Defining the Apoptotic Trigger

THE INTERACTION OF CYTOCHROME c AND CARDIOLIPIN

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Background: Binding of mitochondrial cardiolipin to cytochrome c is thought to trigger apoptosis.

Results: Reverse micelle encapsulation mimics the confinement of the inner mitochondrial membrane allowing binding of cardiolipin to be followed by NMR at atomic resolution.

Conclusion: Cardiolipin interacts with cytochrome c without causing protein unfolding.

Significance: A new model for the role of cytochrome c in apoptosis is required.

The interaction between cytochrome c and the anionic lipid cardiolipin has been proposed as a primary event in the apoptotic signaling cascade. Numerous studies that have examined the interaction of cytochrome c with cardiolipin embedded in a variety of model phospholipid membranes have suggested that partial unfolding of the protein is a precursor to the apoptotic response. However, these studies lacked site resolution and used model systems with negligible or a positive membrane curvature, which is distinct from the large negative curvature of the invaginations of the inner mitochondrial membrane where cytochrome c resides. We have used reverse micelle encapsulation to mimic the potential effects of confinement on the interaction of cytochrome c with cardiolipin. Encapsulation of oxidized horse cytochrome c in 1-decanoyl-rac-glycerol/lauryldimethylamine-N-oxide/hexanol reverse micelles prepared in pentane yields NMR spectra essentially identical to the protein in free aqueous solution. The structure of encapsulated ferricytochrome c was determined to high precision \(< r.m.s. \text{ deviation} \>_{bb} \sim 0.23 \text{ Å} \) using NMR-based methods and is closely similar to the cryogenic crystal structure \(< r.m.s. \text{ deviation} \>_{bb} \sim 1.2 \text{ Å} \). Incorporation of cardiolipin into the reverse micelle surfactant shell causes localized chemical shift perturbations of the encapsulated protein, providing the first view of the cardiolipin/cytochrome c interaction interface at atomic resolution. Three distinct sites of interaction are detected: the so-called A- and L-sites, plus a previously undocumented interaction centered on residues Phe-36, Gly-37, Thr-58, Trp-59, and Lys-60. Importantly, in distinct contrast to earlier studies of this interaction, the protein is not significantly disturbed by the binding of cardiolipin in the context of the reverse micelle.

Cytochrome c is one of a number of mitochondrial proteins whose transfer to the cytosol helps initiate entry into programmed cell death or apoptosis (1). The interaction of cytochrome c with the apoptosis promoting factor Apaf-1 in the cytosol initiates apoptosis via activation of caspases and the formation of apoptosomes (2). Normally, cytochrome c exists in the mitochondrial inter-membrane space where it acts as an electron shuttle between complex III and complex IV, which reside in the inner mitochondrial membrane. The inner mitochondrial membrane contains appreciable levels of the anionic phospholipid cardiolipin (CL), which is thought to interact with the highly basic cytochrome c. Considerable evidence has accrued to suggest that the interaction of cardiolipin with cytochrome c activates a normally suppressed peroxidase activity in this heme protein (2). Cardiolipin is subsequently specifically oxidized and it is postulated that the oxidation products facilitate the leakage of cytochrome c to the cytosol. Many studies utilizing various model lipid bilayers have suggested that the interaction of cytochrome c with cardiolipin involves a strong electrostatic interaction between the negatively charged phospholipid and the positively charged protein that leads to a partial unfolding event (2). The largely electrostatic interactions center on the so-called A- (Lys-72, Lys-73, Lys-86, and Lys-87) and L- (Lys-22, Lys-25, His-26, Lys-27, and His-33) sites of the protein surface and can be attenuated with increasing levels of salt (2). A third interaction, termed the C-site, is thought to be a hydrogen bonding interaction between Asn-52 and a protonated CL headgroup (3). In the partially unfolded state the heme prosthetic group gains the ability to act as a peroxidase that is apparently specific for CL (2). Titration of liposomes into dilute solutions of cytochrome c suggests the promotion of a subpopulation of partially unfolded species with increasing CL

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4 The abbreviations used are: CL, cardiolipin; RM, reverse micelle; CSP, chemical shift perturbation; PCS, pseudocontact shift; HSQC, heteronuclear single quantum coherence; LDAO, lauryldimethylamine-N-oxide; NOE, laboratory frame nuclear Overhauser effect; 10MAG, 1-decanoyl-rac-glycerol; ROE, rotating-frame Overhauser effect; PDB, Protein Data Bank; r.m.s., root mean square.

