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Interaction of Phospholipids with the Detergent-Solubilized ADP/ATP Carrier Protein As Studied by Spin-Label Electron Spin Resonance†

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Received March 22, 1988; Revised Manuscript Received June 14, 1988

ABSTRACT: The interaction of spin-labeled phospholipids with the detergent-solubilized ADP/ATP carrier protein from the inner mitochondrial membrane has been investigated by electron spin resonance spectroscopy. The equilibrium binding of cardiolipin and phosphatidic acid was studied by titration of the protein with spin-labeled phospholipid analogues using a spectral subtraction protocol for the evaluation of the mobile and immobilized lipid portions. This analysis revealed the immobilization of two molecules of spin-labeled cardiolipin per protein dimer. Phosphatidic acid has a similar affinity for the protein surface as cardiolipin. The lipid–protein interaction was less pronounced with the neutral phospholipids and with phosphatidylglycerol. The importance of the electrostatic contribution to the phospholipid–protein interaction shows up with a strong dependence of the lipid binding on salt concentration. Cleavage by phospholipase A_2 and spin reduction by ascorbate of the spin-labeled acidic phospholipids in contact with the protein surface suggest that these lipids are located on the outer perimeter of the protein. At reduced detergent concentration, the protein aggregated upon addition of small amounts of cardiolipin but remained solubilized when more cardiolipin was added. This result is discussed with respect to the aggregation state of the protein in the mitochondrial membrane. It is also tentatively concluded that binding of spin-labeled cardiolipin does not displace the tightly bound cardiolipin of mitochondrial origin, which was detected previously by ^{31}P nuclear magnetic resonance spectroscopy [Beyer, K., & Klingenberg, M. (1985) *Biochemistry 24*, 3821].

The interaction of spin-labeled phospholipids with integral membrane proteins has been extensively studied in reconstituted membranes, aiming at an understanding of the specific lipid composition for protein function and biomembrane stability. As a general result, the electron spin resonance (ESR)[1] spectra revealed strongly and weakly immobilized components which were attributed to lipid molecules in transient contact with the protein surface and to lipids freely diffusing in the membrane. The strength of lipid–protein association was variable among different proteins and lipid classes (Devaux & Seigneuret, 1985). A strong interaction has been frequently observed between negatively charged phospholipids and membrane proteins (Marsh, 1985).

Recently, a tight binding of mitochondrial CL was found by ^{31}P NMR in the detergent-solubilized AAC protein from beef heart mitochondria (Beyer & Klingenberg, 1985). This finding prompted us to employ the spin-label method using phospholipids specifically labeled at carbon atom 12 of stearic acid in the *sn*-2 position. In detergent solution, labeling of this position affords a clear-cut discrimination between protein-associated phospholipids and lipid molecules in detergent micelles. In reconstituted membranes, the best results have been obtained with spin-labels in position 14 of the fatty acid chain (Marsh, 1985).

Solubilization of membrane proteins is usually attributed to the "soap effect" of the solubilizing detergent. The present experiments suggest that charged amino acids on the protein

† The ESR instrument was purchased with a grant from the Deutsche Forschungsgemeinschaft. The work was also supported by the Deutsche Forschungsgemeinschaft (BE 828/2).

[1] Abbreviations: AAC, ADP/ATP carrier; CAT, carboxyatractyloside; CATSL, spin-labeled carboxyatractyloside; CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SA, stearic acid; SASL, 12-doxylstearic acid; CLSL, PASL, PCSL, PESL, and PGSL, phospholipids spin-labeled with 12-doxylstearic acid in the *sn*-2 position; ESR, electron spin resonance; NMR, nuclear magnetic resonance.

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surface may also contribute to the solubility of the protein in the nonionic detergent. The presence of a large excess of positive charges in the primary structure of the AAC results in the binding of negatively charged cardiolipin molecules and eventually leads to a net negative charge of the entire phospholipid–protein complex. These electrostatic phospholipid–protein interactions may have significance also for the proper functioning of the carrier protein in the mitochondrial membrane.

