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Association of Phosphatidic Acid with the Bovine Mitochondrial ADP/ATP Carrier[†]

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ABSTRACT: The beef heart adenine nucleotide carrier protein (Anc) of the inner mitochondrial membrane can be purified in a form stabilized by binding the inhibitor carboxyatractyloside. The protein is copurified with bound lipid. We show for the first time that phosphatidic acid, although a minor component, is one of the lipids bound to Anc. The short spin−lattice relaxation time found by ^{31}P magic angle spinning nuclear magnetic resonance (MAS/NMR) for phosphatidic acid indicates that it is tightly bound to the protein. However, this lipid also has a comparatively small chemical shift anisotropy, suggesting that it can undergo rapid reorientation in space. In contrast, most of the lipid bound to Anc shows anisotropic motion typical of a bilayer arrangement. The phosphatidic acid that is detected in the purified preparation of Anc is also shown to be present initially in the unfractionated mitochondria, prior to the isolation of Anc. In Triton-solubilized mitochondria, phosphatidic acid, cardiolipin, phosphatidylethanolamine, and phosphatidylcholine exhibit resonance lines in the static ^{31}P NMR spectra, but in the purified Anc, only the phosphatidylethanolamine and phosphatidylcholine can be detected by this method, even though the other lipids are still present. This demonstrates that the phosphatidic acid and cardiolipin are interacting with the Anc. The thermal denaturation of the Anc was determined by differential scanning calorimetry. The protein denatures at 74 °C both before and after the NMR studies with the same characteristics.

The ADP/ATP carrier (Anc)[1] is a transporter of the inner mitochondrial membrane. It plays an important role in energy metabolism by allowing the ATP formed by oxidative phosphorylation to pass across the inner mitochondrial membrane to the intermembrane space. The movement of ATP is coupled by an antiport mechanism resulting in the 1:1 exchange of ATP for external ADP. There is also evidence from studies in yeast that CL can stabilize the interaction of Anc with other proteins (*1*).

Anc is capable of facilitating the transport of the large polar molecules of ATP and ADP by undergoing the transition between two distinct conformational states (*2*, *3*). Stabilization of the protein in one of the two conformations can be accomplished with the binding of the high-affinity inhibitor of the transport, carboxyatractyloside (CATR) (*4*, *5*). This has allowed for the purification of the protein and its eventual crystallization in the CATR-bound form (*6*).

The crystal structure demonstrated that there are either two molecules of cardiolipin (CL) bound per monomer of Anc (*6*) or three (*7*). The additional tightly bound CL found in the second crystal structure has been suggested to be important in stabilizing protein−protein interactions in the crystal (*7*). In addition to these X-ray structures, there is evidence from ^{31}P NMR studies of the tight binding of CL to Anc (*8*). Anc copurifies with 16 mol of phospholipid per Anc dimer (*9*). In addition to CL, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are also present in approximately the same molar ratio as they are found in the inner mitochondrial membrane. In the Triton-solubilized preparation, only NMR signals from PC and PE are observed. However, after heat denaturation of the Anc, NMR signals from CL appear (*8*). This indicates that CL is tightly bound to the protein in its native state with restrictions on the motional properties of the lipid. The tightly bound CL does not exchange with added CL over a period of hours. One Anc dimer is associated with four molecules of tetralinoleoyl cardiolipin and two molecules of trilinoleoyl-monolinolenoyl cardiolipin (*10*). The fraction of CL in the trilinoleoyl-monolinolenoyl form in the Anc preparation is enriched several-fold over the fraction in total mitochondrial CL.

CL has been thought to be important for the transport functions of Anc (*11*, *12*). ADP/ATP exchange transport is stimulated by several acidic phospholipids, with cardiolipin being the most effective (*13*) but phosphatidylserine and phosphatidic acid (PA) also being stimulatory (*13*, *14*). Studies of the binding of nitroxide-labeled phospholipids to detergent-solubilized Anc demonstrated binding of both CL and PA with comparable affinity, but not PC or PE (*15*). The binding of spin-labeled lipids to Anc incorporated into bilayers indicated that 50 molecules of phospholipid had restricted motion because of interaction with an Anc dimer, a value slightly greater than that calculated for a protein dimer of this size (*16*). These exchangeable "boundary"

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[1]Abbreviations: Anc, bovine mitochondrial ADP/ATP carrier, isoform 1; CATR, carboxyatractyloside; CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; MAS, magic angle spinning; CSA, chemical shift anisotropy; LAPAO, laurylamido-*N*,*N*′-dimethylpropylaminoxide.

