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ADP/ATP Carrier Protein from Beef Heart Mitochondria Has High Amounts of Tightly Bound Cardiolipin, As Revealed by ^{31}P Nuclear Magnetic Resonance†

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ABSTRACT: An unusual binding of cardiolipin to the ADP/ATP carrier has been found, which is distinguished by the relatively large amount and by the tightness of binding. High-resolution ^{31}P NMR studies on the detergent-solubilized ADP/ATP carrier from beef heart mitochondria revealed narrow signals from phosphatidylcholine and phosphatidylethanolamine and a broadened signal of 30–40-Hz line width, suggestive of cardiolipin. Line broadening of this magnitude is to be expected when tumbling of the whole protein–detergent micelle is the only source of phosphorus spin–spin relaxation. Thus a strong immobilization of the protein-bound cardiolipin is inferred. By sucrose density gradient centrifugation phosphatidylcholine and phosphatidylethanolamine were removed, while approximately six ± one molecules of cardiolipin remained tightly bound in the dimeric protein molecule. The cardiolipin binding was stable against treatment with sodium dodecyl sulfate although release of the inhibitor carboxyatractyloside revealed at least partial protein denaturation. Ca^{2+} ions did not readily interact either with the bound cardiolipin. Complete detachment of the bound phospholipid was achieved by a short heat pulse in the presence of sodium dodecyl sulfate. Denaturation of the carrier protein by guanidinium chloride or $NaClO_4$ also led to release of the bound phospholipid. Thus different stages of protein denaturation must be envisaged.

One of the fundamental problems in biomembrane protein function is the protein–phospholipid interaction. However, the demonstrations of this interaction have been controversial, especially with respect to the specificity to particular phospholipids. A tight binding of phospholipids in isolated membrane proteins would be a most convincing case for a specific phospholipid–protein interaction.

We shall report an unusually high and tight binding of cardiolipin in the isolated ADP/ATP carrier, which represents the highest specific phospholipid binding yet known. The ADP/ATP carrier of the inner mitochondrial membrane constitutes about 9–10% of the total mitochondrial protein. The large abundance as well as the relatively low molecular weight of this dimeric intramembrane protein (67 000) implies that it contributes strongly to the total lipid–protein interaction in the inner mitochondrial membrane.

In a preceding NMR[1] study the interaction of the carrier protein with phospholipids was investigated after reincorporation into phospholipid membranes of defined composition (Beyer & Klingenberg, 1983). It was found that the transmembrane protein causes only subtle perturbations in the bulk phospholipid phase.

In the present work lipid–protein interaction was studied by ^{31}P NMR in detergent micelles instead of recombined membranes. This constitutes the first NMR study of a phospholipid–protein binding in an isolated membrane protein micelle. Phospholipids solubilized in detergents exhibit narrow ^{31}P NMR signals due to the fluidity of mixed micelles (London & Feigenson, 1979; Dennis & Plückthun, 1984). In a detergent-solubilized protein, interaction of a lipid with the protein surface should lead to relaxation enhancement and, correspondingly, to line broadening. This provides a new method for investigating specific interactions of phospholipids

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[1] Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance; CAT, carboxyatractyloside; EDTA, ethylenediaminetetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; PI, phosphatidylinositol; TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate.

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with solubilized membrane proteins.

Materials and Methods

Chemicals. Triton X-100 was obtained from Rohm and Haas and sodium dodecyl sulfate from Sigma. Beef heart cardiolipin was obtained from Sigma. Egg yolk phospholipids were prepared as described previously (Beyer & Klingenberg, 1978; Wells & Hanahan, 1969). [^{3}H]Sucrose was obtained from Amersham.

Sample Preparation and Analytical Methods. The CAT–carrier protein complex from beef heart mitochondria was isolated in Triton X-100 as described earlier (Klingenberg et al., 1979). Pressure dialysis was performed on an Amicon PM-10 ultrafiltration membrane. For removal of excess phospholipid 1 mL of the pressure-dialyzed solution containing typically 10–30 mg of protein was applied to a linear 5–20% w/v sucrose density gradient including 100 mM NaCl, 10 mM MOPS, 0.05 mM EDTA, 0.05 mM NaN_3, and 0.5% w/v Triton X-100, pH 7.2. The gradient was tested for linearity by addition of [^{3}H]sucrose. Centrifugation was performed in an 50 Ti vertical rotor for 24 h at 45 000 rpm. The protein fractions were collected and dialyzed 3 times against 700 mL of the centrifugation buffer without sucrose, followed by a second pressure dialysis. D_2O (10% v/v) was added to the final samples for field/frequency stabilization of the NMR instrument.

