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Reincorporation of Adenosine 5′-Diphosphate/Adenosine 5′-Triphosphate Carrier into Phospholipid Membranes. Phospholipid–Protein Interaction As Studied by Phosphorus-31 Nuclear Magnetic Resonance and Electron Microscopy†

Klaus Beyer* and Martin Klingenberg

ABSTRACT: Combined phosphorus-31 nuclear magnetic resonance (^{31}P NMR) and electron microscopic studies were performed on the ADP/ATP carrier protein from beef heart mitochondria. The protein was incorporated into phospholipids by addition of Triton–protein micelles to a lipid suspension or to the dry lipid. All of the phospholipid (egg phosphatidylcholine or mixtures of egg phosphatidylcholine and egg phosphatidylethanolamine) that contributed to the observed ^{31}P NMR signal under these conditions appeared to be in a bilayer configuration. Freeze–fracturing and negative-staining electron microscopy showed unilamellar vesicles and multilayers. An isotropic signal could be attributed to vesicle rotation, judging from its sensitivity to increasing viscosity. The presence of small vesicles was also noticeable in the ^{31}P NMR spectra of planar oriented membranes. In the presence of phosphatidylethanolamine, aggregation of protein particles was observed. Gel chromatography of the protein–Triton–phospholipid mixture revealed that, before Triton removal, large amounts of protein are associated with multibilayers. Separation of loaded and unloaded membranes by centrifugation in D_2O showed that, upon stepwise addition, protein incorporates preferentially into unloaded liposomes. From these findings a mechanism of protein reincorporation was deduced.

The ADP/ATP carrier protein from the inner mitochondrial membrane can be isolated in highly purified form (Riccio et al., 1975a,b). Incorporation of this protein into vesicular phospholipid membranes enhances the affinity for the inhibitor ligands (Krämer & Klingenberg, 1977) and reconstitutes the specific exchange capability for adenine nucleotides to a portion of the incorporated protein. It has been shown recently (Krämer & Klingenberg, 1980) that the transport activity of the ADP/ATP carrier, when incorporated into phospholipid vesicles, is considerably enhanced in the presence of phosphatidylethanolamine (PE).[1] It may be speculated that this effect is due to a specific interaction of PE with the protein molecule or to a structural rearrangement of the membrane that facilitates the function of the ADP/ATP carrier.

Nuclear magnetic resonance proved to be a valuable tool for the study of lipid–protein interactions (Seelig & Seelig, 1980). In particular ^{31}P NMR affords an insight into the order and mobility of the phospholipid head groups in artificial and

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[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; CAT, carboxyatractylate; Mops, 4-morpholinepropanesulfonic acid; NMR, nuclear magnetic resonance.

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biological membranes (Seelig, 1978). Furthermore, the ^{31}P NMR line shape carries information about the arrangement of phospholipid molecules in membranes due to the large chemical shift anisotropy of the phosphorus nucleus. The possible role of nonbilayer structures in biological membranes has been discussed on the basis of ^{31}P NMR (Cullis & DeKruijff, 1979). This possibility will be examined in the present study.

A small structural perturbation of the fatty acid moiety of several reconstituted membrane systems has been deduced from the quadrupole splitting of ^{2}H-labeled lipids (Seelig & Seelig, 1980). Changes in the effective ^{31}P chemical shift anisotropy in these systems were found to be rather small or even negligible (Rice et al., 1979a,b; Seelig et al., 1981). In the present study this NMR parameter seemed to be reduced, although an exact determination of the shift anisotropy was hampered due to the heterogeneity of the preparation.

Protein reincorporation experiments in the presence of dialyzable detergents such as bile acids or octyl glucoside have been performed frequently with other systems (Racker, 1979). However, the formation of detergent-free lipid–protein complexes by the dialysis method from Triton-solubilized integral membrane proteins is hampered by the low critical micelle concentration of this detergent. Triton may be partially removed by absorption to polystyrene beads (Holloway, 1973) resulting in integration of protein into vesicular lipid membranes (Gerritsen et al., 1978). This technique is inevitable in the case of the ADP/ATP carrier since this protein is best stabilized by Triton X-100 whereas other detergents proved to stimulate more or less rapid protein denaturation (Klingenberg et al., 1979b). Thus the mechanism of protein reincorporation by this method was investigated by electron microscopy, gel chromatography, and a centrifugation technique.

Materials and Methods

The CAT–carrier protein complex was isolated as previously described (Klingenberg et al., 1979a). In the final solution the concentrations of protein and Triton X-100 were 0.9–1.0 mg/mL and 4–5 mg/mL, respectively. Egg yolk phospholipids were isolated as described earlier (Krämer & Klingenberg, 1980; Beyer & Klingenberg, 1978). Triton X-100 was obtained from Sigma. ^{3}H-Labeled Triton X-100 was a gift from Rohm & Haas. Bio-Beads SM-2 were obtained from Bio-Rad and Amberlite XAD-2 beads from Sigma. Poly(vinylpyrrolidone) (Kollidon 60) was obtained from BASF.