Materials and Methods

Chemicals and Enzymes. Cardiolipin was purchased from Sigma as a methanolic solution. Phosphatidylcholine was isolated from egg yolk according to Wells and Hanahan (1969) by silicic acid chromatography. Amberlite XAD-2 beads were obtained from Sigma. Phospholipase A_2 from porcine pancreas was obtained from Boehringer, Mannheim. The crude venom of *Trimeresurus flavoviridis* containing phospholipase A_2 activity was from Sigma. L-Ascorbic acid (analytical grade) was from Merck, Darmstadt. A freshly prepared 200 mM stock solution was neutralized with NaOH before addition to the ESR samples. Spin-labeled maleimide, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, was obtained from Syva.

Analytical Procedures. Protein concentration was determined by the method of Lowry (1951). Triton X-100 was measured photometrically at 274 nm.

Spin-Label Syntheses. 12-Doxylstearic acid (12-SASL) was purchased from Aldrich. PCSL was synthesized by reacylation of lyso-PC from egg yolk with 12-SASL according to Boss et al. (1975). PESL and PGSL were prepared from PCSL by head-group exchange using phospholipase D, as described by Comfurius and Zwaal (1977) for the unlabeled lipids. PASL was synthesized from PCSL by treatment with phospholipase D, according to Ito and Ohnishi (1974). CLSL was prepared by reacylation of monolysocardiolipin with 12-SASL (Cable et al., 1978). Monolysocardiolipin was obtained by cleavage of cardiolipin with the crude venom of *Trimeresurus flavoviridis* as described by Okuyama and Nojima (1965).

All phospholipid spin-labels were purified by preparative thin-layer chromatography using the solvent systems $CHCl_3/CH_3OH/H_2O$, 65/25/4 v/v, or acetone/$CHCl_3$/$CH_3OH/CH_3COOH/H_2O$, 8/6/2/2/1 v/v.

Spin-labeled carboxyatractyloside (CATSL) was synthesized as described by Munding et al. (1983) by acylation of carboxyatractyloside with 2,2,5,5-tetramethyl-3-pyrroline-1-oxycarboxylic acid.

Sample Preparation. The ADP/ATP carrier protein was isolated and purified as the AAC–CAT complex following the procedure of Riccio et al. (1975). The standard protein buffer contained 200 mM NaCl, 10 mM MOPS, 50 μM EDTA, and 50 μM NaN_3. Early fractions from the final column were used for the phospholipid binding studies. Quantitative thin-layer analysis in these fractions showed rather low concentrations of residual PC and PE. Typically, the lipid proportion was CL/PC/PE, 2/1/1 mol/mol, whereas the CL/protein ratio was close to 6/1 mol/mol as determined previously (Beyer & Klingenberg, 1985). The protein was pressure dialyzed to a final concentration of 2–6 mg/mL. In most experiments, the concentration of Triton X-100 was reduced by incubation with 10 mg of XAD 2 beads/mg of Triton for 30 min at room temperature. Spin-labeling was accomplished by shaking the protein solutions with dry films of the spin-labeled lipids for 30 min. Glass beads were added when larger amounts of lipids were to be solubilized.

Spin-labeling of the AAC with the inhibitor CATSL was performed essentially as described by Munding et al. (1983).

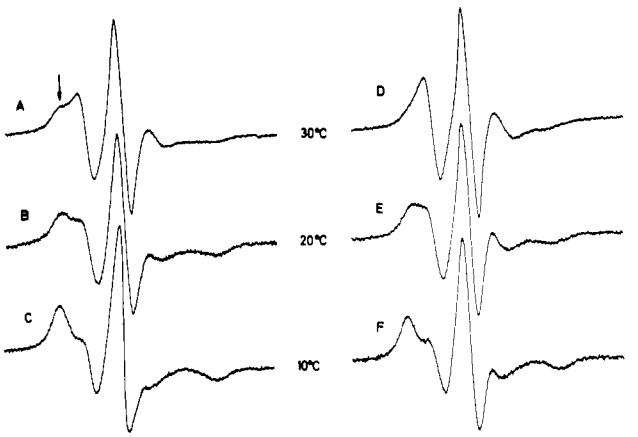

FIGURE 1: Temperature dependence of ESR spectra of CLSL in the presence (A–C) and in the absence (D–F) of AAC protein. Protein concentration, 2.6 mg/mL. Ratio of CLSL/protein, 3 mol/mol. Concentration of Triton X-100, 1.8% w/v. The arrow in spectrum A indicates the immobilized spectral component.