Prior to phospholipid analysis the protein solution was lyophilized and excess Triton X-100 was extracted by use of carefully dried acetone as described by Krämer (1977). The residue was dissolved in methanol–chloroform (2:1 v/v) and applied to TLC plates.

Protein was determined by the method of Lowry et al. (1951) and phosphorus by the method of Chen (1956). The concentration of Triton X-100 was measured photometrically at 274 nm.

NMR and ESR Measurements. ^{31}P NMR experiments were performed with a Bruker SXP 4-100 spectrometer operating at a ^{31}P frequency of 36.4 MHz. Broad-band decoupling was applied at a power output of about 0.15 mT. The pulse repetition rate was 0.2 s^{-1} and the pulse angle was $\simeq$70°. The sample temperature during spectral acquisition was 9–10 °C. ESR spectra were recorded on a Bruker ES 200 D-A spectrometer.

Results

^{31}P NMR of Phospholipids in the Solubilized Carrier. The ADP/ATP carrier protein was isolated as a CAT–protein complex from beef heart mitochondria preloaded with CAT. After the final purification step by gel chromatography on an ACA 34 column, the protein concentration is typically 0.7–1.0 mg/mL. At this stage of purification the preparation contains about 0.2–0.3 mM phospholipid phosphorus (Hackenberg & Klingenberg, 1980). Due to the low phospholipid concentration direct ^{31}P NMR analysis of the purified carrier protein was not feasible. Hence the protein solution was pressure dialyzed to a final protein concentration of 15–20 mg/mL. The total phosphorus concentration in these solutions of about 4–6 mM was sufficient for the correct determination of chemical shifts and line widths of the phospholipid ^{31}P NMR signals after 1 h of signal averaging.

In Figure 1 the ^{31}P NMR spectrum of the Triton X-100 solubilized CAT–protein complex is compared with the spectrum of a standard lipid mixture (5 mg of PC, 5 mg of PE, and 2.5 mg of CL in 8% Triton X-100). Peak assignment in Figure 1 is identical with the assignments made by London & Feigenson (1979). The small difference in Triton X-100 concentrations in the protein solution and in the standard is not critical, since the Triton X-100 concentration had virtually no effect on the ^{31}P chemical shifts in phospholipid solutions containing from 4% to 20% Triton X-100.

