Tightly Associated Cardiolipin in the Bovine Heart Mitochondrial ATP Synthase as Analyzed by $^{31}$P Nuclear Magnetic Resonance Spectroscopy*

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The bovine heart F$_0$F$_1$-ATPase preparation (Serrano, R., Kanner, B., and Racker, E. (1976) J. Biol. Chem. 251, 2453–2461) has been further delipidated. The lipid-deficient preparation contained 2.5 mol of cardiolipin, 1 mol of phosphatidylcholine (PC), and 1 mol of phosphatidylethanolamine (PE) per mol of F$_0$F$_1$. When reconstituted with asolectin the delipidated preparation exhibited an activity of 13 μmol of ATP hydrolyzed/min/mg of protein which was 88% oligomycin-sensitive. The phospholipids in this preparation were analyzed by $^{31}$P NMR spectroscopy to determine if they were immobilized by the enzyme (rendered NMR-invisible). The PC and PE were below the limits of detection under the conditions utilized and the cardiolipin was NMR-invisible until the enzyme was denatured by addition of either 1% sodium dodecyl sulfate or 8 M urea. Addition of cardiolipin to the delipidated preparation and subsequent analysis by NMR spectroscopy revealed that approximately 4 mol of cardiolipin were immobilized per mol of F$_0$F$_1$ ATPase. The enzyme appears to have high affinity for cardiolipin exclusively, since PC (a prominent inner membrane lipid), phosphatidyl serine (an acidic phospholipid), and phosphatidyl glycerol (the precursor to cardiolipin) were not immobilized (rendered NMR-invisible) when added to the delipidated preparation.

The mitochondrial ATP synthase (F$_0$F$_1$-ATPase) from bovine heart is a multisubunit complex (1, 2), which has been isolated relatively free from other polypeptides and endogenous phospholipids (3, 4). It is composed of at least 13 polypeptides (1) which have been subdivided into the catalytic (F$_1$) portion, the membrane sector subunits (F$_0$), and those involved in interfacing the F$_0$ and the F$_1$ portions of the structure. When phospholipids are removed from the F$_0$F$_1$ complex its activity decreases to very low levels, but both ATPase (5–7) and ATP-P$_i$ exchange (3, 4, 8) activities can be restored by reconstitution with a variety of phospholipids.

Several earlier studies (9–12) indicate that acidic phospholipids, including cardiolipin, elicit higher ATPase activities from the F$_0$F$_1$ complex than do those with no net charge. However, in subsequent investigations (7, 13, 14) conditions were established in which high ATPase activities could be obtained with isoelectric phospholipids including PC and PE. In one of these latter studies (7) in which reconstitution was accomplished utilizing octylglucoside as a mediator, it was demonstrated that $V_{max}$ of the phospholipid enzyme complex was slightly higher with PC than with cardiolipin. However, utilizing this reconstitution system it was also established that cardiolipin had more significant effects on other parameters related to catalysis such as the $K_m$ for ATP, the $K_i$ for competitive inhibitors, and ATP-induced conformational changes of the $b$ subunit of the ATPase complex. These latter studies (7, 13, 14) suggest strongly that the $V_{max}$ which could be achieved with a particular phospholipid is dependent on the reconstitution procedure utilized. Nevertheless, the observations made on the catalytic and structural characteristics (7) suggest strongly that cardiolipin is able to influence properties of the enzyme more dramatically than the other major inner membrane phospholipids, PC and PE.

The above mentioned observations suggest that efforts should be made to determine if there is a unique association between the F$_0$F$_1$ ATPase complex and cardiolipin that could be differentiated from interactions with other membrane phospholipids. The probability of a preferential association with cardiolipin has also been inferred by other studies which suggest that this phospholipid binds tightly to, or is required by, other inner membrane components. Earlier studies suggest strongly that cytochrome oxidase (15, 16), the phosphate transporter (17, 18), and glycerol-3-phosphate dehydrogenase (19) require cardiolipin for expression of full catalytic activity. Moreover, Beyer and Klingenberg (20), utilizing NMR spectroscopy, have observed that 5–7 mol of cardiolipin were tightly bound to the dimeric form of the adenine nucleotide carrier, whereas the other inner membrane phospholipids, PC and PE, were readily dissociated from the purified preparation.