Lipid–protein complexes for NMR experiments were prepared in two different ways. In method I, the protein reincorporation was performed in two steps. A total of 150 mg of phospholipid was deposited in a flask from chloroform solution and thoroughly dried under high vacuum. This lipid film was dispersed in 4 mL of buffer containing 200 mM NaCl and 10 mM Mops, pH 7.2. About 12 mg of Triton-solubilized CAT–protein in 12 mL of the same buffer was added corresponding to a molar ratio of phospholipid to protein of 1000:1. The mixture was incubated for 30 min at 4 °C. Triton was removed by gentle shaking of the suspension with 4.3 g of wet polystyrene beads for 1 h. After removal of the beads, the suspension was centrifuged at 100000*g* for 1 h. The pellet was resuspended in 4 mL of buffer, another 12 mL of the protein solution was added, and after incubation, the bead treatment was repeated.

In method II, protein reincorporation was achieved in one step. In contrast to method I, the protein solution was added to the dry phospholipid. In a typical experiment 70 mg of phospholipid was dispersed in 18 mL of Triton-solubilized protein corresponding to a molar ratio of phospholipid to protein of 300:1. Triton was removed by treatment with 6.3 g of wet beads.

In both methods the suspensions remained turbid after protein addition, indicating that the mixtures were not completely transformed into mixed micelles. In method I, the protein and phospholipid yield was typically 40–50%, and the molar ratio of phospholipid to Triton was 5–6. After method II, 60–80% of protein and phospholipid was recovered, and the molar ratio of phospholipid to Triton was 16–20. Protein was determined by the method of Lowry et al. (1951) and phosphorus by the method of Chen (1956).

For NMR analysis the dispersions obtained after the final Triton removal were centrifuged at 100000*g* for 1 h, and the pellets were adjusted with buffer to a total volume of 1.1 mL and a D_2O content of 20% (v/v).

For planar orientation of the protein-loaded membranes, the lipid–protein complex prepared according to method II was suspended in 1 mL of buffer and dialyzed against 50 mL of water for 2 h. Then it was partly dehydrated by a stream of nitrogen in a rapidly spinning NMR tube of 10-mm o.d. The water content of the sample was controlled by weighing from time to time. At a molar ratio of water to phospholipid of 40–50, the sample was pressed between 13 microscope cover slides. The stack was mounted to a NMR goniometer and inserted into a 6.5 mm o.d. quartz tube. For field/frequency stabilization this tube was immersed in D_2O contained in a 10 mm o.d. NMR sample tube. ^{31}P NMR spectra were recorded at 36.4 MHz on a Bruker SXP 4-100 spectrometer. All spectra were broad-band decoupled at a decoupling magnetic field strength of about 0.15 mT. The pulse repetition time was 1 s^{-1} and the pulse angle $\approx 70°$. Freeze–fracturing was done with Balzers freeze–fracturing apparatus. All samples were preincubated with 30% v/v glycerol at 0 °C for 1 h and subsequently frozen from room temperature in liquid Freon. Thin sectioning was performed after osmium fixation.

Results

NMR Spectroscopy. The CAT–protein–Triton complex was incorporated into phospholipid membranes by addition of the Triton-solubilized protein to a phospholipid suspension in two steps. After the first addition followed by Triton removal by Bio-Beads, the dispersion was spun down. The pellet was resuspended in buffer, and the same amount of protein was added and again excess Triton removed (method I, see Materials and Methods). In both steps the amount of Triton added with the protein was low (molar ratio of phospholipid to Triton 2:1 in the first step) so as to avoid complete transformation of the lipid suspension into a mixed micellar solution. This precaution seems to be indicated by the fact that the solubilized carrier protein is prone to self-aggregation upon rapid removal of Triton (Hackenberg, 1979).

The ^{31}P NMR spectrum of the resulting mixture clearly consisted of two components after incorporation into egg PC (Figure 1, upper spectrum). The broad spectral component in Figure 1 exhibits the anisotropic pattern found when phospholipids are arranged in the bilayer configuration (Seelig, 1978). In this case, the signal consists of a broad downfield shoulder and a sharp upfield peak due to the anisotropic nature of head group motions at the bilayer surface. In Figure 1, the broad phosphorus signal is superimposed by an additional narrow symmetric peak. The symmetric line is to be expected when isotropic motion averages the elements of the chemical shift tensor of the phosphorus atom (Seelig, 1978).