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lipids are in addition to the small number of nonexchangeable tightly bound CL molecules.

The only information about the motional properties of phospholipids in purified preparations of Anc in the absence of detergent comes from studies of the binding of spin-labeled lipids (*16*). Paramagnetic resonance spectroscopy of these samples provides information about changes in the very rapid motions of exogenously added lipids on a short time scale on the order of microseconds. In this work, we further explore the composition and properties of the lipids that are copurified with Anc using magic angle spinning NMR (MAS/NMR). In the course of this work, we identified phosphatidic acid as a minor component of the lipids interacting with Anc, and we have determined its motional properties within the macromolecule.

EXPERIMENTAL PROCEDURES

Purification of Anc with LAPAO and with Triton X-100. A suspension of isolated beef heart mitochondria (*17*) corresponding to 50 mg of protein was mixed with 0.5 mL of a medium made of 5 M NaCl, 10 mM EDTA, and 100 mM Tris-HCl (final pH of 7.3), in a total volume of 4 mL. CATR was added at a final concentration of 20 μM and the mixture left to incubate for 10 min at 0 °C. Mitochondria were then lysed by the addition of 1 mL of a 10% solution (w/v) of either Triton X-100 or 3-laurylamido-*N*,*N*′-dimethylpropylaminoxide (LAPAO) (*13*). After standing in ice for 10 min, the lysate was centrifuged at 20000*g* for 10 min. The purification of the CATR−carrier complex was achieved by hydroxyapatite chromatography as previously described (*18*). Briefly, the mitochondrial lysate was layered on a hydroxyapatite column (Bio-Rad, 25 mL settled gel) equilibrated in 10 mM Tris, 100 mM NaCl, 1 mM EDTA, and 0.05% (w/v) Triton X-100 or LAPAO (final pH 7.3). The pass-through fraction was collected and chromatographed on Ultrogel AcA 202 (BioSepra) equilibrated in the same buffer.

Preparation of the Lipid Extract of Intact Mitochondria. Lipids were extracted from isolated beef heart mitochondria with a chloroform/methanol mixture by the procedure of Bligh and Dyer (*19*). The final extract was evaporated to dryness and immediately stored at −20 °C.

Extraction of Detergent with BioBeads. BioBeads (Bio-Rad, SM2 20−50 mesh) were washed with 10 mM Tris, 100 mM NaCl, and 1 mM EDTA (pH 7.3) and evacuated for 15 min. Bovine Anc stabilized with CATR (~1.5 mg of Anc/mL) was solubilized either with 0.04% LAPAO or with 3.6% Triton X-100 in Tris buffer (pH 7.3). LAPAO was extracted with 10 mg of BioBeads/mg of Anc for 1 h at 4 °C, and the extraction was repeated with 100 mg of BioBeads at room temperature for 15 min, until foaming was reduced to a minimum. When protein contained Triton X-100, detergent extraction was conducted with 100 mg of BioBeads/7 mg of protein, for 15 min at room temperature. A control sample devoid of protein, but containing an equal amount of Triton as present in the protein solution, was extracted in a manner identical to that for the protein sample using BioBeads. The amount of residual Triton in both samples was determined by absorbance at 275 nm. Extraction with BioBeads was continued until the Triton concentration was reduced to below its critical micelle concentration (cmc) (0.3 mM). The decreases in the absorbance of both the protein solution and the protein-free sample were found to be similar. Protein was then measured by the additional absorbance at 280 nm (E_{280} = 50000 $M^{-1}\cdot cm^{-1}$). Almost all of the protein was still present after extraction with BioBeads, but most of the Triton had been removed. The protein concentration in this sample was then more accurately determined by the micro-BCA assay (Biolynx, Inc.) using BSA as a standard, and the phospholipid concentration was determined by the method of Ames (*20*).

Preparation of the Sample for ^{31}P MAS/NMR. To obtain a concentrated amount of protein suitable for NMR, we froze the remaining solution and lyophilized it for ~5 h to dryness. Approximately 50 μL of water was added to the lyophilized material. This slurry was then loaded into a 45 μL Kel-F insert of a 4 mm zirconium MAS/NMR rotor. The exact amount of sample in the rotor was not determined but is estimated to contain between 10 and 30 mg of lipid contained in a volume of suspension of 50 μL, depending on the specimen.

