Published in final edited form as:

Chem Phys Lipids. 2014 April ; 179: 57–63. doi:10.1016/j.chemphyslip.2013.11.002.

Structural Transformations of Cytochrome *c* upon Interaction with Cardiolipin

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Abstract

Interactions of cytochrome *c* (cyt *c*) with cardiolipin (CL) play a critical role in early stages of apoptosis. Upon binding to CL, cyt *c* undergoes changes in secondary and tertiary structure that lead to a dramatic increase in its peroxidase activity. Insertion of the protein into membranes, insertion of CL acyl chains into the protein interior, and extensive unfolding of cyt *c* after adsorption to the membrane have been proposed as possible modes for interaction of cyt *c* with CL. Dissociation of Met80 is accompanied by opening of the heme crevice and binding of another heme ligand. Fluorescence studies have revealed conformational heterogeneity of the lipid-bound protein ensemble with distinct polypeptide conformations that vary in the degree of protein unfolding. We correlate these recent findings to other biophysical observations and rationalize the role of experimental conditions in defining conformational properties and peroxidase activity of the cyt *c* ensemble. Latest time-resolved studies propose the trigger and the sequence of cardiolipin-induced structural transitions of cyt *c*.

Keywords

apoptosis; peroxidase; protein folding; heme

Cytochrome *c* and Its Conformational Dynamics

The heme protein cytochrome *c* (cyt *c*) is best known for its role as an electron carrier in respiration and found in all animals, aerobic microorganisms, and plants (Moore and Pettigrew, 1990). The structure of horse heart cyt *c*, by far the best characterized cyt *c* homolog, consists of five α -helices connected by Ω loops; the *N*- and *C*-terminal helices are in contact with each other (Bushnell et al., 1990). The heme is covalently attached to the polypeptide by two thioether linkages at Cys14 and Cys17 (Allen et al., 2003). Four pyrrole nitrogens as well as residues His18 and Met80 are ligands to the heme iron. These structural features are common among all mitochondrial cyt *c* proteins.

The folding and unfolding of cyt *c* in solution have been explored extensively (Winkler, 2004; Maity et al., 2005; Ensign et al., 2008; Chen et al., 2009; Yu et al., 2012). Hydrogen-deuterium amide exchange experiments have suggested that cyt *c* consists of five cooperative folding units (foldons) of varying thermodynamic stability that undergo folding

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and unfolding in a stepwise, sequential mechanism (Roder et al., 1988; Bai et al., 1995; Maity et al., 2004; Krishna et al., 2006). The terminal *N*- and *C*-helices are the regions of greatest stability, while the Met80-containing loop is the least stable substructure. The foldon theory has been probed with experiments and theory, and both showed consistent results (Maity et al., 2005; Weinkam et al., 2005; Krishna et al., 2006; Duncan et al., 2009). However, despite decades of active research, the mechanism of cyt *c* folding has remained controversial, with structural features of kinetic intermediates and alternative folding pathways being the main subject of discussion (Winkler, 2004).

In addition to its native structure, cyt *c* is known to adopt a variety of alternative conformations. At low pH and high salt, the protein becomes a molten globule: a compact state with largely preserved secondary but fluctuating tertiary structure (Ohgushi and Wada, 1983; Jordan et al., 1995; Pletneva et al., 2005; Nakamura et al., 2011). Ligand substitution frequently accompanies conformational changes of cyt *c*. At high pH, the weakly-bound Met80 ligand gets replaced by either Lys73 or Lys79 resulting in increased exposure of the heme group (Rosell et al., 1998; Assfalg et al., 2003; Cherney and Bowler, 2011). In urea- or guanidine hydrochloride (GuHCl)-unfolded protein at near neutral pH, Met80 is replaced by His26 or His33, while His18 remains bound to the heme (Colón et al., 1997). In addition to common denaturants (acid, urea, and GuHCl), many other additives destabilize the native structure of cyt *c*. Detergent micelles (Bertini et al., 2004; Naeem and Khan, 2004; Bhuyan, 2010), anionic polymers (Antalík et al., 2003; Sun et al., 2012), alcohols (Naeem and Khan, 2004; Bágeová et al., 2008; Singh et al., 2011), lipid membranes (Pinheiro et al., 1997), fatty acids (Patriarca et al., 2009), many aliphatic anions (Ibanez and Herskovits, 1976), and even low ionic strength (Banci et al., 1998) or added ATP (Antalík and Bágeová, 1995; Snider et al., 2013) alter structure and/or stability of the protein.