The total incorporated protein–phospholipid mixture could be fractionated by centrifugation in a medium containing D_2O, resulting in a floating upper layer, which contained most of

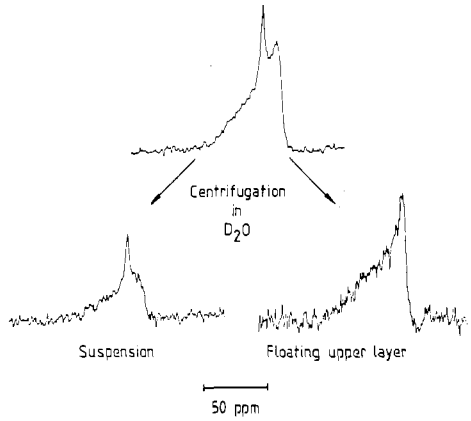

FIGURE 1: 36.4-MHz ^{31}P NMR spectra of egg PC after incorporation of carrier protein and of the suspension and the floating layer after centrifugation in D_2O at 11000*g* for 10 min. 24 mg of protein was added to 150 mg of phospholipid in two steps according to method I. 96% of the H_2O in the original preparation was exchanged for D_2O by dialysis against D_2O buffer. Temperature was 9 °C.

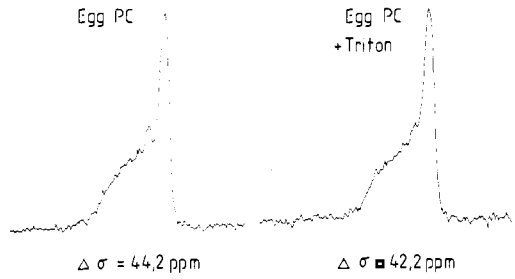

FIGURE 2: ^{31}P NMR spectra of dispersions of pure egg PC and of a mixture of egg PC and Triton X-100. Molar ratio of phospholipid to detergent is 5. The dry lipid or the lipid–detergent mixture was dispersed in buffer as described under Materials and Methods. Temperature was 9 °C. The values of the chemical shift anisotropy were obtained by comparison with computer-generated spectra.

the free phospholipid. The protein was present largely (87%) in the residual suspension where the protein to phospholipid ratio was increased 2-fold. The ^{31}P NMR spectra showed separation of the isotropic and anisotropic spectral components. The symmetric signal was present exclusively in the spectrum of the lower protein containing phase, whereas the upper phase exhibited only the typical asymmetric bilayer line shape (Figure 1, lower spectra).

When the protein was incorporated into a mixture of 60 mol % egg PC and 40 mol % egg PE by the same method, the narrow signal was less pronounced. Nevertheless, the shape of each signal component was virtually indistinguishable from the corresponding signal shapes of the preparation containing only PC. A clear separation of a protein-containing and an almost protein-free fraction by centrifugation in D_2O was impossible because a large part of the protein was found in the floating layer.

Under these incorporation conditions rather large amounts of Triton remained in the final mixture, resulting in a molar ratio of phospholipid to Triton of about 5–6. This may perturb the protein–lipid interaction although a large amount of Triton incorporated into multilamellar membranes of egg PC produces only a very small reduction of the chemical shift anisotropy in the phosphorous spectrum (Figure 2). Alternatively, when the Triton-solubilized carrier protein was added to a dry lipid film (method II), the final ratio after treatment with Bio-Beads was about 16–20 mol of phospholipid/mol of Triton. In this case, only one incorporation step was performed. The molar ratio of phospholipid to Triton before Triton removal was 0.67 mol/mol. The ^{31}P NMR spectra

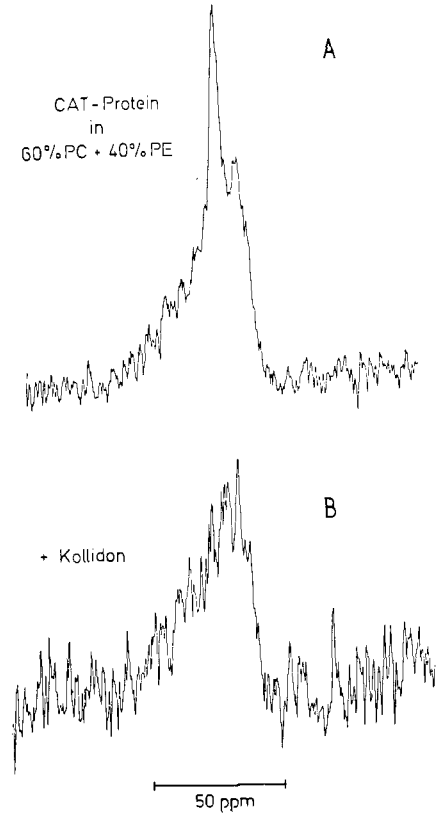

FIGURE 3: ^{31}P NMR spectra of a mixture of 42 mg of egg PC, 28 mg of egg PE, and 18 mg of carrier protein prepared according to method II: (A) dispersion in 1.5 mL of buffer; (B) the same mixture after addition of 500 mg of Kollidon. Temperature was 9 °C. A ^{2}H-lock signal was derived from D_2O in an external capillary.

again were composed of two spectral components, the narrow band being somewhat more pronounced (Figure 3A). Without PE, the narrow peak was yet more enhanced, similarly as observed in the preparation according to method I (not shown).