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membrane content and CL:cytochrome c ratio (4), perhaps in accordance with the cooperative substructure of the protein revealed by an array of biophysical studies (5). These observations, however, present a paradox: considering the enrichment of CL in the mitochondrial membrane, how does this mechanism not constantly result in a substantial fraction of unfolded cytochrome c in vivo, diminishing the ability of cytochrome c to shuttle electrons and potentially causing aberrant apoptosis signaling? Partitioning of CL in the inner mitochondrial membrane away from cytochrome c has been proposed but seems not to have definitive support (2), particularly because CL associates specifically with complex III and complex IV (6) and is essential for proper formation of supercomplexes containing complex III and complex IV (6, 7). As mentioned previously, cytochrome c performs its major function by specific binding to each of these complexes.

Structural characterizations of the interaction of cytochrome c with cardiolipin membranes have generally utilized either planar or positively curved model bilayers. In contrast, the highly invaginated cristae of the inner mitochondrial membrane present a high negative curvature that results in significant confinement. To mimic this condition, we employ the interior water core of a reverse micelle to solubilize cytochrome c and use the surrounding surfactant shell as a host for cardiolipin. Under appropriate conditions, proteins can be encapsulated within reverse micelles with high structural fidelity (8). When prepared in solvents of sufficiently low viscosity (9), one can also obtain high resolution solution NMR data to comprehensively characterize the structure of the encapsulated protein (10) and its interaction with ligands embedded in the surfactant wall or dissolved in the aqueous core (11). We find here that oxidized cytochrome c can be encapsulated within 1-decanoyl-rac-glycerol/lauryldimethylamine-N-oxide (10MAG/LDAO) and hexanol with high structural fidelity. The interaction with cardiolipin can be studied by direct titration with full structural characterization. We find that binding of cardiolipin to the protein occurs at the aforementioned A- and L-sites and a previously undocumented third site centered on residues Phe-36, Gly-37, Thr-58, Trp-59, and Lys-60. The tertiary structure of the protein is fully maintained, as might be predicted by the effects of confinement within the reverse micelle, which mimic the negative curvature and length scale of the inner mitochondrial membrane. There is also no evidence of localized unfolding; because substantial populations of unfolded protein are necessary for peroxidase activity, this suggests that decreased length scales experienced by cytochrome c in vivo can inhibit this activity.

Experimental Procedures

**Protein Purification and Reverse Micelle Encapsulation**—
Wild-type horse heart cytochrome c was isotropically labeled and purified as previously described for a pseudo wild-type version of the protein (12). Yields ranged from 7 to 10 mg/liter of minimal media. Purified cytochrome c was oxidized with KFeCN₆, exchanged into pH 7.4, 25 mM phosphate buffer, and concentrated to ~7 mM for encapsulation. The 10MAG/LDAO surfactant mixture was prepared in a molar ratio of 70/30 and pre-adjusted with HCl to a pH of 7.4 (52). 10MAG/LDAO powder was dissolved in deuterated pentane such that the final total surfactant concentration was 150 mM. A solution of 7 mM cytochrome c was added by direct injection to achieve a molar ratio of water to surfactant (water loading or \(W_o\)) of 12. This corresponds to 32.6 \(\mu\)l of protein solution per ml of reverse micelle solution. Hexanol was added as necessary to improve sample stability and encapsulation efficiency (0–16 mxs). Nuclear Overhauser effect (NOE), rotating-frame Overhauser effect (ROE), and T₂ₑₓperiments were carried out using solutions of ferricytochrome c encapsulated in 75 mM cetrimonium bromide with 450 mM hexanol. Cetrimonium bromide was used as the surfactant system to avoid complications of the glycerol headgroup of 10MAG. The protein was prepared at pH 5 in 50 mM sodium chloride and 50 mM sodium acetate to slow the rate of hydrogen exchange relative to the pH 7.4 samples.