ESR Measurements. ESR spectra were recorded on a Bruker ER-200 D-SRC spectrometer equipped with a variable-temperature accessory. Samples were filled in glass capillaries with an internal diameter of 0.9 mm. Most spectra were obtained in a TE 102 cavity with a modulation frequency of 100 kHz, a modulation amplitude of 1 G_{pp}, a sweep width of 100 G, and a microwave power of 5 mW. The spectra in Figure 1 were obtained in a TM 110 cavity with a modulation amplitude of 2.5 G_{pp} and under otherwise identical conditions. The sweep time was typically set to 500 s. The kinetics of ascorbate reduction were followed by continuous observation of the center peak maximum of the ESR spectrum with the sweep width set to zero. Double integratons and spectral subtractions were performed with a MINC PDP-11/23 minicomputer. Spin densities were determined by using a spin-labeled maleimide as a reference sample. Exact weighing of this powdery compound is more convenient than of the oily spin-labeled lipids. It should be noted that the reference spectra must be run in water at the same temperature as the experimental spectra because of the large, temperature-dependent dielectric loss in aqueous solution (Mohanty & Rifkind, 1984). The potential differential influence of spin–lattice relaxation on the spin intensities of the detergent-bound and protein-bound phospholipid portions was examined by double integration. Spectra obtained with and without the AAC protein under otherwise identical conditions (spin-label concentration, detergent concentration, temperature) always yielded the same result within ±10%, suggesting that the relaxation effect is negligible.

Results

Binding of CLSL to the Solubilized AAC. ESR spectra of CLSL solubilized in Triton X-100 in the absence and in the presence of the purified CAT–AAC complex are shown in Figure 1. The detergent concentration in both samples was adjusted to 18 mg/mL prior to the ESR measurement in order to discriminate the spectral changes due to phospholipid–protein interaction against the immobilization of CLSL in Triton micelles. This adjustment was achieved by treating the protein-containing sample with XAD-2 beads as described under Materials and Methods.

The ESR line shapes in Figure 1 exhibit strong temperature dependence both without and with the AAC protein. At 10 °C, the spectra nearly indiscriminately show strong spin-label immobilization irrespective of the presence of protein. At 30 °C, however, a two-component spectrum was obtaned in the

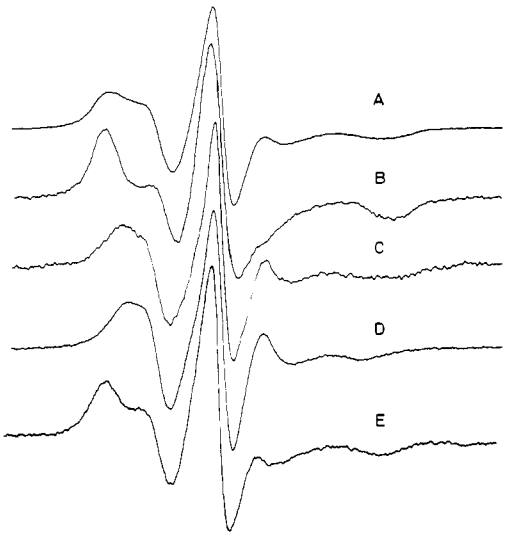

FIGURE 2: Determination of mobile and immobilized CLSL portions by spectral subtraction. (A) CLSL in the presence of AAC protein (5.3 mg in 1 mL of 1.6% w/v of Triton X-100; CLSL/protein, 2.7 mol/mol). (D) CLSL in Triton X-100 (1.5% w/v). (B) Immobilized spectral component as obtained after scaling of spectrum D and subtraction from spectrum A. (C) Mobile spectral component generated by subtraction of spectrum E which is from CLSL in the presence of a 2-fold molar excess of the AAC protein. Spectra were recorded at 20 °C.