It may be suggested that in the presence of PE the narrow line is caused by isotropic intramembrane phospholipid motion (Cullis & DeKruijff, 1979). Alternatively, this signal could originate from the tumbling of rather small phospholipid vesicles combined with lateral phospholipid diffusion in the vesicular membrane (Cullis, 1976). This possibility was examined by increasing the viscosity of the solution and by planar orientation of the protein-loaded membranes. Increasing solution viscosity should slow down vesicle rotation but should hardly influence the intramembrane lipid motion. Increasing solution viscosity by addition of glycerol or sucrose or by lowering the temperature consistently led to increasing line width (experiment not shown). At very high viscosity, caused by addition of 40 wt % poly(vinylpyrrolidone), isotropic phospholipid motion is clearly absent. The remaining signal is typical for the line shape of bilayer lipid (Figure 3B).

The same conclusion emerged when the protein-containing membranes were partly dehydrated and oriented between stacks of glass plates in the magnetic field (Figure 4). The angular dependence of the ^{31}P NMR spectra is in agreement with the bilayer state of most of the membrane lipids. However, there is a considerable amount of nonoriented material that overlaps the single low or upfield lines that reflect the lipid orientation parallel to the plates. The disordered lipids may originate from the molecules in the toroidal edges of small liposomes. Similar observations have been made recently with reincorporated sarcoplasmic reticulum ATPase (McLaughlin

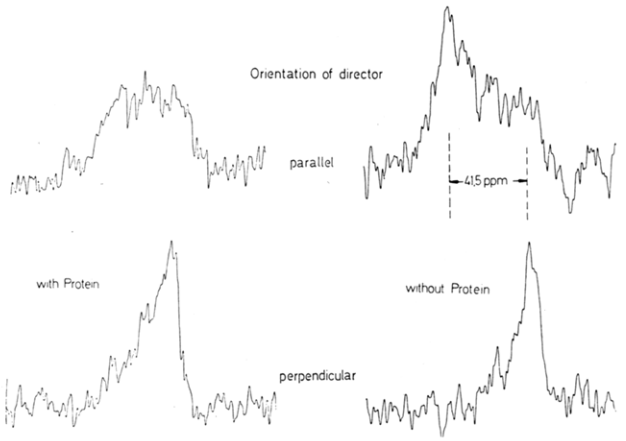

FIGURE 4: ^{31}P NMR spectra of partially dehydrated and oriented membrane. The membranes were prepared according to method II from a mixture of 60% w/w egg PC and 40% w/w egg PE with and without protein. Phospholipid to protein molar ratio was 260 ± 30 mol/mol. Temperature 9 °C. Experimental line broadening was 37.3 Hz. Director: axis perpendicular to the membrane surface.

et al., 1981). Evaluation of the relative share of lipids in the toroidal regions is yet difficult due to the presence of high-power proton decoupling. No prominent orientation-independent signal due to isotropic phospholipid motion can be observed. Thus the narrow spectral component in the suspended preparations seems to be exclusively due to vesicle tumbling.

It is difficult to obtain an effective chemical shift anisotropy from the broad component in the spectra of nonoriented membranes due to the somewhat distorted line shape. Compared to a value of 43–44 ppm found in a dispersion of pure egg lecithin, however, this parameter seems to be reduced in the presence of protein. A reduced shift anisotropy may also be deduced from the ^{31}P spectra of protein-loaded membranes oriented parallel and perpendicular to the magnetic field. In contrast, an oriented membrane preparation obtained in the same way without protein yielded a shift anisotropy of 42 ± 1 ppm (Figure 4), typical for the egg PC–PE mixture in excess water. However, due to the rather poor orientation of the protein-containing membrane, the exact value of the shift anisotropy cannot be extracted from the ^{31}P spectrum.

Electron Microscopy. In order to rationalize the NMR results of the reincorporated phospholipid systems, it was necessary to subject appropriate samples to electron microscopy. Freeze–fracturing of a sample prepared according to method I revealed the presence of two different populations of large and smaller liposomes (Figure 5A). The large liposomes are multilamellar and devoid of particles. In the small liposomes both fracture faces are studded with particles that can be attributed to incorporated carrier molecules. The diameter of the protein-containing liposomes ranges from 500 to 1700 Å, 70% being in the range from 650 to 1300 Å. After separation of the unloaded liposomes by centrifugation in a D_2O medium, as described above, the lower phase contains mainly small vesicles that are shown by thin sectioning to be unilamellar (Figure 5B). This fraction has been correlated to the symmetrical ^{31}P NMR signal (Figure 1).