**Cardiolipin Titration**—
For cardiolipin titration experiments, a solution of 150 mM CL in deuterated hexane was directly injected in defined aliquots. CL titration experiments (\(^{15}N\)-HSQC) were conducted on uniformly \(^{15}N\)-labeled protein samples, with (reduced) and without (oxidized) addition of ascorbate.

**NMR Spectroscopy**—
NMR data were collected at 25 °C at 500 MHz (¹H) on Bruker AVANCE III spectrometers equipped with TXI cryoprobes. Backbone H, N, C, and CA resonance assignments (99%) were confirmed with three-dimensional HNCA, HN(CO)CA, and HNCO experiments (13). Side chain carbon and hydrogen assignments (84% absolute, not discounting proline residues) were obtained using three-dimensional H(CC)(CO)NH-TOCSY and (H)CC(CO)NH-TOCSY experiments, each collected at 2 different mixing times (12 and 20 ms). Assignments were referenced to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), directly for protons and indirectly for nitrogen and carbon (14).

NOE-based distance restraints were obtained from non-uniformly sampled three-dimensional NOESY, \(^{15}N\)-¹H-HSQC, three-dimensional NOE-SY, \(^{13}C\)-¹H-HSQC experiments collected with a 15% sampling density and a Poisson gap distribution (15) and four-dimensional uniformly sampled \(^{15}N\)-¹H-HSQC-NOESY-\(^{13}C\)-¹H-HSQC experiment. NOESY experiments providing structural restraints employed a mixing time of 90 ms. Non-uniformly sampled data were reconstructed using istHMS (15) followed by processing in NMRPipe (16). Uniformly sampled experiments were processed using Felix95 (Accelrys Inc., San Diego, CA). Spectra were analyzed using SPARKY (17). Hydration dynamics experiments were conducted on the cetrimonium bromide RM-encapsulated cytochrome c sample described above. Three-dimensional NOE-SY, \(^{15}N\)-¹H-HSQC and three-dimensional ROESY, \(^{15}N\)-¹H-HSQC experiments were performed with a 20-ms mixing time and a rotating frame spinlock power of 8,333 Hz. Measurements were corrected for autorelaxation as previously described (18) with individual amide T₁ₑₓ values.

**Restraints and Refinement**—
NOEs were binned according to their peak intensity or volume. NOE intensities were calibrated using canonical \(\alpha\)-helical proton-proton distances. Proton-proton distance restraints were then classed as either weak (1.7–5.5 Å), medium (1.7–4.0 Å), or strong (1.7–3.0 Å) for the three-dimensional NOESY experiments, and either weak (1.7–
5.5 Å) or strong (1.7–3.5 Å) for the four-dimensional 15N, 1H-HSQC-NOESY-13C. Restraints for protons in methyl groups were extended by 0.5 Å. Backbone φ and ψ torsion angle restraints were generated using chemical shift assignments for backbone N, C', CA, and CB ("no proton" mode for proteins with a paramagnetic center) from the TALOS+ Web server with a minimum error of 15° (19).

Xplor-NIH (20) was used to incorporate the above experimental distance and dihedral angle restraints into a simulated annealing protocol, starting with cytochrome c in an extended conformation. His-18 and Met-80 coordination geometry was partially defined using invariant bond lengths and angles well known from crystal structures of c-type cytochromes. The bond between the SD of Met-80 and the heme iron was set at 2.3 Å, that between NE2 of His-18 and the heme iron was defined as 2.0 Å, and the angle between the His and heme planes was set at −175°. The protein structure was refined using all experimental NOEs and dihedral angle restraints from TALOS+ using the standard sa.inp script.