The study of Beyer and Klingenberg demonstrates a technique that could be readily applied to the purified F$_0$F$_1$ complex. As discussed by these investigators (20), association of phospholipid with a protein or other immobilized structure is expected to lead to significant line broadening of the NMR signal. If the broadening is sufficiently great no signal will be observed for the immobilized phospholipid. This phenomenon was utilized in the investigation of Beyer and Klingenberg (20) to demonstrate the tight binding of cardiolipin to the adenine nucleotide carrier. In the present study the selective loss of cardiolipin signal in $^{31}$P NMR spectra of phospholipids

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The abbreviations used are: F$_0$F$_1$ ATPase, the oligomycin-sensitive ATP synthase holoenzyme complex; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; SDS, sodium dodecyl sulfate.
associated with F_{2}F_{2} was utilized to investigate the association between the ATPase complex and this acidic phospholipid.

In previous studies of the interaction of the F_{2}F_{2} ATPase complex and phospholipids the enzyme preparations contained from 20 to 65 mol of phospholipid/mol of ATPase (4, 5, 14), and in many cases, higher amounts of phosphoglycerides. In the present study we have succeeded in removing all but 5 mol of phospholipid/mol of enzyme and this degree of delipidation has allowed an investigation of the association between the F_{2}F_{2} complex and cardiolipin. Included in this investigation are experiments verifying the specificity of tight binding between F_{2}F_{2} complex and cardiolipin. Measurements are also reported which allowed estimates of the number of high affinity sites for this acidic phospholipid. A preliminary report of these studies has appeared previously (21).

**EXPERIMENTAL PROCEDURES**

**Materials**

Most of the reagents utilized were purchased from suppliers listed earlier (5, 7, 14). PS (beef brain) was obtained from Serdary Research Laboratories. PG (from egg yolk FC) and Triton X-100 were purchased from Sigma Chemical Company, Inc. (London, Ontario). Utrapure urea was obtained from Schwarz/Mann Biotech.

**Preparations**

Beef heart mitochondria were prepared as described earlier (22). The F_{2}F_{2}-ATPase complex defined by Serrano et al. (3) as 38-45p was prepared as described earlier with the additional cholate extraction step included (14). This preparation was utilized in some of the studies and was also further purified as follows for many of the experiments in this investigation. The adenine nucleotide transport protein and most of the remaining endogenous phospholipids were removed by treatment with lyssolecithin and subsequent purification by centrifugation on a sucrose density gradient, as described by Serrano et al. (3). The material recovered after gradient centrifugation was extracted with 0.5% sodium cholate and precipitated with (NH_{4})_{2}SO_{4} (45% saturation). The pellet was rinsed three times with a solution containing 10 mM Tris-sulfate, 0.5 mM EDTA, 1 mM MgSO_{4}, 0.5 mM dithiothreitol, pH 7.7, to remove residual detergent, (NH_{4})_{2}SO_{4}, and phospholipids. The resulting material is referred to as gradient-purified ATPase. With some gradient-purified preparations the initial cholate extraction step (14) was omitted with no loss in the quality of the preparations.

**Analytical Measurements**

**ATPase Activity, Protein Concentrations, and Electrophoresis—** The ATPase activities of the preparations described above were assayed as outlined previously (5). Asosclentin (100 µg) and/or oligomycin (5 nmol) were included where indicated. A unit of ATPase activity is defined as the amount of enzyme which catalyzes the hydrolysis of 1 µmol of ATP/min under the assay conditions described (5). Specific activities were calculated utilizing protein concentrations determined by the method of Lowry et al. (23). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 10-17% linear gradient gels by the procedure of Laemmli (24) modified to include 2 mM EDTA in the gels and buffers. Gels were stained with 0.5% Coomassie Blue (in 30% isopropanol, 10% acetic acid). NMR Spectroscopy—All ^{31}P magnetic resonance spectroscopy measurements were performed using a Bruker Instruments, Inc. (Dillerka, MA) AM-300 series spectrometer operated at 121.5 MHz. The probehead utilized was a Bruker 10-mm multinuclear unit tuned for ^{31}P; the spin frequency was 17 cycles per s. Field homogeneity was adjusted using the ^{31}P free induction decay obtained from the sample solutions. A pulse width of 15 µs and an 8-s interpulse delay were utilized in all experiments. Under these conditions the phosphorus nuclei in the phospholipids were allowed to fully relax between acquisitions. All samples were maintained in 10% D_{2}O and measurements were carried out utilizing a deuteron field frequency lock and proton broad-band decoupling. In some experiments spectra were generated from 10,000 acquisitions, while in others 2,000 acquisitions were utilized for each spectrum presented. A 2-Hz Lorentzian line-broadening function was applied to the summed free induction decays to improve the signal-to-noise ratio. The areas indicating relative concentrations of individual phospholipids were determined utilizing the integration program included with the software for the Aspect 3000 computer. Chemical shift values are reported relative to 85% H_{3}PO_{4}. All NMR measurements were carried out at 10 °C on samples unless otherwise specified and aliquots of enzyme preparations being analyzed were assayed periodically to verify that the preparation had maintained its enzymatic activity. The amounts of enzyme utilized ranged from 7 to 9 µg of protein.