Determination of Lipid Composition by TLC. The lyophilized material used for NMR was then extracted with a 1:1:1 $CH_3OH/HCCl_3/H_2O$ mixture, and the two phases were allowed to equilibrate at room temperature until they were well-separated. The lower phase was evaporated to dryness under nitrogen, redissolved in a small volume of a 2:1 $HCCl_3/CH_3OH$ mixture, and applied to a silica G (250 mesh) TLC plate. Triton extracts of mitochondria and standards of DOPE, DOPC, TOCL, and egg PA were also run as controls. Plates were developed in TLC glass chambers equilibrated with a $HCCl_3/CH_3OH/28\%$ NH_3 mixture (65:25:5, v:v:v). The lipid spots were visualized in a glass chamber exposed to vapor from iodine crystals. TLC showed a faint spot close to the origin, at the same R_f value as egg PA itself (R_f = 0.096), coinciding with the fact that PA is a minor component of the lipid in purified Anc, in agreement with the NMR results (see below).

^{31}P MAS/NMR. The solid state NMR spectra were recorded on a Bruker AVANCE 500 spectrometer equipped with a standard bore 11.7 T magnet giving a 202.45 MHz frequency for ^{31}P (500.12 MHz ^1H resonance frequency). The spectrometer was equipped with a 4 mm broadband tunable MAS probe. Magic angle spinning was controlled using a Bruker model H2620 pneumatic MAS controller. The chemical shift scale was referenced to an external standard of 85% phosphoric acid in a 4 mm rotor.

The ^{31}P NMR spectra were recorded over a sweep width of 25 kHz. Raw spectra were recorded using 14K data points (acquisition time of 0.3 s), and the data were processed with zero-filling to 16K data points and with exponential multiplication (lb = 3 Hz). The recycle delay was 2.5 s. At least 8000 scans were acquired for the MAS/NMR spectra. The Bruker Topspin solids line shape analysis routine (version 2.1) was used to simulate each of the spectra. The observed peak maxima for the isotropic peaks were at the same chemical shift as those for the calculated isotropic chemical shifts.

^{31}P T_1 Measurements. ^{31}P NMR T_1 times were determined using the saturation−recovery method together with power-gated decoupling of the protons. The samples were spun at rates of 5000 and 3000 Hz. The spectra were recorded at a resonance frequency of 202.45 MHz with a sweep width of 25 kHz, and the acquisition time was set to 0.3 s. Because the saturation recovery method permits rapid repetition, the recycle delay was set to 1 s.

At least eight different variable delay times were chosen, and their order was randomized to eliminate any systematic errors. For each variable delay time, 4096 transients were co-added to give sufficient signal to noise. The raw data were zero-filled to 16K points, and exponential multiplication (broadening factor lb = 3 Hz) was applied. The integrated intensities of the peaks

were plotted versus delay time, and an exponential fit of the data as per eq 1 yielded the T_1 results.

$$I(t) = I(0)\left[1 - \exp\left(\frac{-t}{T_1}\right)\right] \quad (1)$$

^{31}P NMR Static Powder Pattern. The static ^{31}P NMR spectra were recorded over a sweep width of 25 kHz. Raw spectra were recorded using 14K data points (acquisition time of 0.3 s), and the data were processed with zero-filling to 16K data points and with exponential multiplication (lb = 20 Hz). The recycle delay was 2.5 s. The Bruker Topspin solids line shape analysis routine (version 2.1) was used to simulate each of the spectra. The chemical shift anisotropy (CSA) parameters determined from the line shape analysis routine were used to simulate the spectra at different MAS speeds, and these were compared to the experimental spectra. The reported CSA parameters follow the Haeberlen convention as outlined in the IUPAC recommendations (*21*).

Differential Scanning Calorimetry (*DSC*). Measurements were taken using a Nano II Differential Scanning Calorimeter (Calorimetry Sciences Corp., Lindon, UT). The scan rate was 1.0 °C/min with a delay of 5 min between sequential scans in a series to allow for thermal equilibration. Samples were loaded into a calorimeter cell equilibrated at temperatures below 15 °C. DSC curves were analyzed by using the fitting program DA-2 provided by Microcal Inc. (Northampton, MA) and plotted with Origin, version 7.0. DA-2 also estimates the van't Hoff enthalpy by an iterative curve fitting analysis. The concentration of Anc in the calorimeter cell was 3.8 mg/mL before the NMR measurement and 0.5 mg/mL after the NMR measurement. The cell volume is 340 μL.

Circular Dichroism. CD spectra were recorded on an AVIV model 215 spectropolarimeter. A quartz cell with a path length of 0.1 cm was placed in a thermally controlled cell holder. The temperature changes were computer controlled, and points were taken at 5 °C intervals. The machine was equipped with a Peltier junction thermal device and a Thermo Neslab M25 circulating bath.