Pseudocontact shift (PCS) restraints due to the presence of the iron center in the attached heme group were generated by subtraction of the oxidized state protein hydrogen chemical shift values from their respective chemical shifts in the reduced state. Backbone oxidized and reduced HN chemical shifts were directly compared from two RM-encapsulated cytochrome c samples (prepared as above), whereas side chain proton PCS values were obtained from comparing RM assignments with previous aqueous assignments (21). The PCS contribution to the change in the chemical shift of a given nucleus due to the presence of a paramagnetic metal center is a function of both the distance (ri) between the metal and hydrogen of interest, as well as the direction cosines (li, mi, ni) of the position vector of the atom of interest in the reference frame, here described by the overall magnetic susceptibility tensor components (Δχax, Δχan, Δχln, Δχnl).

\[
\delta_{pc} = \frac{1}{12 \pi r_i^4} \left[ \Delta \chi_{ax} (3 m_i^2 - 1) + \frac{3}{2} \Delta \chi_{ln} (l_i^2 - m_i^2) \right]
\]

(Eq. 1)

The change in chemical shift of a nucleus is a function of both PCS as well as a contact shift (CS) contribution. Local effects due to the change in electron density at the metal center are largely responsible for contact shifts, as well as any structural perturbations that may occur in the protein upon change in redox state. The overall magnetic susceptibility tensor components were first determined using the PARA restraints module (22) incorporated into Xplor-NIH. After inclusion of NOE and dihedral restraints, the PCS values were incorporated into a restraint list with an estimated error of 0.1 ppm for HN values and 0.2 ppm for all other hydrogens. The tensor was determined using an iterative fitting procedure using 20% of the lowest energy structures from each round. The final tensor components were 705 ± 26 and −382 ± 21 for Δχax and Δχav, respectively (errors were estimated using an iterative Monte Carlo approach which randomly eliminates 35% of the experimental data for 1000 repetitions). The initial r.m.s. deviation error from this optimization protocol was 0.084 ± 0.054 ppm². Each PCS restraint with a calculated error greater than 0.100 ppm² was discarded, and the same magnetic susceptibility tensor optimization protocol was initiated again. This trimming resulted in a tensor of 716 ± 11 and −393 ± 16 for Δχax and Δχln, respectively, clearly within error of the previously determined tensor. The r.m.s. error for the remaining restraints decreased to 0.016 ± 0.001.

Analysis of a short mixing time (20 ms) NOESY experiment revealed the presence of apparent NOEs between amide hydrogens of the protein and water. Any apparent “water” NOE within 4 Å of a potentially contaminating HA resonance was not considered further. The remaining NOEs clustered into three distinct areas in the structure of the protein, indicating the presence of 3 or 4 waters. Closer inspection of a large patch of water NOEs showed that several of the protons in the cluster were much further than possible for a single water molecule (>10 Å). Additional NOEs were found from methyl groups in the three-dimensional NOESY-13C, 1H-HSQC.

Secondary structure hydrogen bond restraints were added in areas of well defined secondary structure, according to PyMol analysis and TALOS+ results. The majority of non-secondary structure hydrogen bonds described in Brayer et al. (23) were present in the last round of structures, although with somewhat variable geometry. These H-bonds were also included as explicit NOE restraints in the final simulated annealing protocol. There were several hydrogen bonds described previously (23) that were not observed, mainly due to differences in coordinates for solvent-exposed lysine side chains.

For the final simulated annealing run, 1000 total structures were generated for analysis. PCS restraints were included with a force constant of 5.0 and the same tensor components defined above. Dihedrals were scaled at 5 for high-temperature time steps and 350 for temperature minimization. Temperature was scaled from 2500° to 100° over the course of 15,000 2-fs cooling steps after 15,000 high temperature steps. The final family of 32 structures have 0 NOE violations greater than 0.4 Å and no dihedral angle violations greater than 2°. The total system energy was a maximum of −126.0. Statistics for the final 32 structures are provided in Table 1.