Structural Change Dictates New Function

Work by Kagan *et al.* has shown that upon binding to the mitochondrial glycerophospholipid cardiolipin (CL), cyt *c* is able to function as a peroxidase and promote CL oxidation, revealing a previously unknown, early step in apoptosis (Kagan et al., 2005). This activity has been linked to the release of cyt *c* into the cytosol and subsequent caspase activation (Kagan et al., 2005). The discovery has renewed interest in studies of cyt *c* interactions with membranes (Kimelberg and Papahadjopoulos, 1971; Pong and Griffith, 1975; Brown and Wüthrich, 1977; de Kruijff and Cullis, 1980; Rietveld et al., 1983; Hildebrandt and Stockburger, 1989; Heimbürg and Marsh, 1993) and stimulated new research. Disruption of the protein tertiary structure and loss of the Met80-heme coordination have suggested the role of these changes for cyt *c* new function but the exact mechanism of the polypeptide transformations remained unclear. Several excellent reviews about earlier investigations of cyt *c* and CL in apoptosis as well as their interactions exist (Pinheiro, 1994; McMillin and Dowhan, 2002; Bayir et al., 2006; Gonzalez and Gottlieb, 2007; Kapralov et al., 2007; Ow et al., 2008; Kagan et al., 2009). Herein, we focus on the latest discoveries that have shed light on the structural features of CL-bound cyt *c* and its conversion into an apoptotic peroxidase.

The disordered nature of CL-bound cyt *c* has been a challenge to traditional structural methods but many site-specific probes have uncovered details of this elusive ensemble (Figure 1) (Spooner and Watts, 1992; Heimbürg and Marsh, 1993; Kawai et al., 2005; Basova et al., 2007; Kapralov et al., 2007; Kapetanaki et al., 2009; Bradley et al., 2011; Hüttemann et al., 2011; Balakrishnan et al., 2012; Hanske et al., 2012; Silkstone et al., 2012; Sinibaldi et al., 2013). Collectively, these studies have suggested that multiple modes of the protein-lipid interactions (Figure 2) as well as different protein conformations and heme ligation states may be involved. Analysis of distance distributions from time-resolved FRET

(TR-FRET) studies of dye-labeled cyt *c* has overcome the disadvantages of ensemble-averaging techniques and revealed conformational diversity of the CL-bound cyt *c* (Hanske et al., 2012; Hong et al., 2012). Protein structures vary in their degree of protein unfolding and their distribution is dependent on the choice of experimental conditions, including CL content and protein coverage of the membrane surface (Hong et al., 2012). Among these structures, extended conformers with broken contacts between *N*- and *C*-terminal helices likely dominate the peroxidase activity of the ensemble (Hanske et al., 2012). Fluorescence correlation experiments have suggested that compact *C* and extended *E* structures are not independent but undergo conformational exchange related to the break-up and reestablishment of interhelical contacts (Hong et al., 2012).