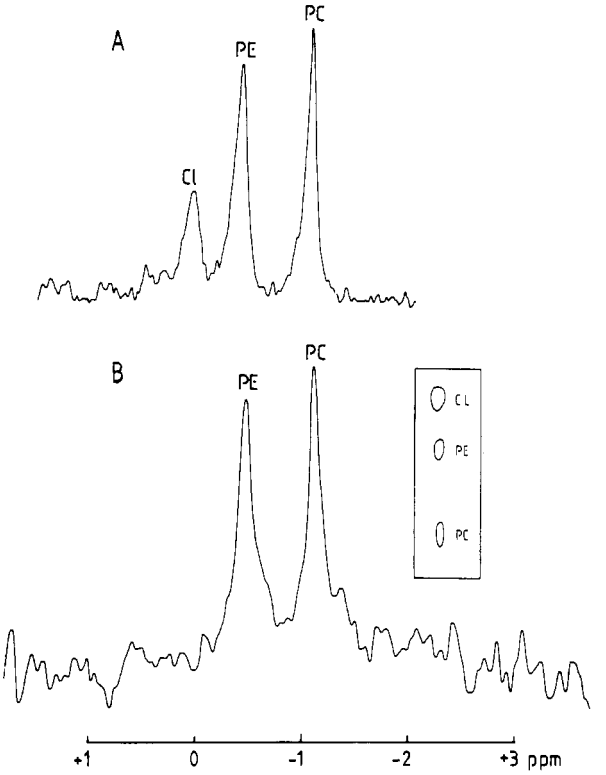

FIGURE 1: ^{31}P NMR spectra of (A) 5 mg of PC, 5 mg of PE, and 2.5 mg of CL in 1 mL of 7.3% w/v Triton X-100 and (B) 8.4 mg of CAT–protein in buffer containing 8.9% Triton X-100, pH 7.2, at 9.5 °C. Chemical shifts are relative to 85% H_3PO_4 in an external capillary. Insert: TLC of a chloroform + methanol extract of the lyophilized CAT–protein. Solvent system: chloroform–methanol–water–acetone–acetic acid (6:2:1:8:2 v/v).

Only signals from PC and PE are visible in the ^{31}P NMR spectrum of the concentrated protein preparation. On the other hand, TLC and phosphorus determination in the TLC spots revealed the presence of PC, PE, and CL in the molar proportion 1.0/0.95/0.8. A minor spot from PI was not evaluated. In the ^{31}P NMR spectrum PI is not detectable, presumably due to the low concentration of this lipid in the solubilized protein.

The absence of a narrow CL signal in the ^{31}P NMR spectra seems to be due to very tight binding to the carrier protein and consequent broadening of the CL signal. An alternative cause, i.e., CL signal quenching by paramagnetic metal ion binding, could be easily excluded, since addition of a large excess of EDTA did not lead to the appearance of a narrow CL line in the spectrum. Also ESR spectroscopy failed to reveal a paramagnetic species, even in the most concentrated protein solutions.

The assumption that CL binds tightly to the Triton X-100 solubilized carrier protein was corroborated by centrifugation of the protein preparation in a sucrose density gradient (Figure 2). The two major fractions containing the solubilized protein and protein-free Triton X-100–phospholipid mixed micelles were collected as indicated in Figure 2. After removal of sucrose by dialysis and volume reduction by pressure dialysis, CL was detected exclusively in the protein fractions by TLC analysis whereas PC and PE were found in the protein-free mixed Triton X-100 micelle fraction migrating at lower buoyant density. ^{31}P NMR spectra and TLC plates of the two

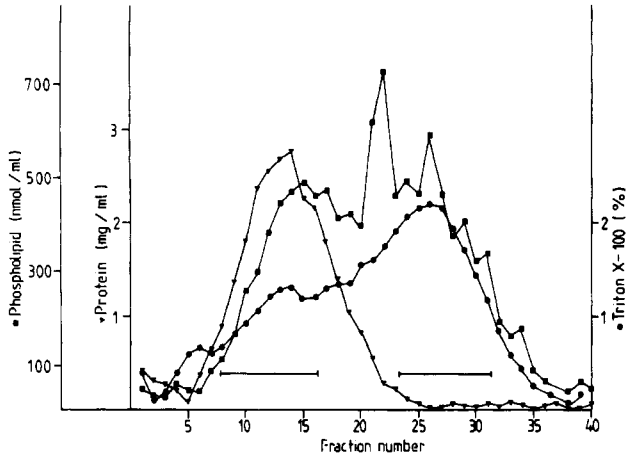

FIGURE 2: Centrifugation of the CAT–protein complex in a linear 5–20% sucrose density gradient. A total of 28 mg of the complex in 1 mL of buffer, containing 22% w/v Triton X-100, was layered on the top of the gradient, followed by 1 mL of the gradient buffer. The gradient buffer contained 0.5% w/v Triton X-100. The sample was centrifuged in an Omega 50 Ti vertical rotor for 24 h at 45 000 rpm.

fractions are shown in Figure 3. After 1000 transients PC and PE signals appear in the nonprotein fraction (Figure 3A). After the same accumulation time in the protein fraction only traces of PC and PE are detectable. There is no narrow signal corresponding to CL. Only after extensive signal averaging does the CL resonance show up as a broad peak of about 30 ± 5 Hz line width (Figure 3B).