**Photon Correlation Spectroscopy—** The size of the ATPase in the presence of 1% Triton X-100 was determined by photon correlation spectroscopy using a Brookhaven Instruments BI-2030AT correlator, operated in conjunction with a BI-200 SM light scattering goniometer and a Spectra Physics model 127 He-Ne laser (35 milliwatts, equipped with a vertical polarization rotator). Light was collected at a right angle to the cuvette wall in 1 cm square cuvettes at 293 °C in a 0.1 s duration time of 2-15 min, with a detector optics aperture chosen to optimize both the count rate and signal-to-noise ratio (which averaged 0.28 ± 0.05 for these experiments). Data were analyzed by the method of cumulants; the results reported here were obtained with a second-order cumulant analysis (25).

**Phospholipid Analyses—** Samples analyzed by NMR spectroscopy were lyophilized and then extracted with acetone to remove excess Triton X-100 which otherwise interfered with lipid analyses. Lyophilized samples were vortexed with 2 ml of carefully dried acetone, and then centrifuged at 27,000 × g for 15 min. The pellet was extracted with 2 ml of CHCl_{3}/methanol (1:2, v/v) followed by centrifugation as before. This extraction with CHCl_{3}/methanol was repeated two additional times and the combined supernatants were concentrated under a stream of nitrogen. The lipid extract was then subjected to preparative thin layer chromatography on silica gel H in a solvent system consisting of CHCl_{3}/methanol/H_{2}O/acetic acid (85:17:2.3, v/v). This solvent system resulted in an excellent separation of Triton X-100 (R_{f} = 0.81), cardiolipin (R_{f} = 0.62), PE (R_{f} = 0.53), FC (R_{f} = 0.14), and lyssolecithin (R_{f} = 0.04). The regions corresponding to cardiolipin, PE, PC, and LPC, were scraped from the plates and the silica gel was extracted with 2 ml of CHCl_{3}/methanol/H_{2}O (1:2:0.3, v/v). The recovered phospholipids were concentrated under nitrogen and analyzed quantitatively by the procedure of Chalvardjian and Rudnicki (26).

**Determination of Stoichiometry of Phospholipids in ATPase Preparations—** The amount of enzyme in moles was calculated assuming a molecular mass of 500,000 daltons. In the 38-45p preparation the presence of the adenine nucleotide transport protein was estimated to be 2.3 mol/mol of the ATPase utilizing densitometry analyses of polyacrylamide gel scans; this value was utilized in converting mg of protein to moles of enzyme. This conversion assumed equal staining intensities by the adenine nucleotide transporter and the γ subunit of the ATPase with Coomassie Blue. In samples which were purified as described above, phospholipids were extracted from a predetermined amount of enzyme and determined quantitatively as described above to obtain the phospholipid-to-enzyme ratios. In experiments in which the enzyme was titrated with cardiolipin, the areas under the cardiolipin and lyssolecithin signals were utilized to determine the amount of cardiolipin bound to the enzyme. In these experiments the areas under the lyssolecithin signal remained relatively constant throughout the titration procedure and were used as standards to normalize the area for cardiolipin in each spectrum taken. The cardiolipin signal increased as the amount added was incrementally increased (see Fig. 3). The total cardiolipin in the sample was that added plus the endogenous cardiolipin. The area under the cardiolipin signal was determined after the enzyme was denatured with 1% SDS and this value was utilized to obtain the magnitude of the signal per mol of cardiolipin. After excess cardiolipin was added, the areas associated with the cardiolipin signal before and after denaturation were determined. The difference in these areas was divided by the area per mol of cardiolipin to determine the moles of cardiolipin immobilized by the ATPase complex (NMR-invisible cardiolipin).