Modes of Cyt *c*-CL Interactions

Analyses of the effects of ionic strength and mutations suggest that cyt *c* binding to CL-containing membranes is guided by electrostatic forces (Sinibaldi et al., 2008; Abe et al., 2011; Hanske et al., 2012; Hong et al., 2012; Sinibaldi et al., 2013) but hydrophobic interactions also play a role, perhaps after the initial docking configuration is established. Three distinct sites on the cyt *c* surface have been suggested for interactions with CL: the A site, formed by Lys72, Lys73, Lys86 and Lys87; the C (or P) site, located near Asn52 (Rytömaa and Kinnunen, 1994; Rytömaa and Kinnunen, 1995); and the L site involving Lys22, Lys 27, His33, Lys25 and His26, that operates at low pH (Kawai et al., 2005). A recent mutational study ascribed a pivotal role to Lys72 and Lys79 for binding to CL (Sinibaldi et al., 2013).

Repeatedly, the region near the highly conserved residue Arg91 has been implicated in binding interactions with membranes. EPR experiments using anionic liposomes suggested that the nearby residues Lys72, Lys86 and Lys87 face the membrane surface, but argued against protein insertion into the lipid bilayer (Kostrzewa et al., 2000). Other studies indicated the protein penetration into CL-containing lipid bilayers (Gorbenko, 1999) as well as other anionic membranes (Heimbürg and Marsh, 1995; Choi and Dimitriadis, 2004). Analysis of the emission maxima of dye-labeled cyt *c* variants pointed to the hydrophobic environment of the labeling site at residue 92 close to Arg91 (Hong et al., 2012; Snider et al., 2013); however, given the ease of cyt *c* dissociation, the insertion is likely not deep (Hanske et al., 2012). The interaction of the Arg guanidinium group with the phospholipid head groups is known to facilitate formation of membrane pores (Tang et al., 2007). Interestingly, an early study found that a semisynthetic cyt *c* in which Arg91 is replaced by norleucine failed to rupture membranes (Tuominen et al., 1997). The mutation Arg91Ala dramatically affects the protein stability and solvent accessibility of the heme group (Rajagopal et al., 2012), suggesting that if Arg91 indeed inserts into a bilayer, associated structural changes may contribute to opening of the heme pocket in cyt *c*.

In the lipid anchorage model, one or two CL acyl chains have been proposed to insert into hydrophobic channels of cyt *c*. One suggested insertion cleft is located close to the C site and involves the conserved residue Asn52 (Rytömaa and Kinnunen, 1995; Banci et al., 1999; Tuominen et al., 2002). Another channel may be between the two parallel polypeptide strands formed by residues 67-71 and 82-85, close to the Met80 loop (Kalanxhi and Wallace, 2007; Rajagopal et al., 2012). In addition, occupation of both insertion sites has also been proposed (Sinibaldi et al., 2010).

A possible explanation to the puzzling variation in binding modes could be the difference in experimental conditions. The lipid/protein ratios influence the mode of cyt *c* binding to anionic liposomes: high ratios lead to primarily electrostatic (peripheral) binding, while low ones favor cyt *c* anchoring to the membrane (Oellerich et al., 2004). Higher concentrations

of CL in lipid mixtures and large available membrane surface areas promote massive unfolding of cyt *c* (Hong et al., 2012). Furthermore, the relative population of *E* structures depends strongly on the CL content of the membrane.

Heme Environment and Radical Intermediates

An open coordination site and facile access of substrates are important for peroxidase activity of heme enzymes. The folded cyt *c* with Met80-ligated heme is a poor peroxidase but protein unfolding dramatically activates this function (Diederix et al., 2002). The hallmark of structural alterations of cyt *c* upon interaction with CL is the disruption of the Met80 ligation. The residue is located at the least stable red foldon (Maity et al., 2004) and the affinity of a Met sidechain for the ferric heme is relatively low (Tezcan et al., 1998), both explaining the ease of breaking this interaction. Lowered heme redox potential (Basova et al., 2007), Soret band shifts (Hanske et al., 2012), disappearance of the diagnostic 695 nm charge transfer band (Belikova et al., 2006; Kapralov et al., 2007; Sinibaldi et al., 2011), circular dichroism (CD) (Sinibaldi et al., 2008; Sinibaldi et al., 2011) and magnetic circular dichroism (MCD) (Bradley et al., 2011) experiments consistently indicated Met80 displacement and changes in heme ligation.