Estimation of CL Mobility. An upper limit of the line broadening to be expected in the case of CL binding to the Triton X-100–protein micelle may be obtained by neglecting all internal motions of the complex. The rigid body tumbling rate can be calculated from the Stokes–Einstein relation by use of the diameter of the protein–detergent micelle as determined earlier by ultracentrifugation (Hackenberg & Klingenberg, 1980):

$$\tau_c = 4\pi\eta R^3/3kT$$

(η, viscosity of the medium; R, radius of the mixed micelle; k, Boltzmann constant; T, temperature). With η_{H_2O} = 1.347 cP at 9 °C (*Handbook of Chemistry and Physics*, 1969) and R = 65 Å, the rotational correlation time of the micelle is $\tau = 4.0 \times 10^{-7}$ s.

The observed line broadening $\Delta\nu$ most probably results from both dipolar phosphorus–protein interaction and chemical shift anisotropy relaxation. The contribution from the dipole term is difficult to assess, since the intramolecular proton–phosphorus distances in the bound CL molecule are not known. Moreover, intermolecular dipole interaction and cross-relaxation cannot be excluded. Neglecting these effects an order of magnitude calculation of the expected line width may be performed, assuming proton–phosphorus internuclear distances of 2.6–3.2 Å for the four nearest-neighbor methylene protons according to the corresponding values in the crystal structure of glycerophosphocholine (Abrahamson & Pascher, 1966). Under the simplifying conditions assumed here (isotropic motion; four nearest-neighbor protons at equal distances; $\omega^2\tau_c^2 \gg 1$, where ω is the Lamor frequency of the phosphorus nuclear spin), the line broadening $\Delta\nu$ follows from (Abragam, 1961; Hull & Sykes, 1975)

$$\pi\Delta\nu = 1/T_{2,DIP} + 1/T_{2,CSA} = \left[\frac{4}{5}\frac{\gamma_H^2\gamma_P^2\hbar^2}{r_{HP}^6} + \frac{2}{15}\omega^2\sum_i(\sigma_{ii} - \sigma_{av})^2\right]\tau_c$$

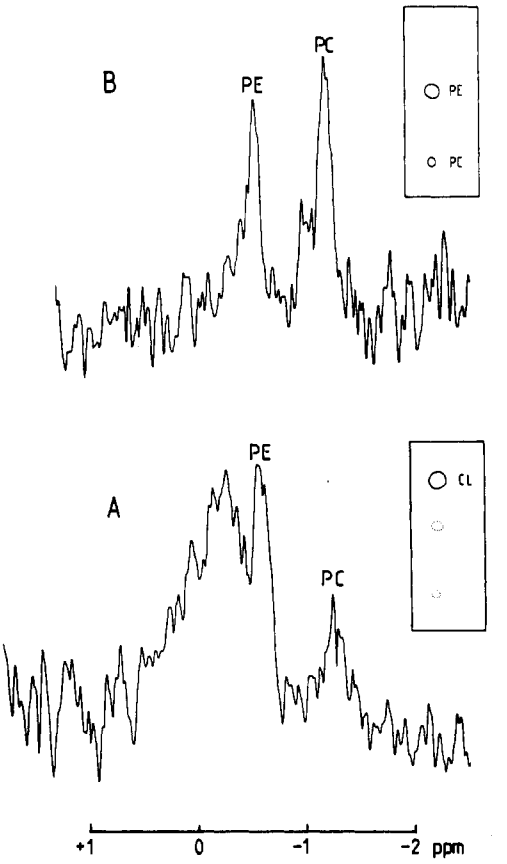

FIGURE 3: ^{31}P NMR spectra of the concentrated (1-mL) gradient fractions as indicated in Figure 2: (A) protein fraction, 13.3 mg of protein/mL, 5600 transients; (B) low-density lipid fraction, 1000 transients. Inserts: TLC of the two fractions. For details, see Figure 1.

where $1/T_{2,DIP}$ and $1/T_{2,CSA}$ are the dipolar and chemical shift anisotropy contributions to the spin–spin relaxation rate, γ_H and γ_P are the gyromagnetic ratios for the ^{1}H and ^{31}P nuclei, r_{HP} is the proton–phosphorus distance, σ_{ii} are the principal values of the ^{31}P chemical shift tensor, $\sigma_{av} = (1/3)\sum_i\sigma_{ii}$, and i = 1, 2, or 3.