Results

Reverse Micelle Encapsulation of Ferricytochrome c—The encapsulation of oxidized cytochrome c within the aqueous core of a reverse micelle is particularly challenging due to its high positive charge at neutral pH (24). We have recently introduced a new surfactant system based on mixtures of the zwitterionic surfactant LDAO, the neutral surfactant 10MAG mixture and hexanol acting as a co-surfactant (25). Cytochrome c is stably encapsulated in this surfactant system over a wide range of pH values (5.0–7.4), enabling the recapitulation of conditions used in many previous studies of the cytochrome c/CL interaction (3, 4, 26–30). The 13N-HSQC spectra of the free solution and encapsulated proteins are essentially superimposable. Standard triple resonance assignment experiments (13) implemented using non-uniform sampling (15, 31) were used to obtain comprehensive resonance assignments, which have been deposited to the BMRB (accession number 25640). Backbone assignments were essentially complete (99%), and 85% of
side chain protons and carbons were successfully assigned. Comparisons of RM-encapsulated and previous aqueous (21) assignments show that chemical shifts are highly correlated (all $R^2 > 0.99$).

The High-resolution Structure of Encapsulated Ferricytochrome c—The structure of encapsulated oxidized cytochrome c was determined using NMR-based methods. Structural restraints included NOE-derived distances, backbone torsion angles determined using chemical shifts (19), and distance and orientation restraints relative to the paramagnetic heme iron based on analysis of pseudocontact shifts (22). Hydrogen bond restraints were introduced following analysis of an initial round of structural refinement. Individual water molecules were introduced in the latter stages of refinement based on NOEs to the water resonance as described in more detail below. There were an average of 20 experimental restraints per residue, of which 3.3 were long-range NOEs, and another 3.8 were derived from pseudocontact shifts. Further information about the restraint set and refinement statistics are provided in Table 1.

One thousand structural models were individually refined and the 32 lowest-energy structures were chosen to represent the final ensemble, which has been deposited to the PDB under accession number 2N3B.

The final 32-structure ensemble has an overall backbone r.m.s. deviation of 0.23 Å and an all heavy-atom r.m.s. deviation of 0.65 Å (Fig. 1a). This excellent precision can be attributed to the very large number of restraints per residue and the long-range angular and distance restraints provided by the pseudocontact shift analysis. The regions of the structural ensemble with the highest variance (as shown in Fig. 1b) are largely made up of the 20s loop and regions close to the paramagnetic center, which contain fewer overall restraints because of the extensive line broadening experienced by nuclei close the iron. The structure of encapsulated ferricytochrome c is highly homologous to published crystal structures (all backbone heavy atom r.m.s. deviation of 1.2 Å) with most variation localized to three small regions of the protein (Fig. 1c). Some of this deviation of the backbone between the two models may be attributed to internal motion, particularly in the 20s loop where hydrogen exchange (32) and NMR relaxation (33) indicate that this region is particularly mobile in the oxidized form of the protein.

Structural Water and Hydration Water—Extensive crystallographic and NMR studies have indicated that cytochrome c contains a number of structural waters and changes in the number and position of these waters contribute to the electron transfer properties of the protein (23, 34–36). Detection of protein/water interactions using solution NMR can be potentially quite problematic, especially waters located near exchangeable hydrogens (37). Encapsulation of proteins within the water core of reverse micelles largely eliminates artifacts introduced by hydrogen exchange (18). When combined with heteronuclear NMR methods to provide spectral resolution, the nuclear Overhauser effect can be confidently employed to probe for protein/water interactions (8, 18). Analysis of $^1$H-$^1$H NOEs involving the water resonance allowed the identification of four distinct