RESULTS

^{31}P MAS/NMR. The advantage of MAS/NMR is that resolvable signals can be obtained from slowly tumbling species that have high molecular weights. This allows us to identify all of the phospholipid species that are copurified with Anc. Similar spectra were obtained with samples of Anc extracted with LAPAO or with Triton X-100. ^{31}P MAS/NMR spectra from samples prepared with LAPAO exhibited better-resolved peaks and were used to obtain information about the nature and relative amounts of the lipids present in the preparations of the purified protein (Figure 1A). The preparations after extraction of Triton exhibit a broader CSA in the static ^{31}P NMR spectra and higher-intensity spinning side bands with MAS/NMR (Figure 1B). This is likely the consequence of the more complete removal of detergent in the case of Triton X-100. In the sample obtained by extraction of Triton X-100, detergent elimination was likely more complete because its removal could be monitored by spectrophotometric measurements. Data from the Triton-extracted samples are used for other results presented and were collected several times. The peak for CL is clearly identified in MAS/NMR, although it does not appear in static spectra of solutions of Anc in detergent, even after the addition of 1% SDS and incubation for 4 days (*8*). In addition, we observe a minor peak at a chemical shift expected for phosphatidic acid (PA) around neutral pH at 2.7 ppm (*22*). The identity of this peak as PA is confirmed by increasing the pH approximately 2 units, to pH 9, resulting in a change in the chemical shift of this peak to 4.2 ppm, without affecting the other two peaks. The observed pH dependence of the chemical shift is characteristic of PA and is in general agreement with the published ^{31}P NMR titration curve (*22*).

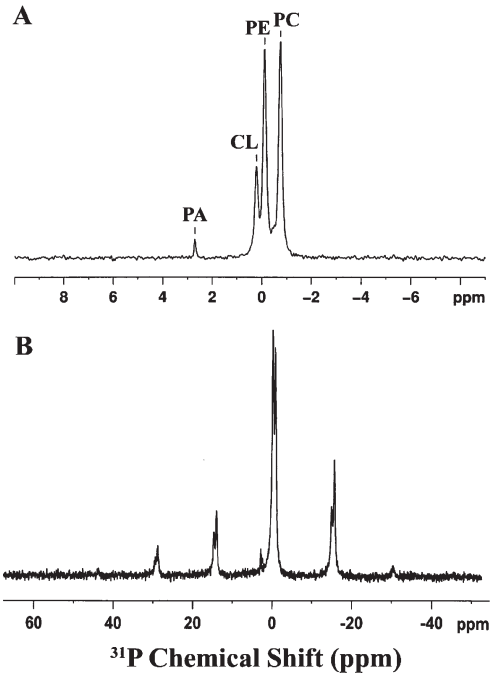

FIGURE 1: ^{31}P MAS/NMR spectrum of Anc. (A) Sample prepared from LAPAO-solubilized protein and the detergent removed with BioBeads. The plotted region focuses on the isotropic peaks only. The spinning speed was 5 kHz. Similar spectra were obtained using a 3 kHz spinning speed. (B) Sample prepared from Triton X-100-solubilized protein and the detergent removed with BioBeads. The spinning speed was 3 kHz, and the full spinning sideband pattern is plotted. Parameters obtained from a series of such spectra run at different spinning speeds are listed in Table 1.

The Anc purified from Triton X-100 extracts exhibited strong spinning side bands in ^{31}P MAS/NMR (Figure 1B) that could be used to analyze various parameters (Table 1). Values in Table 1 are averages of measurements made at spinning speeds of 3 and 5 kHz, which were in good agreement with each other. The values correspond to those for the sample prepared from Triton-solubilized material unless otherwise indicated. The relative areas, chemical shifts, and T_1 values were similar for samples prepared from LAPAO and from Triton X-100. The CSA and line width were less well determined for the samples from LAPAO because of the weaker spinning sidebands possibly caused by the presence of residual detergent.

^{31}P Static Powder Pattern. The shape and CSA of the static ^{31}P NMR powder pattern are sensitive to the phase of lipid (*23*). There are examples of this parameter being used in studies of peptide−lipid interactions (*24*). In the case of purified Anc, the static ^{31}P NMR powder pattern is indicative of the arrangement of the lipid as a bilayer in the liquid crystalline state (Figure 2). Unlike the MAS/NMR spectra or spectra of lipids solubilized by detergent, this sample is a turbid suspension of lipid that shows a broad CSA and therefore a low intensity and low signal-to-noise

Table 1: Parameters from the ^{31}P MAS/NMR Spectra of Anc Preparations

assignment	relative area	isotropic chemical shift (ppm)	CSA (ppm)	line width (Hz)	T_1 (s)
PA	2.0	2.7	1.0	50	0.05
CL	13.7	0.22	ND[a]	ND[a]	0.6[b]
PE	40.6	−0.12	23	110	0.6
PC	43.7	−0.8	28	90	0.6

[a]Not determined. [b]Measured with the sample from LAPAO.