In the above expression the dipolar term yields line broadenings of 8.3 and 30.7 Hz, respectively, for the limiting ^{1}H–^{31}P distances (3.2 and 2.6 Å). With the principal values of the ^{31}P shielding tensor as given by Herzfeld et al. (1978) and by allowance for the applied field strength of 2.115 T, the line broadening due to chemical shift anisotropy relaxation was found to be 17 Hz. The overall line widths of 25.8–47.7 Hz obtained from this simple estimate are in the same order of magnitude as the line broadening observed experimentally.

Quantitation of CL Content. The total amount of phospholipid cosolubilized with the carrier as well as the amount of CL associated with the carrier protein was determined routinely by phosphorus analysis according to Chen (1956) in protein preparations after pressure dialysis and in the protein fraction after sucrose density centrifugation. A substantial contribution to the analytical values from inorganic phosphorus seems very unlikely, since there was no ^{31}P NMR signal due to phosphate in the carrier solutions before and after protein denaturation. The phosphorus/protein ratios varied when the samples had not been subjected to sucrose density gradient centrifugation. After gradient centrifugation the amount of phospholipid phosphorus was in the range of 0.18–0.25 μmol/mg of protein. These values are slightly lower than the ratios reported in an earlier paper (0.28 ± 0.04 μmol of phosphorus/mg of protein; Hackenberg & Klingenberg, 1980).

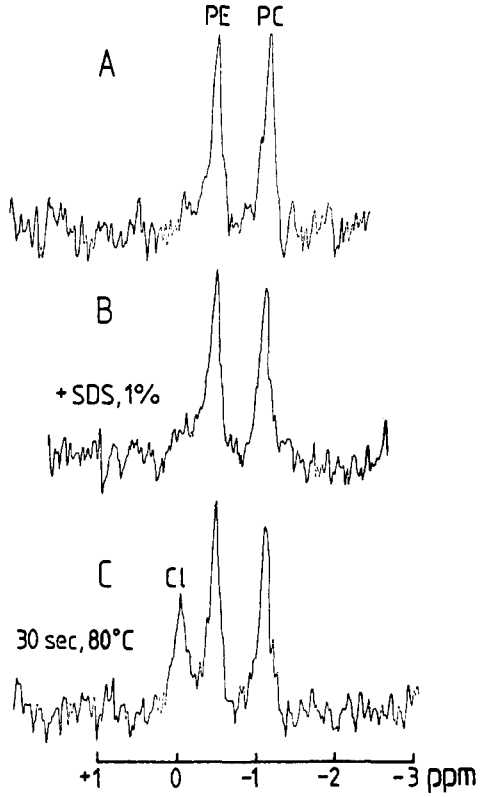

FIGURE 4: Release of bound CL by protein denaturation: (A) 10.0 mg of protein in 900 μL of buffer containing 11.1% w/v Triton X-100; (B) same sample after 4 days in the presence of 1% w/v SDS; (C) same sample after heating to 80 °C for 30 s. A total of 2000 transients were accumulated at 9.5 °C in spectra A–C.

When the analytical ratios in the centrifuged samples were above 0.22 μmol of phosphorus/mg of protein, small signals from PE and PC appeared in the ^{31}P NMR spectra. Thus our data indicate that the CL phosphorus bound to the ADP/ATP carrier amounts to about 0.19 ± 0.03 μmol/mg of protein. Assuming that all the protein in the samples is ADP/ATP carrier, this corresponds to 6 ± 1 mol of CL/mol of carrier protein when the known molecular weight of the protein dimer is taken into account (Aquila et al., 1982).

Gradual Release of CL on Protein Unfolding. The tight binding of CL to the carrier protein suggests that this phospholipid interacts strongly with the protein surface. It can be assumed that the CL–protein interaction is mainly of electrostatic origin. Thus an attempt was made to weaken this interaction by charged perturbants. First, the negatively charged detergent SDS was added to the Triton X-100 solubilized protein. It may be expected that SDS competes for CL binding sites, eventually resulting in partial displacement of the bound CL and, accordingly, in a narrow ^{31}P NMR signal from CL solubilized in detergent micelles. This effect should be differentiated from SDS-induced protein denaturation.