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**TABLE 1**

NMR and refinement statistics for oxidized RM-encapsulated cytochrome c

| NMR distance and dihedral constraints |  |
|--------------------------------------|--|
| Distance constraints                 | 1,553 |
| Intra-residue                        | 441  |
| Inter-residue                        |     |
| Sequential ([i,j] = 1)               | 426  |
| Medium-range ([i,j] < 5)             | 280  |
| Long-range ([i,j] > 5)               | 290  |
| Water                                | 15   |
| Heme                                 | 35   |
| Hydrogen bonds                       | 66   |
| Total dihedral angle restraints      | 145  |
| $\phi$                               | 72   |
| $\psi$                               | 73   |
| Pseudocontact shifts                 | 399  |
| Total restraints                     | 2097 |
| Restraints per residue               | 20.2 |

**Structure statistics**

| Violations (mean ± S.D.)             | 0.0336 ± 0.0007 |
| Distance constraints (Å)             | 0.287 ± 0.023   |
| Dihedral angle constraints (*)        | 0.060 ± 0.006   |
| Pseudocontact shifts (ppm)           | 2.00            |
| Max. dihedral angle violations (*)    | 0.330           |
| Max. distance constraint violation (Å)| 2.0             |
| NOE violations >0.3 Å                | 0.0             |
| NOE violations >0.4 Å                | 0.0             |
| Dihedral violations >2°              | 0.0             |
| Deviations from idealized geometry   | 0.0022 ± 0.00005|
| Bond lengths (Å)                     | 0.4319 ± 0.003  |
| Improper constraints (*)              | 0.366 ± 0.010   |
| Average pairwise r.m.s. deviation (Å)| 0.639 ± 0.076   |
| All heavy atoms                      | 0.228 ± 0.039   |

$^a$ Pairwise r.m.s. deviation across the 32 final structures.

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**FIGURE 1.** The structure of oxidized horse heart cytochrome c encapsulated in reverse micelles. a, the 32 lowest energy structures from the final round of the simulated annealing protocol in Xplor-NIH (20) are aligned and the secondary structure elements are colored with different shades of blue. The four internal water molecules are displayed as blue dots, and the overlaid heme moieties are highlighted in salmon. b, precision of the NMR-determined model is illustrated by the thickness of the backbone trace as a function of the local r.m.s. deviation, which is also colored from lowest (blue) to largest (cyan) r.m.s. deviation. c, overlays of the backbones of crystal structures of ferricytochrome c determined in low salt (PDB code 1CRC, blue) (39) and high salt (PDB code 1HRC, cyan) (23), a solution structure of cytochrome c (PDB code 1AKK, orange) (40), and that determined by solution NMR methods of the RM-encapsulated protein (PDB code 2N3B, green). All residues were used for the alignment.
Interaction of Cytochrome c and Cardiolipin

Comparing RM-encapsulated Cytochrome c with Other Structural Models—A comparison of the RM-encapsulated cytochrome c with two previous crystal structures (23, 39) and a previous solution NMR structure (40) is shown in Fig. 1. Although very good agreement (1.2 Å backbone atom r.m.s. deviation) is seen between the RM-encapsulated cytochrome c and each of 2 crystal structures (PDR codes 1HRC and 1CRC), a much higher r.m.s. deviation (2.1 Å) is observed with a previous NMR structural model of cytochrome c (40). The regions of largest deviations between the RM-encapsulated cytochrome c and the crystal structures include residues 43–47, 54–57, and 82–84. The loop from 43 to 47 is more extended in the crystal structures, where it is closer to the 20s loops in the RM structure, showing extensive packing between Phe-46 and the 20s loops residues, including the backbone of Gly-29 and Pro-30. The end of the 50s helix lacks a final turn present in the crystal structures, orienting Lys-55 differently due to several NOEs to the Thr-40 side chain. Finally, Ala-83 and Gly-84 are slightly extended in the RM structure, where they are more closely packed to the end of the 60s helix in the crystal structures. These structural deviations may be due to lack of restraints, true differences between the room temperature solution structure and the cryogenic crystal structure, or a result of spatial restriction inside of the RM. Comparison of hydrogen bonding in the present structural ensemble and a previous crystal structure (23) reveals that the overwhelming majority of these interactions are present in at least a significant fraction of the members of the RM-NMR structural ensemble. Finally, the side chain of Arg-38 is not forming a direct hydrogen bond to the heme, as it is mediated by a structural water (Fig. 2).