In experiments where the tightly bound cardiolipin was 38-45p ATPas was estimated, the same amount of F_{2}F_{2} ATPase in moles was utilized as in the experiments with the gradient-purified ATPase preparation and the NMR measurements were performed with exactly identical parameters as those utilized with the gradient-purified preparations. This allowed use of the value previously derived for the signal area per mol of cardiolipin as described in the preceding paragraph in calculating the moles of cardiolipin immobilized by the
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In 38-45p preparations. NMR spectra were derived before and after denaturation of the 38-45p preparation and the difference in the magnitudes of the cardiolipin signals was divided by the signal area per mol of cardiolipin (see above) to calculate the moles of cardiolipin immobilized by the ATPase complex.

RESULTS

Characterization of ATPase Preparations—The F$_1$F$_0$ ATPase preparations described under "Methods" were characterized with respect to enzymatic activity, polypeptide, and phospholipid composition. The ATPase activities and oligomycin sensitivities of the preparations are illustrated in Table I. All preparations had low ATPase activity in the absence of added phospholipid. The slightly higher activities in the gradient-purified preparations were due to residual lysolceithin not removed in the gradient centrifugation step. Lysolceithin is known to elicit oligomycin-sensitive ATPase activity in the F$_1$F$_0$ complex at moderately low concentrations (22). Addition of asolectin resulted in a significant increase in ATPase activity in all the preparations. Oligomycin sensitivity was maintained in all preparations of the enzyme, verifying the functional integrity of the F$_1$F$_0$ complex. The preparations utilized in the NMR experiments described below were the reextracted 38-45p preparation and the reextracted, gradient-purified ATPase. These will be designated as 38-45p ATPase and gradient-purified ATPase in the remainder of the manuscript.

Treatment of the 38-45p preparation with lysolceithin and further purification utilizing sucrose density gradient centrifugation (3) were very effective in removing the adenine nucleotide transport protein. Based on densitometry analyses of gel patterns of the 38-45p and gradient-purified preparations of the ATPase, it was calculated that only 4% of the transport protein present in the 38-45p preparation remained after treatment with lysolceithin. This compares favorably with the success of Serrano et al. (3) who were able to remove at least 90% of the adenine nucleotide transport protein present in the 38-45p preparation by the same procedure. The phospholipid content of the 38 45p preparation ranged from 20 to 40 mol of phospholipid/mol of F$_1$F$_0$ ATPase with a cardiolipin/PC/PE ratio of 1:3:3 (5). The phospholipid content of gradient-purified ATPase preparation was much lower, with average $\pm$ S. E. values for cardiolipin, PC, and PE of 2.6 $\pm$ 0.2, 1.4 $\pm$ 0.2, and 1.2 $\pm$ 0.2 mol/mol of ATPase, respectively ($n = 6$ gradient-purified samples) for a total of 5 mol of phospholipid/mol of ATPase complex.

Spectroscopy Measurements on the ATPase Complexes—The three prominent phospholipids in the 38-45p preparation of the ATPase are readily detected by NMR spectroscopy, as illustrated in Fig. 1. The signals for PC and PE are approximately equivalent, whereas the signal for cardiolipin was somewhat less which confirms its lower relative concentration in the 38-45p ATPase. This preparation was denatured by suspending it in 8% Triton X-100 at room temperature. The gradient-purified enzyme (Fig. 1), likewise denatured, demonstrated a signal for the small amount of lysolceithin retained after the gradient-purification step and a signal from cardiolipin. No signals were observed for either PC or PE, which demonstrates that they are in concentrations below the limits of detection under the conditions utilized for spectral accumulation (10,000 acquisitions).

The oligomycin-sensitive ATPase activity of the preparations can be retained for periods up to 24 h in the spectrometer if the samples are maintained in the probe at 10 °C in 1% Triton X-100. As determined by light scattering measurements (see "Experimental Procedures"), the enzyme combines with Triton X-100 to form a particle with a diameter of 250 nm. Electron micrographs of F$_1$F$_0$ (27) have suggested that the ATPase complex has the dimensions of 12 by 20 nm. The particle observed in the presence of 1% Triton X-100 could therefore accommodate no more than 12-13 F$_1$F$_0$ complexes. This represents a significant dispersion as compared with the large aggregates of the delipidated enzyme that exist when it is suspended in the absence of detergent (14).