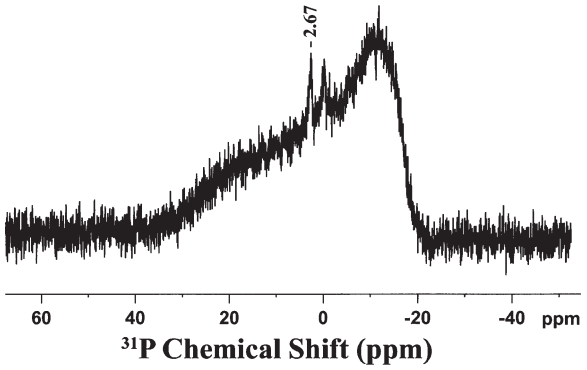

FIGURE 2: Static ^{31}P NMR spectrum of Anc. The sample was prepared from Triton X-100-solubilized protein and the detergent removed with BioBeads. The number of scans acquired was 24000. A feature at 2.67 ppm, corresponding to the resonance of CL, is shown. There is also a small signal at ~0 ppm.

ratio. Distinct features at 2.7 and ~0 ppm are observed in the broad powder pattern arising from species with narrower peaks.

^{31}P NMR Spectra of Detergent-Solubilized Preparations. We compared the nature and amounts of free lipid that was not firmly bound to protein in intact mitochondria with the lipid in the isolated Anc. This was done by measuring the liquid state static ^{31}P NMR spectra of mitochondria solubilized with Triton X-100 in comparison with a preparation of purified Anc in Triton (Figure 3). In addition to the PE and PC in both samples, the material solubilized from mitochondria also had resonance peaks corresponding to PA and CL. We showed above, using MAS/NMR, that PA and CL are present in the purified Anc but do not give rise to resonance lines in the static NMR spectrum because they are too firmly bound to the protein. In addition, since a significant amount of PA is observed in the mitochondrial preparation, the PA found by MAS/NMR in the samples of purified Anc did not arise from lipid degradation during the isolation of the protein but was present in the original preparation of the mitochondria.

Differential Scanning Calorimetry (*DSC*). The acquisition of the MAS/NMR spectra to determine T_1 required several days of continual measurements at ambient temperature and under a strong centrifugal field. It was therefore important to assess whether the protein remained in its native state during the measurement. To the best of our knowledge, there has been no study of the characteristics of the thermal denaturation of this protein. Preparations of Anc purified from Triton X-100 and treated with BioBeads to extract the detergent showed a thermal transition at 74 °C (Figure 4). The calorimetric enthalpy of this transition is 48.5 kcal/mol of monomer and the van't Hoff enthalpy 130 kcal/mol. The analysis was performed on samples both before and after the material had been used to acquire the T_1 data and shows that the protein remains in its native state during the NMR measurements and is capable of thermal