Redox State-dependent Structure Change—The presence of a paramagnetic center, such as the iron(III) of the heme in cytochrome c, will influence the magnetic environment of surrounding NMR-active nuclei (41, 42). Paramagnetic shifts result from both through-bond contact shift and through-space pseudocontact shift interactions (42). The pseudocontact shift is rich in long-range geometric information relative to the paramagnetic center and thus provides a powerful constraint for structure determination and detection of structural changes.

Refinement of the magnetic susceptibility tensor that defines the pseudocontact shift ideally proceeds by the chemical shift differences of probe nuclei in the presence of a paramagnetic center and in its absence under the assumption that the structural context does not change. Conversely, with the availability of a large set of independent structural restraints, one is able to use deviations of the observed pseudocontact shift from the consensus structure to locate regions of structural change upon a change in redox state (which is accompanied by a change in spin state) in heme proteins. We use this approach here. Fig. 3 depicts the structure of cytochrome c with violations of the observed PCS value versus that predicted from the optimized magnetic susceptibility tensor components. The PCS violations are largely localized to the 50s helix, the 20s loops, and Phe-82 to Ala-83, whereas the rest of the protein fits well to the refined tensor and structural model. The violations can be interpreted as either locations of errors in the structural model or as possible sites of structural perturbations upon redox state change. Except for Phe-82 and Ala-83, which are under-restrained due to the effects of relaxation by the paramagnetic center, the precision of the model is high even without use of the PCS restraints. Thus violations of PCS restraints are due to chemical shift changes arising from subtle structural rearrangements that occur upon change in heme oxidation state. Analyses of
The interaction of ferricytochrome c with mitochondrial CL has been studied extensively over the past decade (2). The initial interaction has been shown to involve at least 2 distinct binding modes. The availability here of a high-resolution structural view of these interactions confirms the location and identity of residues involved in the largely electrostatic interaction between the A- and L-sites of cytochrome c and cardiolipin (2–4, 28, 45). The previously proposed C-site (the side chain of Asn-52) is not supported by our results. Structural analysis indicates that the presentation of the C-site would require partial unfolding. Instead, a novel CL binding site, here termed the N-site, is apparent.

Previous studies of the interaction of CL with cytochrome c have generally concluded that CL destabilizes the protein and leads to extensive unfolding. Most studies of this kind have used the exterior surface of liposome membranes to follow the interaction of cytochrome c with cardiolipin. An impressive number have been conducted on the mechanism of this unfolding phase (4, 29, 45, 47, 48) and this gross structural perturbation leads to the activation of the peroxidase activity that subsequently initiates critical modifications of cardiolipin (26, 28, 29, 49, 50).

Although liposomes represent a convenient system in which to present a lipid bilayer to cytochrome c, several issues arise. First, liposomes present a relatively small, positive curvature (51). As cytochrome c is largely present on the inside of these structures, it mostly encounters this large, negative curvature. Although there are certainly several aspects of RM encapsulation that do not recapitulate native conditions (i.e. single “lipid” layer), encapsulated cytochrome c is confined to a more native-like curvature ($k_{RM} \approx -4.8 \times 10^9$ m$^{-1}$).

### Discussion

The interaction of ferricytochrome c with mitochondrial CL observed in reverse micelles—The interaction of cytochrome c with the mitochondrial lipid CL is thought to be a pivotal first step in the apoptosis cascade, resulting in cytochrome c unfolding, activation of peroxidase activity, and subsequent export from the mitochondrial intermembrane space (2). This interaction has largely been probed using FRET to follow structural changes of the protein upon binding the exterior of liposomes doped with cardiolipin (e.g. Ref. 4). Mutational studies followed by FRET and other general spectroscopic techniques have also been useful in this regard (e.g. Ref. 26 and 45).