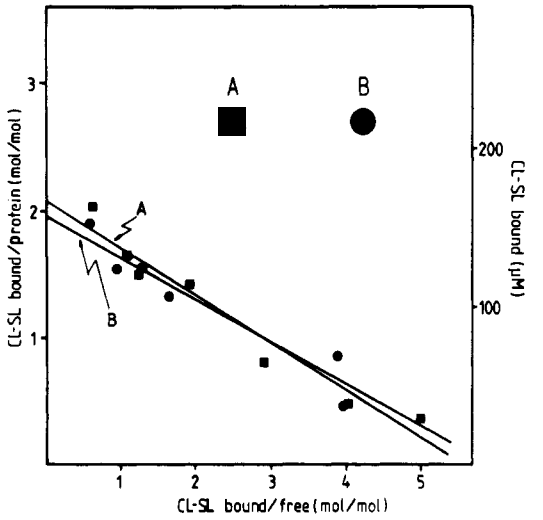

FIGURE 3: Evaluation of CLSL binding by the Scatchard method. ESR spectra at different CLSL/protein ratios were decomposed by spectral subtraction (cf. Figure 2). (A) Subtraction of the mobile spectral component as obtained from a solution of CLSL in Triton X-100. (B) Subtraction of a virtually immobilized spectrum as obtained from a CLSL solution in the presence of a 2-fold molar excess of the AAC protein. For further experimental details, see Figure 2. Linear regression analysis yields an apparent dissociation constant K_d of 29.7 μM and a number of binding sites (n) of 2.06 for (A) and 26.2 μM and 1.93 for (B), respectively. Spectra were recorded at 20 °C.

protein-containing sample whereas the spectrum of CLSL in Triton alone appears to be homogeneously broadened. The immobilized spectral component in the presence of the carrier protein (indicated by an arrow in Figure 1, spectrum A) can therefore be attributed to a CLSL portion interacting with the protein surface. It has been shown previously that the AAC protein when stabilized with the inhibitor CAT will not denature during the time required for obtaining an ESR spectrum (typically 10 min), even at about 50 °C (Munding et al., 1983). Thus, the present experiments, aiming at the observation of specific interactions of phospholipids with the detergent-solubilized AAC, were performed at 20 or 30 °C.

The quantitative evaluation of CLSL binding is shown in Figures 2 and 3. The immobilized and mobile components were separated by digital subtraction of appropriate spectra from the composite spectrum in Figure 2A. An "immobilized endpoint" spectrum (Figure 2B) yielding the relative amount of protein-associated CLSL was obtained by subtracting a spectrum of CLSL in Triton alone (Figure 2D). In order to separate the corresponding mobile component, a spectrum of CLSL in the presence of a 2-fold molar excess of the AAC protein was subtracted (Figure 2C,E).

The $CLSL_{bound}/CLSL_{free}$ ratios at a number of different total CLSL/protein molar ratios were further evaluated by a Scatchard representation. The two data sets obtained by determination of the immobilized and the fluid components (cf. Figure 2B,C) are in good agreement and can be reasonably approximated by linear regression as shown in Figure 3, suggesting that the Scatchard analysis is applicable to the observed lipid binding equilibrium. The apparent dissociation constants and the total number of binding sites obtained from the different subtraction procedures (K_d = 29.7 and 26.2 μM, n = 2.06 and 1.93; see Figure 3) indicate that the solubilized AAC protein is capable of binding about two molecules of cardiolipin with rather high affinity. The extent of phospholipid binding to the protein (but not the number of binding sites) will depend on the detergent concentration, since the added phospholipid easily distributes among the available detergent micelles. Thus, it was important to keep the detergent concentration strictly constant throughout the titration.