When method II was applied for the protein incorporation, particles appeared both in unilamellar small liposomes as well as in large multibilayers (Figure 5C,D). Again no preference of the particles for the inner or outer fracture face could be observed. In the presence of PE, small vesicles become less frequent. Under this condition, protein particles in the plane of the membrane seem to aggregate as judged from a characteristic particle network (Figure 5C). This structural feature is not observable in the absence of PE (Figure 5D). The aggregation is distinct only in large fracture faces, but this may be due to the dense packing of the particles in the small liposomes.

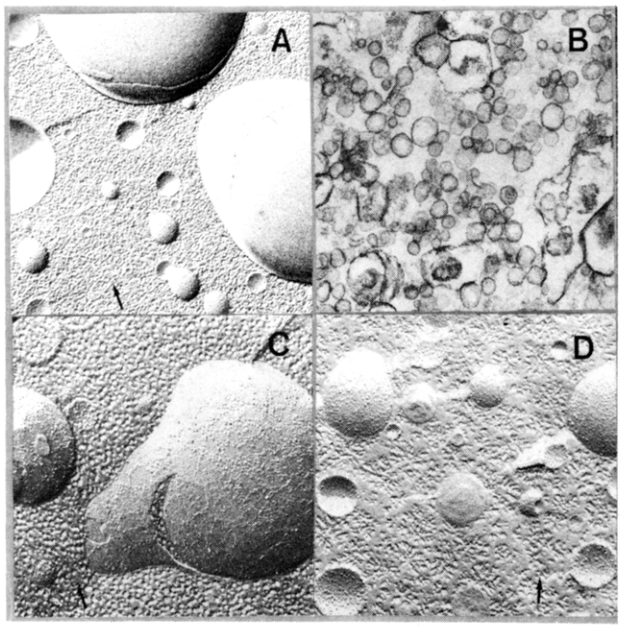

FIGURE 5: Electron microscopy of membranes after protein reincorporation: (A) freeze–fractured sample prepared with egg PC according to method I; (B) same preparation after centrifugation in D_2O (thin-sectioned sample of the protein-containing vesicles); (C) freeze–fractured sample prepared with 60% egg PC and 40% egg PE according to method II; (D) same as (C) without PE. The phospholipid to protein molar ratios were about 300 in (A) and (B) and 200 in (C) and (D). Magnification: (A) ×36 000; (B–D) ×54 000.

Particles were counted in 65 liposomes, and the calculated lipid to protein ratio was compared to the ratio found by chemical analysis. If one assumes an area of 70 ($Å^2$) for each phospholipid molecule in the liquid-crystalline state (Luzatti, 1968) and identifies each particle with a protein dimer of molecular weight 60 000, an average ratio of 376 lipid molecules per protein dimer was obtained, with a standard deviation of 258, compared to the ratio of 200 mol/mol found by chemical analysis. This result may be explained by assuming that the observed particles are aggregates of two dimeric carrier molecules. However, due to the large standard deviation, quantitative conclusions about the aggregation state can be drawn only with precaution.

Mechanism of Protein Incorporation. The mode of formation of protein-containing lipid membranes upon detergent removal may shed light on the physical properties of the lipid–protein complexes. These experiments were performed only with egg lecithin. The distribution of protein, phospholipid, and Triton after mixing of the components was examined before Triton removal by chromatography on Sepharose 2B. In the first stage, both preparations, as obtained by addition of the solubilized protein to a preformed phospholipid dispersion (method I) or to a dry lipid film (method II), revealed large particles eluting at the void volume of the column, in coexistence with smaller mixed micelles (Figure 6). This is in agreement with observations reported by Dennis (1974) in Triton–lecithin mixtures. Interestingly, the protein is mainly associated with the larger structures, whereas Triton is largely in the smaller micelles. In both experiments about 50% of the protein elutes with the large peak at the void volume. Among this peak the Triton to protein and phospholipid to protein ratios remain fairly constant, whereas among the mixed mi-