Contrary to the expected SDS–CL competition, the CL–protein interaction proved to be quite stable against SDS, as shown in Figures 4 and 5. In the experiment depicted in Figure 4, the CAT–protein complex was incubated at 5 °C for 4 days with 1% SDS. The presence of excess Triton X-100 in this experiment prevents SDS from crystallization. The ^{31}P NMR spectrum after this treatment is nearly identical with the spectrum before SDS addition. However, after a short heat pulse (80 °C, 1 min) a narrow signal appeared at the expense of the broad hump mentioned above (see Figure 4). The assignment of the new signal to CL was confirmed by

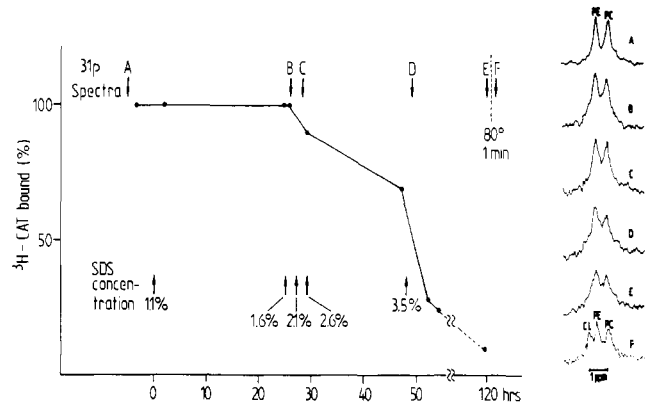

FIGURE 5: Effect of SDS on the binding of [^{3}H]CAT and CL to the solubilized ADP/ATP carrier. The concentrations of protein and Triton X-100 were 41 mg/mL and 22% w/v, respectively. The [^{3}H]CAT binding was determined by gel chromatography of small (20- or 40-μL) samples taken from the protein solution (≃1 mL at the beginning of the experiment) on a 1 × 10 cm Sephadex G75 column. Additions of SDS and the start of data acquisition (1200 scans for each spectrum) are indicated by arrows. The temperature between NMR experiments was 4 °C and during spectral accumulation 10 °C.

addition of commercial CL from beef heart. A further increase in intensity of the CL signal could not be obtained by incubation of the sample at the elevated temperature. Obviously, the CL release was complete after the first heating.

It may be argued that the new signal in Figure 4C is due to partial formation of lysophospholipids, since removal of the *sn*-2 acyl chain in phospholipids leads to a downfield shift in the ^{31}P NMR spectrum by about 0.4–0.5 ppm (Plückthun & Dennis, 1982). However, the integrated intensities of PC and PE signals remained unchanged after the heat pulse, indicating that the signal was not caused by phospholipid degradation. During the heat step the solution became turbid due to the precipitation of denatured protein. Interestingly, short heat treatment in the absence of SDS was not sufficient to liberate the protein-bound CL although protein precipitation occurred (spectra not shown).

The inability of SDS to displace the carrier-bound CL certainly does not rule out protein denaturation caused by the ionic detergent. Rapid loss of CAT binding results when solubilization of the CAT–protein complex is attempted in anionic detergents such as SDS or sodium cholate (Riccio et al., 1975). To keep control of the protein intactness, SDS was added stepwise to isolated carrier protein labeled with [^{3}H]-CAT. The change in CAT binding and the corresponding ^{31}P NMR spectra were followed simultaneously over 50 h (see Figure 5). In the presence of 1.1% SDS the CAT binding is stable against denaturation for at least 30 h. Partial [^{3}H]CAT release occurs when the SDS concentration is raised to about 2% w/v. The ^{31}P NMR spectrum shows that the inhibitor release is not accompanied by the detachment of bound phospholipid, indicating that the release of either CAT or CL represents different levels of protein denaturation.

It must be noted that in this experiment the Triton X-100 and protein concentrations were much higher than in the experiment shown in Figure 4. In the concentrated Triton X-100 solution the addition of SDS probably leads to the formation of nonspherical micelles or aggregated micelles (Wennerström & Lindman, 1979). The broadening of the ^{31}P NMR signals in spectra B–F of Figure 5 may be due to this SDS-induced micelle growth.