It should be noted that the two spectral subtractions are not totally independent of each other. The "immobilized spectrum", which was used to separate out the pure mobile component, itself contained a small residue of the fluid spectrum. This contribution was taken into account when computing the fluid CLSL component in the composite spectra of the CLSL–protein mixtures.

During the stepwise CLSL addition, slow protein aggregation was observed when the phospholipid/protein molar ratio exceeded unity, although the total detergent concentration was by far sufficient to solubilize the added phospholipid (see below). Thus, in the Scatchard titration, a large molar excess of CLSL was avoided, and all ESR experiments were completed as rapidly as possible. In spite of these precautions, increasing turbidity at higher CLSL concentrations was indicative of some protein aggregation. However, the observation that bound CLSL is easily displaced by unlabeled CL even in slightly aggregated samples (see below) suggests that the protein-bound CLSL portion is in equilibrium with CLSL in Triton micelles.

An analogous titration of the protein with PA also yielded about two binding sites. The evaluation of an apparent binding constant was hampered, however, due to the strong deviation of the Scatchard plot from linearity at low lipid/protein ratios. Slow protein precipitation was also observed upon addition of increasing amounts of PA (experiments not shown).

Phospholipid molecules associated with the protein surface may exchange with phospholipids in the same detergent–protein–phospholipid mixed micelle or in excess detergent micelles. This is borne out when measuring the line width of the mobile low-field component. At a lipid to protein ratio of 2 mol/mol, this signal component was broadened by about 10% as compared to the spectrum obtained at the same temperature without protein. At still higher lipid to protein ratios, the variation in line width was within the experimental error which was presumably introduced by the subtraction procedure (see Discussion).

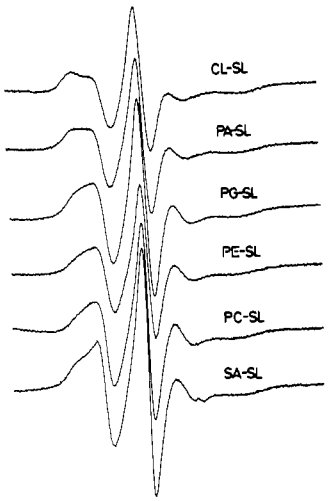

FIGURE 4: Binding of different lipids to the solubilized AAC. Spin-labeled lipids were added to a solution of 6.1 mg/mL AAC protein in 2.05% w/v of Triton X-100 at a lipid to protein ratio of 1 mol/mol. The spectra were recorded at 30 °C.

Selectivity of Phospholipid Binding. It may be surmised that the rather strong interaction of CLSL with the AAC protein is at least partially due to electrostatic attraction of the negatively charged phospholipid head groups by positively charged amino acid residues on the protein surface. This notion is qualitatively corroborated by addition of a series of spin-labeled phospholipids with different head-group structures to the AAC protein. Throughout the series, the detergent concentration, the lipid to protein ratio, and the spectrometer settings were carefully kept constant (Figure 4). The low-field shoulder in the ESR spectra, which is attributed to the immobilized lipid portion in contact with the protein surface, is much less pronounced with the neutral phospholipids PCSL and PESL than with PASL and CLSL. At pH 7.2, PA and CL have more than one negative net charge (Abramson et al., 1964). An intense low-field component is also exhibited by the spin-labeled stearic acid derivative 12-SASL. The line width of the mobile component is narrower than in the phospholipid spectra, presumably due to the greater mobility of 12-SASL in detergent micelles. The negatively charged PG analogue PGSL, which may be schematically derived from CLSL by removing a PA fragment, does not exhibit a clear-cut low-field signal, indicating that interaction of this phospholipid with the AAC is rather weak. It may be assumed, however, that the inhomogeneously broadened low-field lines of PGSL, PESL, and PCSL reflect the superposition of just two spectral components as shown above for CLSL and PASL.

