported with regard to the conformation of a polyalanine peptide with lysine segments at its termini. With the lysine's charged the protein was found to be in extended state. Upon deprotonation, it collapsed into a compact, molten globule like state.[25]

The most striking consequence of the observed results is that it sheds some light on the biological role of NaCl in the space between inner and outer membrane. It is generally thought that salt reduces the number of cytochrome c molecules bound to the inner membrane surface thus ensuring an equilibrium between a majority of unbound and a minority of bound proteins. Our results suggest an additional role: it prevents the protein from predominantly switching into a misfolded state. If this happened the protein could no longer serve effectively as an electron transfer protein.

In conclusion, our data reveal two catalytic processes as being involved in cytochrome c – cardiolipin interactions. First, the liposome surface promotes a conformational transition of the protein. Second, Cl^- binding reverses this transition in solution. Our discovery should prompt investigations on the functionality of cytochrome c in mitochondria that are deficient with regard to their $NaCl^-$ concentration in the inner membrane space. We predict that this will cause a major perturbation of the mitochondrial electron transfer process.

We like to thank Dr. Joe Foley for providing us access to his dynamic light scattering apparatus, Dr. Elias Spilotis for providing us access to his centrifuge and Dr. Jonathan B. Soffer for producing the TOC figure. L.A.P. received fellowship support from the College of Arts and Sciences.

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† Electronic Supplementary Information (ESI) available.
See DOI: 10.1039/b000000x/

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