Cyt *c* misligation by His26 or His33 is encountered upon protein unfolding (Colón et al., 1997) but smaller-scale conformational rearrangements in the alkaline form of the protein involve nearby Lys73 or Lys79 (Rosell et al., 1998). Resonance Raman (RR) experiments with anionic DOPG liposomes suggested that Met80 is replaced by His33, His26 or water (Oellerich et al., 2003). A recent NMR study with SDS micelles employed to mimic CL identified His33 as the preferred ligand to the heme (Simon et al., 2013). On the other hand, MCD studies demonstrated that Met80 is replaced by a Lys or OH⁻ rather than a His (Bradley et al., 2011). Analysis of a series of Lys mutants suggested that both bis-His and Lys-His species can be formed (Sinibaldi et al., 2013), arguing for large-scale protein rearrangements in more destabilized variants. A minor blue shift of the Soret band in our experiments that involve both *C* and *E* species is consistent with the low-spin character of the new ligand set but the identity of the ligand has not been established (Hanske et al., 2012). The protein unfolding opens up the heme crevice in cyt *c*, however, the misligating residue may have the counteracting effect on peroxidase function. Studies have demonstrated that His misligation inhibits peroxidase activity of the denatured cyt *c* (Diederix et al., 2002). The coordinated Lys may be more easily displaced compared to His but the question remains which of these two species is of the primary functional significance as they also likely vary in the degree of protein unfolding.

The coordination position at the heme occupied by Met80 in the native protein is presumably the site for binding of H₂O₂ (Barr and Mason, 1995; Diederix et al., 2003). Analysis of the peroxidase function of unfolded cyt *c* has suggested the role of the peroxo-iron species in the reaction mechanism (Diederix et al., 2003). While cyt *c* is believed to cleave H₂O₂ homolytically (Barr and Mason, 1995), a heterolytic cleavage has been observed for fatty acid hydroperoxides (Belikova et al., 2009). Docking simulations suggested that fatty acid hydroperoxides can bind close to Arg38 and His33 and this proximity may assist in the heterolytic cleavage of the peroxide bond (Belikova et al., 2009).

Various experiments have consistently demonstrated that CL and other anionic lipids not just displace Met80, but also open up the heme crevice. Importantly, these structural changes increase peroxidase activity of cyt *c* for subsequent oxidation of CL (Kagan et al., 2005; Hanske et al., 2012). Early RR experiments with anionic liposomes revealed an increase in the population of the high-spin five-coordinate species associated with decreased hydrophobicity of the heme environment and release of the protein constraints (Heimburg et

al., 1991). EPR studies of heme nitrosylation (Kapralov et al., 2007) as well as CO (Kapetanaki et al., 2009) and NO photolysis experiments (Silkstone et al., 2012) suggested an opening of the heme crevice in cyt *c* upon CL binding. The *E* species revealed in TR-FRET experiments imply that in these “open” species the heme does not have extensive tertiary contacts with the surrounding polypeptide (Hanske et al., 2012).

Protein-bound radicals formed during the peroxidase reaction of CL-bound cyt *c* offer additional insights about the altered protein structure and CL binding sites. The porphyrin radical cation of Compound I has not been detected for CL-bound cyt *c* but several EPR studies have demonstrated the presence of protein-bound tyrosyl radicals (Kagan et al., 2005; Tyurina et al., 2006; Kapralov et al., 2011; Rajagopal et al., 2013). For the interaction of CL with horse heart cyt *c*, the radical residing on the highly conserved Tyr67 has been suggested to be important for the oxygenase function since mutation of the four tyrosine residues (Figure 1) to phenylalanine showed a significant decrease in CL hydroperoxides for Tyr67Phe only (Kapralov et al., 2011). A recent study of human cyt *c* provided evidence for more than one radical contributing to the composite EPR signal and implicated Tyr46 and Tyr48 as possible radical sites. This observation of multiple protein-immobilized radical species is consistent with the heterogeneous nature of the CL-bound cyt *c* ensemble. Tyrosyl radicals can in turn support the generation of radicals on fatty acyl chains (Rajagopal et al., 2013). Tyr67 is positioned close to proposed insertion sites of CL acyls into the protein interior (Figure 2) making this radical site a particularly strong candidate for reaction with CL.