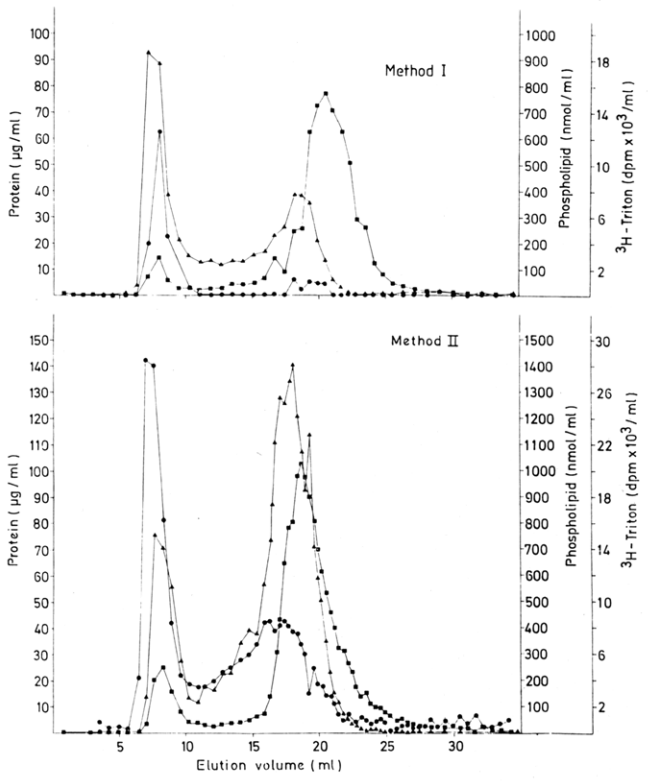

FIGURE 6: Chromatography of mixtures of protein, phospholipid, and Triton on Sepharose 2B prior to Triton removal: (A) 0.5 mg of protein added to a suspension of 5 mg of egg PC in 850 µL of buffer (method I); (B) 1.35 mg of protein added to 5 mg of dry egg PC (method II). (●) Protein; (▲) phospholipid; (■) ^{3}H-labeled Triton.

cellar peak the ratios vary strongly. It seems that after addition of the Triton-solubilized protein to phospholipids, the protein–detergent micelles have largely fused with phospholipid multibilayers. Nonionic detergent micelles readily incorporate into phospholipid vesicles, followed by redistribution of the detergent molecules in the vesicular membrane and among the vesicle population (Beyer & Klingenberg, 1978). This mechanism may lead to rapid displacement of detergent by phospholipid molecules in the vicinity of the protein.

The effect of Triton removal by Bio-Beads from the primary protein–egg lecithin mixture was followed (Figure 7) according to stepwise incorporation (method I). [^{3}H]CAT–protein complex, phospholipid, and Triton distributions were determined. After centrifugation of the incubation mixture, about 40–50% of the added protein was found in the pellets with binding of 12–14 nmol of CAT/mg. Also, the phospholipid content in the sample decreased considerably when increasing amounts of protein were added. On the other hand, the molar ratio of lipid to protein decreased to a final value of about 90 mol/mol. Rather low amounts of protein (determined by the Lowry method) as well as released [^{3}H]CAT were found in the supernatants after the centrifugation steps.

Obviously, after contact with the polystyrene surface, very few mixed Triton–protein–phospholipid micelles coexist with the membraneous fraction. Furthermore, some protein and phospholipid are removed after binding to the beads. In a parallel experiment, protein was omitted from the Triton-containing buffer. In this case, phospholipid was bound yet more effectively (not shown). Thus the presence of protein in the reassembled membranes seems to prevent the extraction of phospholipids.

This conclusion could be verified by the above-mentioned separation of the protein–phospholipid vesicles from the unloaded phospholipid. Repetition of this procedure leads to a

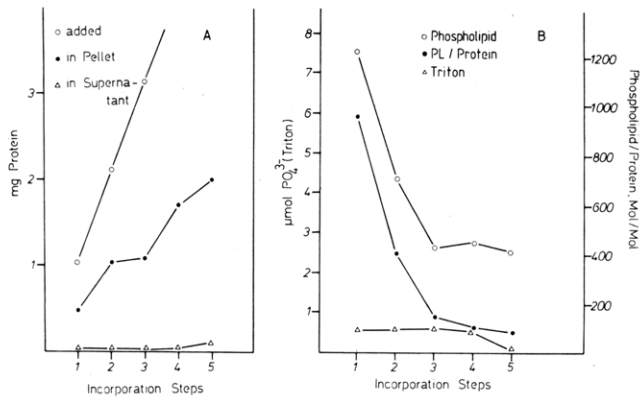

FIGURE 7: Ten mixtures of 1.05 mg of [^{3}H]CAT–protein and 10 mg of PC were incubated and treated with Bio-Beads according to method I. After centrifugation, the supernatants and two pellets were set aside for analysis. The protein incorporation was repeated with the residual pellets 4 times. (A) Protein content as determined in the pellets and supernatants, respectively; (B) phospholipid and Triton content in the pellets. The Triton content was determined in a parallel experiment with ^{3}H-labeled Triton and unlabeled CAT.