A strong selectivity for CLSL binding may also be inferred from competition experiments (Figure 5). Stepwise addition of unlabeled CL to an equimolar mixture of AAC protein with CLSL in Triton X-100 results in the gradual release of immobilized CLSL as can be seen qualitatively from the spectra. In contrast, addition of PC to the same preparation leads to an appreciable CLSL displacement only when PC is added in large excess. Addition of PA also resulted in CLSL displacement. However, PA was somewhat less effective than CL (not shown). These findings are in good qualitative agreement with the lipid binding selectivity as demonstrated in Figure 4.

The amino acid sequence of the AAC contains an excess of 18 positively charged residues, which are candidates for the interaction with negatively charged lipids. Thus, it is to be expected that increasing ionic strength leads to weakening of electrostatic interactions and thereby to reduced binding of

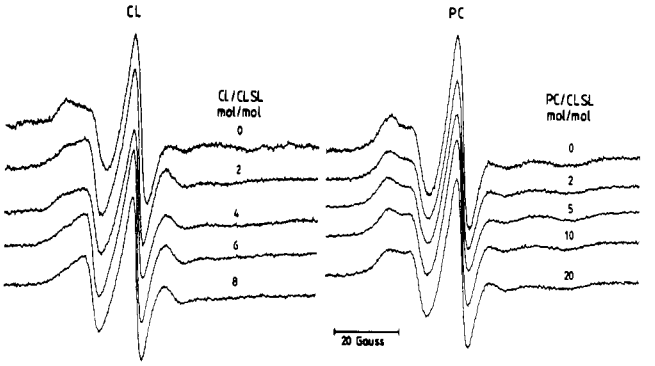

FIGURE 5: Competition of unlabeled phospholipids with CLSL for binding to the AAC protein. The protein (2.3 mg/mL in 1.25% w/v of Triton X-100) was incubated with an equimolar amount of CLSL prior to addition of increasing amounts of unlabeled CL or PC. The samples were equilibrated for 30 min at 0 °C before the ESR measurement at 30 °C.

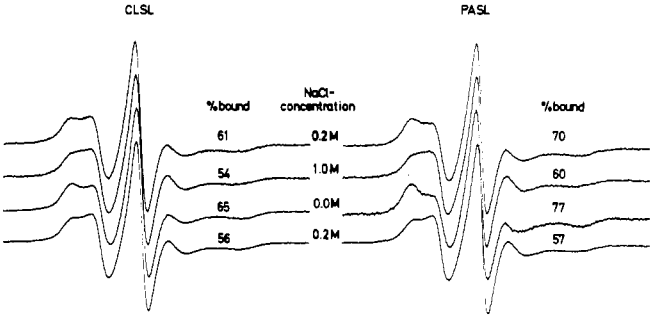

FIGURE 6: Dependence of CLSL and PASL binding to the AAC on salt concentration. The protein (5.5 mg/mL) was preincubated in a buffered solution containing 2.2% w/v of Triton X-100 and 0.2 M NaCl with CLSL and PASL at a lipid/protein ratio of 0.73 and 0.56 mol/mol, respectively. After ESR spectra were recorded (upper traces), the salt concentration was enhanced to 1 M by addition of solid NaCl, and the ESR measurement was repeated. The lower traces were obtained after extensive dialysis of the solution against NaCl-free MOPS buffer and against the standard protein buffer containing 0.2 M NaCl, as indicated. The percentage of immobilized lipid was determined by spectral subtraction as described in Figure 2.

CLSL and PASL to the protein molecule. The effect of changes in charge screening on phospholipid immobilization is shown in Figure 6. The NaCl concentration (200 mM) in the standard protein solution was enhanced to 1 M by addition of solid salt. After the ESR measurement, the salt was extensively dialyzed against NaCl-free MOPS buffer containing the same detergent concentration as the dialysate. Finally, the solution was brought back to the initial salt concentration by dialysis against standard protein buffer. The percentage of immobilized lipid was again evaluated by spectral subtractions as outlined above. The changes in the immobilized fraction of PASL and CLSL (Figure 6) are in qualitative agreement with the expected ionic strength effect. The loss of spin-label binding observed after restoring the original NaCl concentration (cf. the upper and lower spectra of each series in Figure 6) may be due to protein denaturation caused by the extreme changes in ionic strength.