As mentioned above, the ATPase, when suspended in 1% Triton X-100 and maintained at 10 °C for 24 h, can be reconstituted to full oligomycin-sensitive activity if combined with asolectin under normal assay conditions (10 μg of enzyme protein). Furthermore, if 1% Triton X-100 is added to a

TABLE I

| ATPase specific activity | Oligomycin-sensitive ATPase activity | % |
|-------------------------|------------------------------------|----|
| No added lipid With asolectin |                                   |    |
| 38-45p ATPase             | 2.1 14.5                           | 96 |
| 38-45p ATPase, reextracted | 1.4 15.9                           | 95 |
| Gradient purified ATPase  | 3.0 10.6                           | 99 |
| Gradient-purified ATPase, reextracted | 3.8 13.0 | 88 |

FIG. 1. $^{31}$P NMR spectra of the 38-45p ATP synthase (upper tracing) and the gradient purified ATP synthase (lower tracing). The amounts of protein utilized for these spectra were 8.4 and 6.9 mg of the 38-45p and the gradient-purified ATP synthase, respectively. The preparations were maintained in 10 mM Tris-sulfate, pH 7.4, 8% Triton X-100. The chemical shifts in this and the following figures are reported relative to 85% H$_3$PO$_4$. 10,000 acquisitions were accumulated for each spectrum. The spectrum for the gradient-purified enzyme was multiplied 1.25 times relative to that from the 38-45p in order to emphasize the signal from cardiolipin (DPG).
normal assay mixture (10 μg of enzyme protein) it has no effect on the specific activity and has minimal effect on oligomycin sensitivity (62% of original oligomycin sensitivity). When assayed at a concentration of 3 mg/ml (the maximum concentration in the NMR tube) and in the presence of asolectin, F,F, demonstrated a low specific activity (0.9 μmol/min/mg of protein) which was 73% oligomycin-sensitive; the stimulation of activity by asolectin was 5-fold under these conditions. If the assay mixture containing high amounts of protein was adjusted to contain 1% Triton X-100 there was no change in either the activity or the oligomycin sensitivity, which indicated that the detergent at this concentration had little effect on the catalytic properties of the enzyme. The above observations demonstrate that the enzyme retains its native properties in the presence of 1% Triton X-100.

In Fig. 2 the NMR spectra of two separate preparations of the gradient-purified ATPase are shown. Aliquots taken from the NMR tube demonstrate high oligomycin-sensitive activity when assayed in the presence of asolectin. The only significant signal in either of the preparations is that from residual lysolecithin. If the enzyme is denatured irreversibly by addition of either 8 M urea (Fig. 2A) or 1% SDS (Fig. 2B) a strong signal for cardiolipin becomes evident. These observations indicate that the cardiolipin present in the gradient preparation is immobilized such that it is NMR-invisible until the ATPase is denatured irreversibly with either urea or SDS. In this and the following NMR measurements the addition of SDS resulted in a chemical shift to more positive values for all phospholipids. The magnitude of the SDS-induced chemical shift was a function of the particular phospholipid. The magnitude of the signal from phospholipid was depressed in the presence of 8 M urea as indicated by a loss in the lysolecithin signal, but no change in the resonance position of the signal was noted (Fig. 2A).

The gradient-purified preparation of the ATPase complex was titrated with exogenously added bovine heart cardiolipin to determine how many moles of the lipid are immobilized by the enzyme. This titration, shown in Fig. 3, demonstrates that a portion of the cardiolipin was rendered NMR-invisible after addition of 3 mg/ml (the maximum concentration in the NMR tube) and in the presence of asolectin, F,F, demonstrated a low specific activity (0.9 μmol/min/mg of protein) which was 73% oligomycin-sensitive; the stimulation of activity by asolectin was 5-fold under these conditions. If the assay mixture containing high amounts of protein was adjusted to contain 1% Triton X-100 there was no change in either the activity or the oligomycin sensitivity, which indicated that the detergent at this concentration had little effect on the catalytic properties of the enzyme. The above observations demonstrate that the enzyme retains its native properties in the presence of 1% Triton X-100.