In another attempt to detach the carrier-bound CL, the effect of Ca^{2+} addition was investigated. CL immediately precipitates when 5 mM $CaCl_2$ is added to a solution of 5 mg

of the phospholipid in 8% w/v Triton X-100. Under high-resolution conditions (600-Hz spectral window) the ^{31}P NMR signal of the CL–Ca^{2+} complex is broadened beyond detectability. Full restoration of the CL signal can be achieved by addition of excess EDTA. No phospholipid precipitation occurred in the protein solution upon Ca^{2+} addition in contrast to detergent-solubilized CL. After incubation of the protein with 5 mM $CaCl_2$ at 4 °C for 20 h, addition of EDTA resulted in the appearance of a small CL signal (spectra not shown). This signal increased by about 70% when 1% SDS was added at room temperature. Thus, during the Ca^{2+} incubation only 30% of the protein-associated CL interacted with Ca^{2+} whereas about 70% was inaccessible to Ca^{2+} ions. Subsequent addition of 1% SDS led to full CL release, suggesting that Ca^{2+} destabilizes the carrier protein so that the phospholipid is no longer protected against replacement by SDS molecules.

From these findings it follows that CL release requires rather harsh conditions. This was further elaborated by denaturing the carrier protein with $NaClO_4$ or guanidinium chloride (^{31}P NMR spectra not shown). When 2 M $NaClO_4$ was added to the concentrated protein solution, the high ionic strength and the chaotropic nature of perchlorate resulted in rapid protein aggregation and, at the same time, in a narrow ^{31}P NMR signal from CL. After centrifugation of the aggregated protein the supernatant contained virtually the total phospholipid including CL but less than 10% of the protein. It must be assumed that virtually all CL was liberated immediately upon addition of sodium perchlorate since no change was observed in the integrated intensity of the CL signal after storage of the perchlorate-treated sample for 24 h at 4 °C.

Addition of 6 M guanidinium chloride also led to a narrow CL signal in the phosphorus spectrum. In this case the protein did not aggregate, not even after 24 h. However, in contrast to $NaClO_4$, guanidinium chloride caused an increase in the intensity of the CL signal by about 35% within 24 h, suggesting that unfolding of the carrier protein by guanidinium chloride is a rather slow process.

Discussion

The present study indicates that about six CL molecules are strongly bound in the isolated ADP/ATP carrier. This lipid–protein interaction obviously resists the high Triton X-100 concentrations obtained by centrifugation of the complex in a Triton X-100 containing sucrose density gradient and after ultrafiltration.

The high content of positively charged amino acids in the ADP/ATP carrier (Aquila et al., 1982) and, in particular, the indication of a collar of lysine groups opposing negatively charged phospholipid head groups in the membrane (Bogner et al., 1982) suggest that the protein surface provides a sufficiently large number of potential binding sites for cardiolipin. Moreover, the earlier observation that negatively charged lipids stimulate the transport activity in the reconstituted carrier (Krämer & Klingenberg, 1980; Brandolin et al., 1980) indicates that neutralization of positive amino acid charges is a prerequisite for proper function of the carrier.

The finding that protein-bound CL is not readily displaced by a large excess of SDS or Ca^{2+} ions underscores the tight binding of CL to the protein. This agrees with the observation that the SDS-induced release of [^{3}H]CAT clearly precedes the release of CL (cf. Figure 5). The inhibitor binding site seems to be more sensitive to early stages of denaturation than the binding of CL.

The ^{31}P NMR line widths of PC and PE were never significantly different in Triton X-100 solution and in the protein preparations. This indicates that, in contrast to CL, PC and PE are not immobilized by lipid–protein contact. Most probably, the neutral lipids are partitioned among detergent micelles in the final protein solution. Excess CL solubilized in Triton X-100 micelles seems to have been lost during the purification procedures (cf. Figure 1). The variation in the amounts of PC and PE copurified with the carrier protein may be due to slight differences in the quality of the hydroxylapatite used in the first step of protein enrichment.

These results may be compared to earlier reports on binding of CL and on its importance for full electron transport activity in several respiratory enzymes of the inner mitochondrial membrane (Awasthi et al., 1971; Fry & Green, 1980, 1981; Robinson et al., 1980). Notably in cytochrome oxidase two to three molecules of CL are tightly bound to the protein complex. The importance of CL binding to the ADP/ATP carrier is particularly striking, if one compares the ratios of bound phospholipid per milligram of protein. Thus, in the carrier, CL binding is 10–20 times higher than in cytochrome oxidase.