Encapsulation of ferricytochrome c into reverse micelles allows the interaction of cardiolipin to be directly examined in atomic detail. Titration of cardiolipin into solutions of RM-encapsulated cytochrome results in localized perturbations in the $^{15}$N-HSQC spectrum (Fig. 4a). Importantly, the overall tertiary structure of the protein is maintained, even at a 25-fold molar excess of CL to cytochrome c. No unfolding of the protein is observed, although the long-term stability of the reverse micelle solution is reduced from months to weeks. When individual weighted $^{15}$N/H CSP for each residue (Fig. 4b) are plotted on the structure three distinct interaction sites are illuminated (Fig. 4c). The first region contains residues Val-20, Lys-22, Gly-24, Lys-25, His-33, Asn-103, and Glu-104, which largely correspond to residues previously proposed to be in the so-called “L” site of CL interaction (46). A second distinct interaction site is composed of Thr-78, Lys-79, Ile-81, and Tyr-48 and corresponds to the previously proposed “A,” or anionic, binding site for CL (3). A large third site, which has not been described previously, is comprised of Phe-36, Gly-37, Thr-58, Trp-59, and Lys-60 (Fig. 4c). We term this the “N” or novel site. We see no evidence for the interaction of CL with Asn-52, previously termed the C-site (2). The side chain of this residue points directly into the interior of the protein and is involved in hydrogen bond formation with both the side chain oxygen of Thr-49 and an oxygen of the propionate group of the heme, both of which must be broken and significant structural rearrangements including unraveling of the 50s helix must occur before any peripheral interaction with the CL headgroup. Several other residues that are not clearly localized on the protein surface display changes in chemical shift (e.g. Val-3, Gly-41, Gly-45, Lys-73, Lys-88, and Thr-89).

Each of the well defined cardiolipin interaction sites (L, A, and N) are sufficiently separated that one CL molecule cannot simultaneously bind to more than one site and thus several cardiolipin molecules are involved in the interaction, or possibly that the protein interacts with the lipid surface as a whole. In contrast to earlier studies that identified the L and A interaction sites, we find no evidence for global, subglobal, or even local unfolding of the protein upon binding CL. Several individual peaks display line broadening upon introduction of CL, including residues Thr-58, Lys-88, and Thr-89.
importantly, the exterior surface of the liposome lacks the features of confinement that are likely present in the intermembrane space of mitochondria. Theoretical and experimental work shows that confinement of protein molecules within "cages" of the dimension of the folded native state can impart considerable stability to the native state by steric exclusion of the more extensive (partially) unfolded states (52–54). For confinement within a sphere, the extent of stabilization is sensitive to the relative diameters of the confining sphere and the protein, but can range up to \(\frac{1}{20}kT\) (52). The inner diameter occupied by the protein in the RM approaches this upper limit (\(\frac{1}{18}kT\)) and is sufficient to prevent global unfolding of the protein upon interaction with CL. The diameter of the interior space of mitochondrial cristae ranges from 4 to 18 nm, after subtracting the thickness of 2 lipid bilayers present in the electron microscopic tomography measurements of Frey and Manella (51). This is 1.3 to 6 times the diameter of cytochrome c, resulting in a predicted stabilization of the native state by \(~20 kT\) (52). The inner diameter occupied by the protein in the RM approaches this upper limit (\(~18 kT\)) and is sufficient to prevent global unfolding of the protein upon interaction with CL. The diameter of the interior space of mitochondrial cristae ranges from 4 to 18 nm, after subtracting the thickness of 2 lipid bilayers present in the electron microscopic tomography Measurements of Frey and Manella (51).

Thus, under native mitochondrial conditions, confinement effects likely serve to keep cytochrome c properly folded and capable of carrying out its primary electron transfer function. Electrostatic "tethering" interactions with the negatively charged lipid bilayer presented by CL, including at the newly discovered N binding site, would then seem to serve as a molecular recognition device rather than resulting in a change in functional state. Clearly, an additional event is required to activate the peroxidase activity that is proposed to initiate modification of cardiolipin and subsequent transfer into apoptosis (2).

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