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The above experiment suggested that only cardiolipin was immobilized by the enzyme in the presence of 1% Triton X-100. In contrast, no portion of either PC or PE was NMR-invisible, suggesting that the enzyme may bind cardiolipin more tightly than other phospholipids. To test this possibility the gradient-purified ATPase was combined with other phospholipids. As demonstrated in Fig. 5, there is no evidence of any immobilization of PC, even at a ratio of 10 mol of PC/mol of enzyme. The magnitude of the NMR signal before and after addition of SDS was not significantly different in either of two separate experiments. As in previous measurements...
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**FIG. 3.** Titration of gradient-purified ATP synthase with cardiolipin. The acquisition parameters were as shown in Fig. 1 and 2000 transients were accumulated for each spectrum. Sample conditions were as in Fig. 2. Activities of the enzyme were monitored periodically during the experiment as described in Fig. 2. Spectra were obtained after addition of 0, 1, 2, 3, 4, 5, and 10 mol of cardiolipin (DPG) per mol of ATP synthase, and after addition of SDS (1% final concentration).

**FIG. 4.** SDS denaturation of the 38-45p ATP synthase preparation. The acquisition parameters were as designated in Fig. 1 and 10,000 transients were accumulated for each spectrum. Sample conditions and activity measurements were as in Fig. 2. The spectrum demonstrating the cardiolipin (DPG) signal of higher magnitude (upper tracing) was obtained after addition of SDS (1% final concentration) to denature the enzyme.

**FIG. 5.** 31P NMR spectrum of the gradient-purified ATP synthase combined with phosphatidylcholine. The acquisition parameters were as designated in Fig. 3. Sample conditions and activity measurements were as in Fig. 2. Spectra were obtained after addition of 0, 1, 2, 3, 4, 5, and 10 mol of PC per mol of ATP synthase, and after addition of SDS (1% final concentration).

The signal for cardiolipin was noted when the enzyme was denatured by addition of detergent.

Since cardiolipin is an acidic phospholipid the gradient-purified enzyme was analyzed in the presence of another acidic species, phosphatidyl serine. Spectral analyses were carried out in the presence of PS, added at a concentration of 10 mol/mole of ATPase complex. The spectra obtained before addition of SDS demonstrated a strong signal for PS, but no signal for cardiolipin. Upon addition of SDS a prominent signal for cardiolipin was noted, but no increase in the magnitude of the resonance for PS was observed (data not shown). This observation suggests strongly that no PS was immobilized by the gradient-purified ATPase.

Spectral analyses of phosphatidyl glycerol in the presence of the gradient-purified ATPase were also carried out since PG is a precursor molecule to cardiolipin and is also an acidic phospholipid. This lipid, added at a concentration of 10 mol/mole of ATPase complex, provided a strong signal which was not increased when the enzyme was irreversibly denatured by addition of SDS (data not shown). Again, a prominent signal for cardiolipin appeared when SDS was added. These data indicate that the lipid-depleted ATPase did not immobilize the added PG.

**DISCUSSION**

The gradient-purified ATPase preparation utilized in these studies is considerably more lipid-depleted than other FoF1 preparations employed in studies of the interactions of the ATP synthase with phospholipids (5, 14). On the average only 2.6 mol of cardiolipin remained in gradient-purified FoF1, whereas the observations from the cardiolipin titration of the gradient-purified preparation (Fig. 3) and NMR analysis of the 38-45p ATPase (Fig. 4) indicated a stoichiometry of 4 mol of tightly bound cardiolipin/mol of the enzymatically active FoF1 complex. Apparently the purification procedure removed a portion of the cardiolipin which when added back to the enzyme bound tightly enough to be NMR-invisible.
Most of the residual endogenous phospholipid in the gradient-purified F_{0}F_{1} was the tightly associated cardiolipin. The 1 mol each of PC and PE per mol of enzyme were beyond the detection limits of the spectrometer in any reasonable time frame so it was not possible to determine whether they were tightly bound to F_{0}F_{1} with the amounts of enzyme available. NMR analyses of the 38-45p preparation of the ATPase did not reveal any reproducible increase in the magnitudes of the PC and PE signals upon denaturation of the enzyme with 1% SDS, but a release of only 1 mol of each may have been beyond the limits of sensitivity since the small increase in signal would have been added to an appreciable preexisting signal for these two phospholipids. At this point it is uncertain whether the small amounts of PC and PE remaining with the gradient-purified enzyme are tightly associated and/or required for maintenance of catalytic capacity.