In NMR studies of natural or reconstituted membranes, distinct spectral components from mobile and immobilized phospholipids are generally not observable. This is usually traced to the exchange of lipids in the "boundary layer" and in the bulk phase, which is rapid on the NMR time scale. There have been reports by one group that in sarcoplasmic reticulum membranes (Yeagle, 1982), in bovine retinal rod outer segments disks (Albert et al., 1982), and in glycophorin/phospholipid reconstituents (Yeagle & Romans, 1981) a certain portion of the ^{31}P NMR intensity of the phospholipids is missing. Subsequently, however, improved NMR methods allowed the missing ^{31}P intensity to be visualized as a broadened resonance underlying the ^{31}P NMR spectrum of relatively unperturbed lipids (Albert & Yeagle, 1983). From these experiments it follows that in membranes the visualization of tightly bound phospholipids by ^{31}P NMR is rather difficult due to the restricted motion of both protein-bound and bulk phospholipid species.

In the high-resolution spectra presented here, the "missing" ^{31}P signal from CL is made visible by the transfer of the protein into a detergent micelle and appears as a broadened line of about 30–40-Hz line width at half-height (cf. Figures 1 and 3). This line broadening must be due to at least partial immobilization of the CL phosphorus in the detergent–protein–phospholipid mixed micelle. However, it is to be expected that in mitochondria the signal due to CL binding to the ADP/ATP carrier is also extremely broadened.

Acknowledgments

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^{1}H NMR Characterization of Metastable and Equilibrium Heme Orientational Heterogeneity in Reconstituted and Native Human Hemoglobin†

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ABSTRACT: A proton nuclear magnetic resonance study of the reaction of apohemoglobin A with both oxidized and reduced hemes reveals that at least two slowly interconverting species are initially formed, only one of which corresponds to the native proteins. Reconstitutions with isotope-labeled hemes reveal that the hyperfine-shift patterns for heme resonances in the metazido derivatives differ for the two species by interchange of heme environment characteristic of heme orientational disorder about the α,γ-meso axis, as previously demonstrated for myoglobin [La Mar, G. N., Davis, N. L., Parish, D. W., & Smith, K. M. (1983) *J. Mol. Biol. 168*, 887-896]. Careful scrutiny of the ^{1}H NMR spectrum of freshly prepared hemoglobin A (Hb A) reveals that characteristic resonances for the alternate heme orientation are present in both subunits, clearly demonstrating that "native" Hb A possesses an important structure heterogeneity. It is observed that this heterogeneity disappears with time for one subunit but remains unchanged in the other. This implies that a metastable disordered state in vivo involves the α subunit and an equilibrium disordered state both in vivo and in vitro is involved within the β subunit. The presence of metastable disorder in fresh blood suggests an in vivo hemoglobin assembly from apoprotein and heme that is similar to the in vitro reconstitution process. The slow equilibration and known lifetimes for erythrocytes provide a rationalization for the presence of detectable metastable states. The implications of such heme disorder for Hb function are discussed.

Human adult hemoglobin (Hb A)[1] is at present the best, albeit incompletely, understood allosteric protein in terms of its biosynthesis, structure, and function (Dickerson & Geis, 1983). It is frequently taken as the model protein on which to test hypothetical structure–function relationships because of the detailed knowledge of its properties. The X-ray structures have provided details on the unique structures for the two functional forms of the proteins as well as for numerous nonfunctional derivatives (Perutz, 1970, 1976; Fermi, 1975; Baldwin & Chothia, 1979). In each case, a single form of the protein is obtained, at least with respect to functional consequences. Rapid interconversions among various substrates differing only slightly from that of the ground-state structure are likely, but do not significantly alter the picture derived from X-ray diffraction (Karplus & McCammon, 1981).

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[1] Abbreviations: NMR, nuclear magnetic resonance; Hb A, human adult hemoglobin; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; ppm, parts per million; met-Hb, ferric hemoglobin; Mb, myoglobin.

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