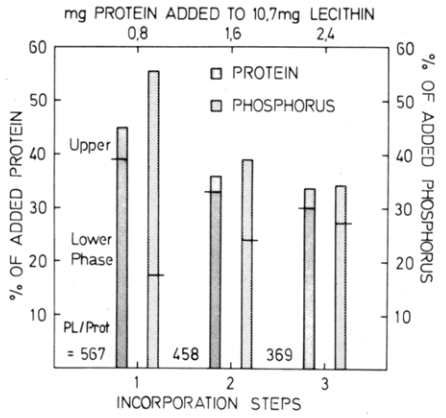

FIGURE 8: Distribution of phospholipid and protein after separation of protein-loaded and unloaded liposomes by centrifugation in D_2O. Protein at a concentration of 1 mg/mL was added to suspensions of 10.7 mg of egg PC in 160 µL of buffer. After Triton removal by Bio-Beads, the suspensions were pelleted, resuspended in D_2O buffer, and centrifuged as in Figure 1. The incorporation procedure was repeated twice with the same amount of protein.

decrease of the amount of floating phospholipid whereas the ratio of phospholipid to protein in the lower phase changes only slightly (see Figure 8). Again the delipidation by the Bio-Bead treatment seems to be most effective when the amount of protein-free lipid is high, i.e., after the first incorporation step, suggesting that phospholipid associated with the carrier protein withstands the interaction with the hydrophobic surface of the beads.

Conversely, it can be concluded from the rather small decrease in lipid to protein ratio in the protein-containing lower phase that newly added protein scarcely incorporates into previously formed lipid–protein liposomes (Figure 8). At a lipid to protein molar ratio of about 100:1, obviously, no additional protein incorporates into the membranes (cf. Figure 7).

Discussion

The study by magnetic resonance of the lipid–protein interactions in natural membranes is complicated by the lipid and protein composition. The reincorporation approach using purified proteins offers a means to circumvent these difficulties, since it permits one to work with specific lipid and protein compositions. A prerequisite for studies of specific lipid–protein interactions by magnetic resonance methods is the

removal of the detergent used to isolate the protein. The order of the hydrophobic lipid core and the hydrophilic interface as detected by ^2H and ^{31}P NMR may be perturbed even by traces of ionic detergents (Rice et al., 1979a,b).

In the present study, the distorted line shape of the anisotropic ^{31}P NMR signal of the lipid–protein complexes (Figures 1 and 3) as compared to the lipid dispersions alone (Figure 2) may result from homogeneous as well as inhomogeneous line broadening. Reduction of ^{31}P spin–spin and spin–lattice relaxation times and thereby homogeneous line broadening has been observed recently as the result of lipid–protein interaction (Rice et al., 1979b; Seelig et al., 1981). Additional inhomogeneous broadening is probably caused by the broad vesicle size distribution found in the present preparations.

Comparison of oriented membranes with and without the reincorporated ADP/ATP carrier protein suggests a perturbing effect of the protein as shown by the poor orientational order and the apparent reduction in the effective ^{31}P chemical shift anisotropy in Figure 4. A reduced ^{31}P shift anisotropy has recently been observed in reconstituted sarcoplasmic reticulum membrane preparations (Seelig et al., 1981). However, in the present case the lack of a clear-cut downfield ^{31}P signal upon orientation of the glass plates normal parallel to the magnetic field prevents the determination of the exact value of the chemical shift anisotropy. Indeed, the presence of small vesicles may be responsible for the line-shape distortion also in the dehydrated and oriented material and thereby produces an apparent reduction in this parameter. Attempts to enlarge the small vesicles by freezing and thawing of the samples yielded even more of them as indicated by an increased "isotropic" ^{31}P signal (experiment not shown). Thus, the only firm conclusion to be drawn from the orientation experiment is that the carrier protein favors the formation of membrane fragments with high curvature. This bears resemblance to the situation in the inner mitochondrial membrane that contains regions of high curvature due to its extreme folding.

The question whether nonbilayer lipid plays a role in certain biological membranes has been discussed recently on the basis of ^{31}P NMR results. Isotropic phospholipid motion was found in membranes containing large amounts of PE (Cullis & DeKruijff, 1978; Stier et al., 1978; DeKruijff et al., 1978). In the inner mitochondrial membrane, similar structures have been suggested to exist (Cullis et al., 1980) since about 40% of the phospholipids in this membrane consists of PE. The present results, however, argue against a substantial contribution of nonbilayer lipid in the reconstituted membrane system, even after protein incorporation. Although Kollidon was used for immobilizing the vesicles, a conversion to the bilayer structure could be excluded. Addition of the same amount of Kollidon to a lipid mixture consisting of dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, and cholesterol in a molar ratio of 3:1:2 (DeKruijff et al., 1979) or to a mixture of cardiolipin and Ca^{2+} in a molar ratio 1:0.6 (Cullis et al., 1978) did not convert the isotropic ^{31}P signal into a bilayer line shape.

A striking enhancement of the transport activity by phosphatidylethanolamine after reconstitution was reported earlier (Krämer & Klingenberg, 1980). The present work suggests that—at least in the artificial system—nonbilayer structures are not essential for the function of the carrier protein.