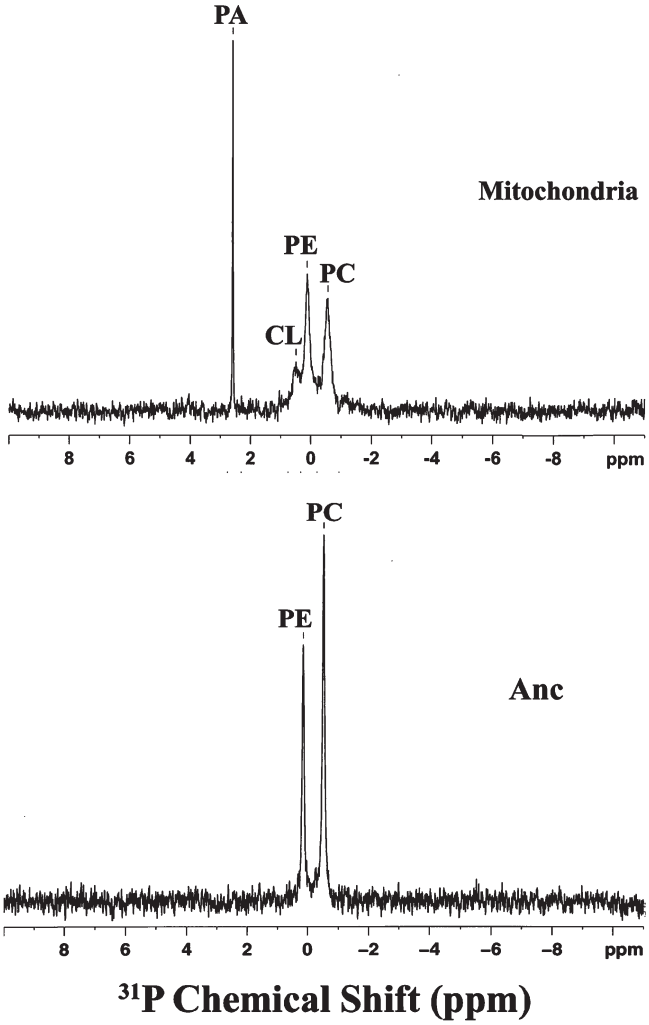

FIGURE 3: Static ^{31}P NMR spectra of solutions of Triton-solubilized mitochondria or Triton-solubilized purified Anc. The Triton X-100 is still present in these solutions. The number of scans acquired is approximately 4000 for each of the two spectra. Note that the PE peak in the Triton-solubilized mitochondria also has signals from phosphatidylserine that has a similar chemical shift but comprises only 9% of the mitochondrial lipids.

denaturation with the same characteristics as a fresh sample (Figure 4). Similar results were obtained by DSC analysis before and after acquisition of the NMR spectra of the preparation of Anc purified using LAPAO. For this sample, the denaturation temperature was also 74 °C, was irreversible, and remained unchanged after the NMR studies (not shown).

CD. The CD spectrum of a fresh Anc sample, similar to the one used for DSC, shows a high content of helical structure (Figure 5). This is in agreement with the structure determined by X-ray crystallography (*6*). The protein exhibits a loss of secondary structure at temperatures above ~75 °C that is irreversible on

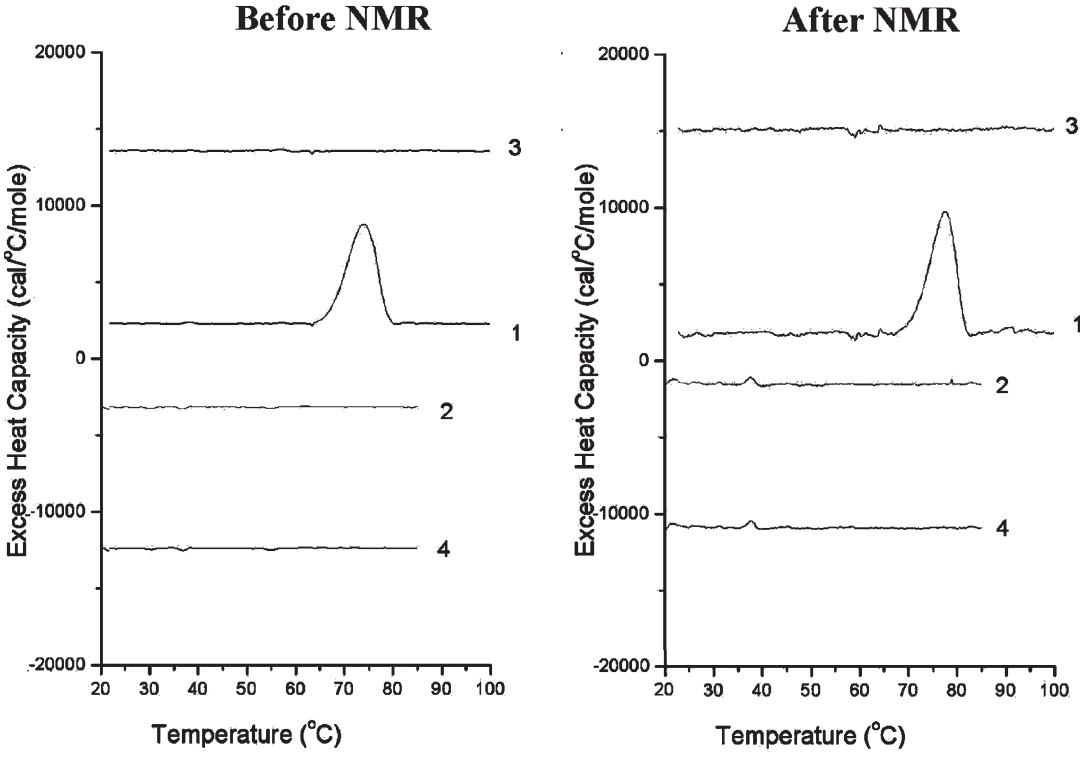

FIGURE 4: DSC of Anc. The sample of Anc was purified from Triton X-100-solubilized protein and the detergent removed with BioBeads. The scan rate was 1 °C/min. Odd-numbered scans are heating scans and even-numbered scans cooling scans. Curves have been separated for the sake of presentation.