Sequence of Unfolding Events and Membrane Permeabilization

Elegant work of Spiro *et al.* has identified a possible trigger for CL-induced rearrangements: the breakup of the His26-Pro44 hydrogen bond (Figure 3) (Balakrishnan et al., 2012). T-jump/UV RR experiments at pH 3 have uncovered that the loss of this hydrogen bond initiates rapid (μ s) formation of the β -sheet structure along the protein's 40's Ω loop, followed by extension of the β -sheet into the adjacent 60's and 70's helices. These changes lead to the heme displacement and disruption of the Met80 ligation to the heme. Trp59 is a useful reporter of cyt *c* conformational state; as a result of these rearrangements, its mobility increases, indicating loosening of the protein structure.

Measurements of intrinsic Trp59 fluorescence have been used to demonstrate an increase in the average Trp59-to-heme distance and thus, cyt *c* unfolding upon interaction with CL (Pinheiro et al., 1997; Belikova et al., 2006; Kapralov et al., 2007; Stepanov et al., 2009; Hanske et al., 2012). Introduction of extrinsic fluorophores at other locations, coupled with stopped-flow measurements, has allowed us to resolve the sequence of steps (Figure 3) and explore the roles of the native-structure foldons in the mechanism of cyt *c* unfolding (Muenzner et al., 2013). Binding of cyt *c* to CL liposomes in these experiments occurs during the instrument dead-time (<5 ms). From the loosened nature of the burst-phase species, we infer that formation of β -sheets (Balakrishnan et al., 2012) and rupture of the Fe-Met80 bond have already taken place within this time. The protein's 50's helix (near C site) and the region next to residue 92 (A site) partially insert into the membrane (Hanske et al., 2012; Snider et al., 2013) within the first minute of the interaction (Muenzner et al., 2013). Upon membrane anchoring, major changes in the protein's tertiary structure start to occur. The full extent of lipid-induced protein unfolding is reached after an hour, yielding a sizeable population of *E* species (Hanske et al., 2012; Muenzner et al., 2013). While early rearrangements depend on a hierarchy of foldons in the native structure, the later process of large-scale unfolding is influenced by the protein interactions with the membrane surface.

A latest fluorescence microscopy study by Groves *et al.* (Bergstrom et al., 2013) monitored the cyt *c*-induced permeabilization of giant unilamellar vesicles (GUVs) (Beales et al., 2011) containing CL. Analysis of fluorescent images has revealed the intriguing ability of cyt *c* on its own to trigger formation of pores in CL liposomes and mediate its relocation through membranes. The pores are formed minutes after binding of cyt *c* to vesicles and estimated to be around 2 to 20 nm in size. Cyt *c* interactions with CL membranes cause negative membrane curvature and the authors propose that unfolded *E* conformers may facilitate formation of the pores. In another study, the induction of lipid pores was demonstrated to be dose-dependent, leading to lysis of the membranes at high cyt *c* concentrations. The establishment of a highly “leaky”, but non-lytic state of the membrane occurred at very high cyt *c* concentrations, which again points to a role of cyt *c* itself in the formation of membrane pores (Xu et al., 2013). Pores with a diameter of 3.5 nm were also observed by monitoring electric properties of the CL-containing membranes after their incubation with cyt *c* and hydrogen peroxide (Puchkov et al., 2013).