Whereas ^{31}P NMR did not show appreciable differences between the protein phospholipid mixture in the presence and absence of PE, freeze–fracture electron microscopy revealed particle aggregation in the presence of this phospholipid. This effect is not ascribed to freezing in liquid Freon, which normally does not lead to protein aggregation (Zingsheim & Plattner, 1976). It can be due to a specific influence of PE at the high protein concentration used in this case. Whether this aggregation can be correlated with the activating effect of PE on the transport in the much more dilute reconstituted system (Krämer & Klingenberg, 1980) deserves further investigation.

The occurrence of particles on both fracture faces demonstrates that the ADP/ATP carrier is an intramembrane protein. This agrees with the high amount of detergent bound to the isolated protein (Hackenberg & Klingenberg, 1980). Asymmetric location within the membrane of the carrier in the CAT-stabilized c conformation as reported recently (Brandolin et al., 1980) cannot be excluded. The fact that particles are nearly equally distributed among concave and convex fracture faces may be due to the random incorporation process. The shape and dimensions of this protein as disclosed by ultracentrifugal study (Hackenberg & Klingenberg, 1980) argue in favor of an extension only through the hydrophobic core of the bilayer not protruding to the hydrophilic space.

On the basis of electron microscopy and model experiments, the following membrane reassembly process can be visualized. Just after addition of the detergent-solubilized protein to a lipid dispersion, the protein creeps into the outer shell of the multilayered phospholipid liposomes. Some phospholipid is extracted by the excess detergent to form mixed phospholipid–Triton micelles, whereas the protein remains associated with lipid multibilayers of different size. Contact with the polystyrene surface then peels off the outer multibilayer shell, resulting in formation of unilamellar protein-loaded liposomes and unloaded multibilayers as seen in the electron microscope after freeze–fracturing (Figure 5A). Subsequently added protein mainly incorporates into the remaining multibilayers, and treatment with polystyrene beads again produces lipid–protein vesicles. This process may be repeated until all multibilayers are used up, resulting in an almost constant lipid to protein ratio of about 100 mol/mol.

The inability of protein-loaded membranes to take up additional protein may be compared to the observation by Conrad & Singer (1981) that much lower amounts of amphipathic molecules bind to biological membranes than to protein-free artificial membranes. This finding was explained in terms of "internal pressure" in biological membranes, which precludes the partition of amphiphiles into the bilayer interior. A more conclusive geometric reasoning shows that the free lipid area available for protein uptake may reach a critical value at a lipid to protein molar ratio of 100:1. If one assumes a hexagonal distribution of cylindrical protein molecules embedded in the membrane, at this ratio the nearest neighbor distance is below the protein diameter of $\simeq$50 Å, as estimated from an earlier ultracentrifugal study (Hackenberg & Klingenberg, 1980).

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Registry No. ADP/ATP carrier, 9068-80-8.

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A Factor in Sea Urchin Eggs Inhibits Transcription in Isolated Nuclei by Sea Urchin RNA Polymerase III†

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ABSTRACT: Isolated nuclei from sea urchin embryos synthesize RNA at a rate comparable to other animal cell nuclei. All three RNA polymerases are active as judged by α-amanitin sensitivity and hybridization to specific cloned DNAs. Extracts were prepared from sea urchin eggs and embryos by extraction with 0.35 M KCl. None of the crude extracts had a large effect on total RNA synthesis. However, extracts from sea urchin eggs inhibited RNA polymerase III activity in nuclei from blastula and gastrula embryos. There was no effect on the synthesis of ribosomal RNA by RNA polymerase I or on the synthesis of two RNA polymerase II products, histone mRNA and the sea urchin analogue of U1 RNA. The inhibitor is present in two different species of sea urchin and has been 50-fold purified by diethylaminoethylcellulose and hydroxylapatite chromatography. The inhibitor is not present in extracts prepared from sea urchin blastula embryos.

During oogenesis, the sea urchin synthesizes and stores a large portion of the RNA molecules that will be used subsequently in the developing embryo [reviewed by Davidson (1976)]. In addition, the products of RNA polymerase II, messenger RNA and heterogeneous nuclear RNA, continue to be synthesized at high rates in the mature sea urchin egg (Levner, 1974; Brandhorst, 1980; Dworkin & Infante, 1978). The structural RNAs which are synthesized by RNA polymerases I and III are probably not synthesized in the egg (Levner, 1974). The synthesis of these RNAs is activated early in embryonic development (Nijhawan, 1978; O'Melia, 1979a,b; Nijhawan & Marzluff, 1979; Griffith et al., 1981). The differences in expression of these major classes of RNA molecules might arise from independent regulation of the three RNA polymerases involved in their synthesis [reviewed by Roeder (1976)].

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