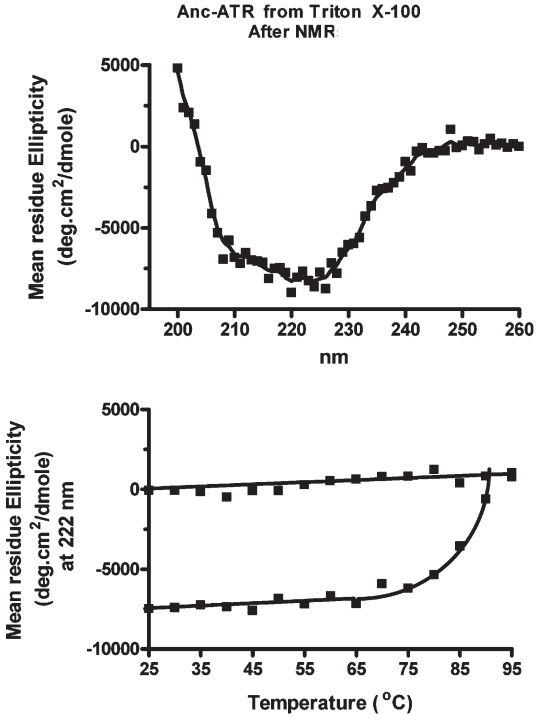

FIGURE 5: CD of Anc. The sample of Anc was purified from Triton X-100-solubilized protein and the detergent removed with BioBeads. The top panel shows the spectrum of the unheated protein at room temperature after the NMR experiment. The bottom panel shows the temperature dependence of the mean residue molar ellipticity at 222 nm. The top curve with values close to zero is the cooling scan, the while bottom curve is the heating scan.

cooling. The CD data are consistent with the DSC results showing that the Anc is still in its native state after several days at room temperature and that it exhibits a thermal denaturation that is irreversible. The helical unfolding extends to a higher temperature than does the DSC denaturation endotherm.

DISCUSSION

Our results demonstrate that PA is copurified with Anc during the chromatographic separations. The identity of this material as PA is confirmed by the chemical shift of the phosphate that is different from other common phospholipids, by the pH dependence of the chemical shift, and by the TLC results. We believe that this lipid has not been previously detected in preparations of Anc because it is not bound to the protein in a fixed position and therefore does not appear in the crystal structure. It is not seen in liquid state NMR in detergent-solubilized solutions (Figure 3) (*8*) presumably because it is bound to the protein and has a short T_2. PA is also difficult to observe by TLC using common solvent systems because of overlap with other lipids present in the purified protein preparations and because PA is present in such small amounts. With several solvent systems used for phospholipid TLC analysis, CL has approximately the same R_f as PE. With the solvent system used in this work, the R_f of PA is low and does not move much from the origin, but it can be clearly distinguished from the other lipid components present in purified Anc, mainly PC, PE, and CL. For these reasons, the presence of PA in purified preparations of Anc may not have been previously detected.

The PA we detect in the samples of purified Anc is not likely to have arisen by lipid degradation. Phospholipase D is present in mitochondria, and it is upregulated in certain disease processes (*25*, *26*) and could be responsible for producing PA. However, we show (Figure 3) that PA is present in detergent-solubilized mitochondria that have had minimal treatment or exposure to room temperature. In addition, a major lipid component of the inner mitochondrial membrane, CL, is synthesized in the mitochondria from PA.

Thus, there must be a constant steady state level of PA in the mitochondrial membrane.