Impact of Additives on the Ensemble

Binding of cyt *c* to membranes, unfolding and increase in peroxidase activity are not only dependent on the composition of the lipid bilayer (Belikova et al., 2006; Stepanov et al., 2009; Abe et al., 2011), but can also be influenced by diverse additives. High ionic strength solutions impede electrostatic interactions between the A-site of cyt *c* and CL (Rytömaa and Kinnunen, 1994; Salamon and Tollin, 1996; Belikova et al., 2006; Sinibaldi et al., 2008; Hong et al., 2012; Perhirin et al., 2013) and the peroxidase activity of cyt *c* decreases (Belikova et al., 2006; Abe et al., 2011; Hanske et al., 2012). The nucleotide ATP has been shown to compete with CL for binding to cyt *c* and is able to dissociate cyt *c* from CL liposomes (Rytömaa and Kinnunen, 1994; Sinibaldi et al., 2008; Sinibaldi et al., 2011; Snider et al., 2013). Consistent with these results, a microscopy study showed a reduction of GUV leakage upon addition of ATP (Bergstrom et al., 2013). In the same study, yet another factor influencing the cyt *c* interactions with CL, cholesterol, was assessed: GUVs consisting of both CL and cholesterol were leakier compared to non-cholesterol membranes, which could be attributed to easier pore formation due to a more pronounced negative membrane curvature in the cholesterol-containing membranes.

New Areas of Research

Diverse new studies concerning structural changes of cyt *c* are emerging. At mM physiological concentrations of cyt *c* in mitochondria (Forman and Azzi, 1997), cyt *c* oligomerization and aggregation are important to consider. Partially unfolded protein forms may exacerbate these phenomena: at high cyt *c* concentrations protein aggregation does take place during refolding and slows down this process (Nawrocki et al., 1999). Hirota *et al.* have demonstrated that oligomerization of cyt *c* is caused by intermolecular swapping of the protein's C-terminal region, a process that also leads to disruption of the Met80 ligation to the heme (Hirota et al., 2010). These intermolecular interactions may also occur during cyt *c* interactions with CL and thus influence peroxidase activity of the protein. Another investigation has revealed formation of β -sheets and protein fibril patterns after adsorption of cyt *c* to lipid films (Sankaranarayanan et al., 2013) but peroxidase activity of these alternative cyt *c* structures has not been assessed.

The first steps to explore the connections between cyt *c* structural properties and its peroxidase activity *in vivo* have been undertaken. Alteration in Met80 coordination has been modeled with a Met80Ala mutant in HeLa cells (Godoy et al., 2009). The mutant showed increased peroxidase activity and, surprisingly, was found to spontaneously release from mitochondria and translocate to the cytoplasm and nucleus even in nonapoptotic cells.

Structure and interactions with CL of two *Caenorhabditis elegans* cyt *c* proteins have been characterized (Vincelli et al., 2013), providing a foundation for future *in vivo* studies with this model organism.

Conclusions

Analysis of cyt *c* interactions with CL has been aided by many powerful spectroscopic methods. The possible trigger of CL-induced changes, subsequent loosening and opening of the protein structure as well as the role of cyt *c* in the formation of membrane pores have recently been revealed. The heterogeneous CL-bound cyt *c* ensemble consists of polypeptides that vary in the degree of protein unfolding and their interconversions impact the protein's peroxidase activity. Experimental conditions have a strong influence on the cyt *c* – CL interaction and their variations may explain apparent discrepancies in existing models of this protein ensemble.

Acknowledgments

The studies of CL-bound cyt *c* in our laboratory are supported by NIH RO1-GM098502 (E.V.P.).