The tightly bound cardiolipin appears to be associated with the ATPase only when the enzyme has not been irreversibly denatured. However, at this point we cannot rule out the possibility that cardiolipin could be associated tightly enough with the irreversibly inactivated enzyme to render it NMR-invisible. Both the denaturants utilized could have disrupted hydrophobic interactions between cardiolipin and the enzyme sufficiently (28) to allow the phospholipid to partition into the detergent environment of the enzyme (1% Triton X-100). Under these conditions denaturation and release of the cardiolipin could have been two separate events, neither being dependent on the other.

The observation that approximately 4 mol of cardiolipin/mol of F_{0}F_{1} associate tightly enough to be NMR-invisible suggests the possibility of four high affinity sites on the enzyme surface for cardiolipin. Further evidence supporting this view was the observation that no other phospholipids tested were immobilized by the enzymatically active enzyme. This indicates strongly that in the native enzyme there are approximately four sites which have a unique and high affinity for cardiolipin. The role of cardiolipin in the functioning enzyme has not been defined, however. It has been previously demonstrated that this phospholipid, when added back to the 38-45p preparation, elicits more dramatic alterations on the catalytic properties of the ATPase complex (7). The prevailing evidence (29–31) indicates that these effects are mediated by the F_{1} portion of the ATPase complex. Therefore, these high affinity sites for cardiolipin may exist on one or more of the F_{0} subunits. If this is the case, the tightly bound cardiolipin molecules are unlikely to be involved directly in catalytic events. It is more likely that these phospholipids participate in stabilizing those conformations of the enzyme that are required for expression of activity. It is recognized that these conformations will also be modulated by other effectors of the enzyme complex.

The observations in this study with the F_{0}F_{1}-ATPase are similar to those made by Beyer and Klingenberg (20) with the adenine nucleotide carrier. Utilizing essentially the same approach they were able to demonstrate tight binding of cardiolipin to the carrier and were unable to demonstrate any appreciable association of PC and PE with the detergent-solubilized protein. However, they did not observe any mobilization of cardiolipin from the adenine nucleotide carrier in the presence of 1% SDS. In contrast, this concentration of detergent resulted in release of approximately 4 mol of NMR-invisible cardiolipin from the 38-45p preparation of the ATPase. Presumably the cardiolipin associated with the adenine nucleotide carrier that is present in the 38-45p preparation was not released upon addition of SDS since the stoichiometry for tightly associated cardiolipin in this latter preparation was very similar to the value obtained with the gradient-purified F_{0}F_{1} in the presence of added cardiolipin.

As mentioned earlier, the inner mitochondrial membrane contains enzymes or carriers that bind cardiolipin tightly and/or require it for maximal catalytic capacity. These include cytochrome oxidase (15, 16), glycerol-3-phosphate dehydrogenase (19), the phosphate transporter (17, 18), and the adenine nucleotide carrier (20). Evidence has also been provided that cardiolipin comprises the binding site on the inner membrane for the mitochondrial form of creatine phosphokinase (32). These observations, in addition to those made in the present investigation with the ATP synthase, suggest that cardiolipin may be an integral component of several enzymes and/or carriers associated with the inner mitochondrial membrane. However, it is not clear at this time whether all these proteins have high affinity sites for cardiolipin. Apparently this is the case with cytochrome oxidase (15, 16), the adenine nucleotide carrier (20), and the F_{0}F_{1}-ATPase complex (this study). More studies will be necessary to determine if the cardiolipin associated with these high affinity sites is important in maintaining the catalytic capacity of these mitochondrial components.

The concept that there are a few high affinity sites on mitochondrial membrane components suggests there are specific areas on the surface of these proteins that interact with cardiolipin via both hydrophobic and ionic interactions. Since cardiolipin exhibits a strong negative charge (11) and is in both leaflets of the bilayer (33) it is likely that any binding site for cardiolipin on a polypeptide would contain a positively charged residue oriented close to either the cytoplasmic or matrix surface of the inner membrane. With respect to the transmembrane enzymes and carriers, it is possible that other inner membrane phospholipids interact with the polypeptides, but remain more freely exchangeable with bulk phase lipids. In addition to PC and PE, any cardiolipin not tightly bound to high affinity binding sites on polypeptides would presumably interact with lowered affinity and thus exchange with bulk phase phospholipids at the protein surface more readily. Therefore, it is possible that there are populations of cardiolipin in the inner membrane: that which is tightly associated to specific binding sites on polypeptides and that which is randomly distributed throughout the bilayer. However, if there are several membrane-associated proteins with specificity for cardiolipin, it is likely that there is little free cardiolipin in the inner membrane.

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