The motional properties of PA in the preparations of Anc are unusual and demonstrate that the PA is not free but is interacting with the protein. We find the T_1 for PA to be 0.05 s (Table 1). This compares with 1.1 s for DOPA in a 9:1 or 8:2 DOPC/DOPA mixture (E. Kooijman, personal communication). This is a very dramatic difference and indicates a strong interaction of PA with the protein. The T_1 for PA is also 10-fold smaller than for PC or for PE in the same preparation of Anc (Table 1). The shorter T_1 of PA is a result of strengthened dipolar interactions between the PA and the protein because of the tight binding. The CSA and overall shape of the powder pattern of most of the lipid in a static ^{31}P NMR spectrum of the purified Anc correspond to those of a bilayer (Figure 2). The unusual aspect of the motional properties of PA is that although the T_1 is short, the CSA is also small (Table 1). The CSA measures the ability of the phosphorus moiety to reorient in space within the lifetime of the excited spin state. The sharper feature in the static ^{31}P NMR spectrum at a chemical shift close to that of PA (Figure 2) is also consistent with PA having a small CSA when bound to Anc. If the motion of the PA were slowed, one would a priori expect a large value of the CSA, since the time would be too short for the molecule to access all orientations corresponding to a time-averaged isotropic orientation. The most likely explanation for the small CSA is that there is more than one site for PA binding to Anc and that the lipid can undergo rapid chemical exchange between different sites. Other less likely possibilities include that in which the Anc is bound to the lipid bilayer in a particular orientation and the PA binds to the protein in a manner in which it becomes oriented at the "magic angle" with respect to the bilayer, or that in which although the bulk of the lipid is in a bilayer arrangement (Figure 2), there may be local domains that are enriched in protein and PA that form nonlamellar local regions such as micellar, sponge phase, or cubic phase. Because of the high local curvature of such structures, the PA could reorient rapidly in space by lateral diffusion.

The calorimetric enthalpy for the thermal denaturation of Anc corresponds to an enthalpy of 1.5 cal/g. This is much lower than the enthalpy of denaturation of water-soluble proteins at ~70 °C which is ~8 cal/g (*27*). It is known that integral membrane proteins have a low enthalpy of denaturation, perhaps because they do not unfold completely. For example, another integral membrane protein, the Na^+/K^+-ATPase, has a denaturation enthalpy of 1.3 cal/g (*28*). This finding is in accord with Anc being largely embedded in the membrane and having only a small extramembranous domain.

The ratio of the van't Hoff enthalpy to the calorimetric enthalpy gives the cooperative unit size, which is 2.7 for Anc. This indicates that there is interaction among monomers in our preparation. There is much evidence for Anc forming dimers (*2*). Our DSC evidence indicates that the two monomers are in intimate association so that they undergo a thermal transition in a cooperative manner. The cooperative unit size being larger may also be a consequence of the high concentration of Anc in the lipid of the preparation we used as well as the possibility that there is some aggregation of the dimers with increasing temperature.

From the protein and lipid concentrations in these samples, we calculate that there are 340 phospholipid molecules per mole of protein dimer. Given the ratio of the four different lipids, this corresponds to 150 PC, 140 PE, 47 CL, and 7 PA molecules. The number of CL molecules per dimer agrees well with the amount of motionally restricted lipid determined with spin-labels (*16*). The amount of PC and PE is much larger, and these lipids appear not to be bound tightly to the protein since they give rise to signals in the static NMR spectra of the detergent-solubilized purified Anc preparation (Figure 3). The 7 molecules of PA per dimer is close to the value of 3 tightly bound lipids per monomer (*7*). However, these lipids have been shown by the diffraction studies to be CL. Interestingly, both PA and CL are the only two major phospholipid components of mammalian membranes that have more than one negative charge at neutral pH, and they can both form intramolecular hydrogen bonds in the headgroup (*22*, *29*). A major difference between the structures of these two lipids is the fact that CL has the negative charge distributed over a larger headgroup having two phosphates separated by a glycerol. It is possible that the headgroup structure of CL allows it to bind optimally to sites on Anc, while PA interacts more transiently and exchanges among the several CL-binding sites. From the crystal structure of Anc, both of the phosphate groups of each CL are involved through hydrogen bonding with main chain nitrogens and with carbonyls of symmetry-related residues, while the acyl chains of CL interact with aromatic or hydrophobic residues. PA could also simulate these bonding interactions between Anc and lipid (*7*). Perhaps under the conditions required for crystallization CL replaces the entire PA content, although when Anc is in the mitochondrial membrane PA can compete with CL. The conditions of crystallization require detergent concentrations reaching more than 10% (w/v), which may alter the relative affinities of CL and PA for binding to Anc, resulting in only CL remaining bound in the crystal, PA being removed by the detergent.

The presence of PA is of particular interest because it is known to bind specifically to a large number of diverse proteins (*30*, *31*) and to play an important role in cell signaling and membrane dynamics (*32*), including specifically in the mitochondria (*33*). PA is unique among lipids in having a p*K* near physiological pH, in having the capability of being an H-bond acceptor, and in having a marked tendency to form structures with negative curvature. This has led to the development of the electrostatic−hydrogen bond switch model for describing some of the unique properties of PA and its interactions with proteins (*32*).

The role of lipids facilitating the self-association of Anc has been illustrated with the finding of crystallographic dimers of Anc (*7*). It is tempting to propose that both tightly bound PA and CL mediate the functional dimerization of Anc.

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