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Abbreviations

ATP	adenosine triphosphate
CD	circular dichroism
CT	charge transfer
CL	cardiolipin
cyt <i>c</i>	cytochrome <i>c</i>
DOPG	dioleoyl phosphatidylglycerol
EPR	electron paramagnetic resonance
FRET	fluorescence resonance energy transfer
FTIR	Fourier transform infrared
GUV	giant unilamellar vesicles
GuHCl	guanidine hydrochloride
MALDI	matrix-assisted laser desorption/ionization
MCD	magnetic circular dichroism
NMR	nuclear magnetic resonance
RR	Resonance Raman
SDS	sodium dodecyl sulfate
TR-FRET	time-resolved FRET

- Cyt *c* binding to CL membranes promotes dissociation of Met80 and opening of the heme crevice.
- Many powerful spectroscopic probes have been employed to study cyt *c*-CL interactions.
- Three different modes of cyt *c* interactions with CL membranes have been proposed.
- The heterogeneous CL-bound cyt *c* ensemble consists of species that vary in the degree of protein unfolding.
- Experimental conditions have a strong influence on the species populating the CL-bound cyt *c* ensemble.

Figure 1. Site-specific probes of the interaction of cyt *c* with CL and other anionic membranes
The heme environment (Basova et al., 2007; Kapetanaki et al., 2009; Silkstone et al., 2012), the protein secondary structure (Heimburg and Marsh, 1993; Balakrishnan et al., 2012) and single residues probing substructures of cyt *c* have been investigated with a variety of methods. Loss of Met80 ligation to the heme was illustrated by multiple spectroscopic techniques (Belikova et al., 2006; Sinibaldi et al., 2008; Bradley et al., 2011; Sinibaldi et al., 2013). The motional characteristics of ^{13}C -labeled Met65 and Met80 were probed by NMR (Spooner and Watts, 1992). The aromatic residue Trp59 was a reporter for both fluorescence (Belikova et al., 2006; Hanske et al., 2012) and RR studies (Balakrishnan et al., 2012). EPR studies and analysis of reaction products for Phe mutants explored the role of Tyr residues as radical sites (Kapralov et al., 2011; Rajagopal et al., 2013). MALDI mass spectrometry was employed to reveal sites of modification with diethylpyrocarbonate (yellow); these alterations prevented vesicle fusion induced by cyt *c* at low pH (Kawai et al., 2005). Labeling of Lys residues by succinimidyl-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-

carboxylate (*blue*) were used to probe their proximity to the membrane by EPR (Kostrzewa et al., 2000). Modifications of installed Cys residues by small fluorescent dyes (*green*) allowed examination of conformational properties and kinetics of protein unfolding by FRET (Hanske et al., 2012; Hong et al., 2012; Muenzner et al., 2013).

Figure 2. Proposed modes of cyt *c* –CL interaction

Insertion of the protein into the membrane (Gorbenko, 1999), insertion of acyl chains into the protein (Rytömaa and Kinnunen, 1995; Tuominen et al., 2002; Sinibaldi et al., 2010), and major unfolding of cyt *c* on the membrane surface (Hanske et al., 2012; Hong et al., 2012) have been described. Colors depict foldon units of cyt *c*.

Figure 3. Sequence of CL-induced structural rearrangements of cyt *c*

Electrostatic attraction of positively-charged cyt *c* to anionic CL docks the protein on the membranes surface. Initial structural changes in cyt *c* include breaking of the hydrogen bond between His26 and Pro44 and subsequent conversion of the 40's Ω loop and adjacent regions to β -sheets, as well as the disruption of the Fe-Met80 bond (Balakrishnan et al., 2012). Protein “sinking” into the membrane or insertion of a CL acyl chain into the protein follows. Large-scale unfolding with the breakup of interhelical contacts between *N*- and *C*-terminal helices takes place later (Muenzner et al., 2013). The compact and extended conformers are in exchange with each other and partitioning between these forms depends on the CL content of the membrane (Hong et al., 2012). Interactions of the *C*-terminal helix

with the membrane could aid in the formation of membrane pores (Bergstrom et al., 2013). Colors depict foldon units of cyt *c*.