A similar result was obtained with 12-SASL (spectra not shown). However, the determination of ionic strength effects on the 12-SASL–protein interaction is hampered, since the line shape of the spin-labeled fatty acid in Triton alone strongly depends on the ionic strength. This makes spectral subtractions as performed with the neutral phospholipids extremely difficult.

Accessibility of the Protein-Bound Phospholipid. It may be argued that the protein-bound phospholipid molecules are shielded by the irregular protein surface against chemical or

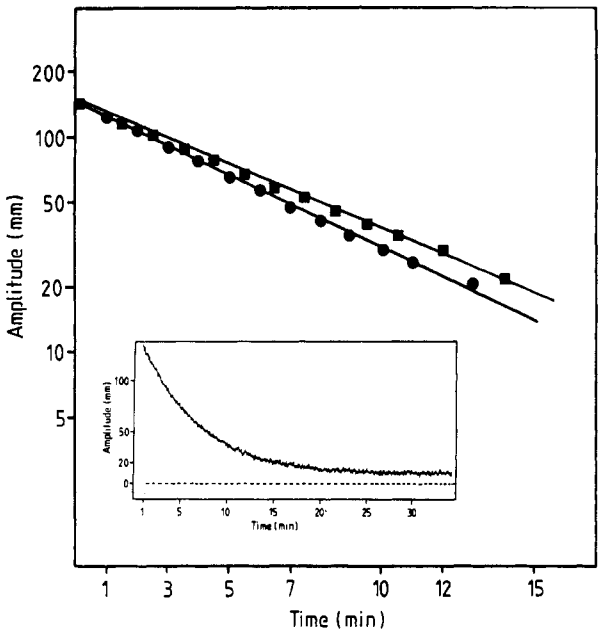

FIGURE 7: Spin reduction of PASL by ascorbate. Ascorbate (100 mM) was added to a solution of PASL (138 μM) in 1.5% w/v of Triton X-100 in the absence (●) or in the presence (■) of 3.6 mg/mL AAC protein. The spin reduction was followed at 30 °C by continuously recording the center-field line height (see insert).

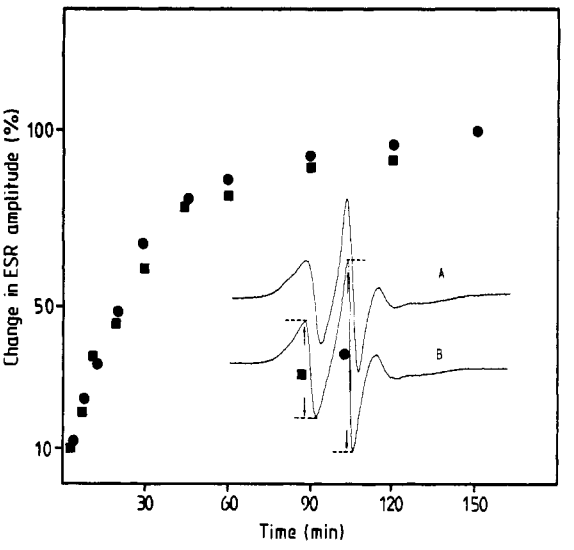

FIGURE 8: Cleavage of CLSL by phospholipase A_2 in the presence of AAC protein. The protein (5.3 mg/mL) in 1.6% w/v of Triton X-100 was incubated with a 5-fold molar excess of CLSL. After addition of 5 mM $CaCl_2$ and phospholipase from pig pancreas (at about 600-fold excess of the substrate), the cleavage reaction was followed at 30 °C by measuring the low-field (■) and center-field (●) peak to peak amplitudes as indicated by the arrows. Spectrum A was recorded immediately before and spectrum B 150 min after addition of the enzyme.

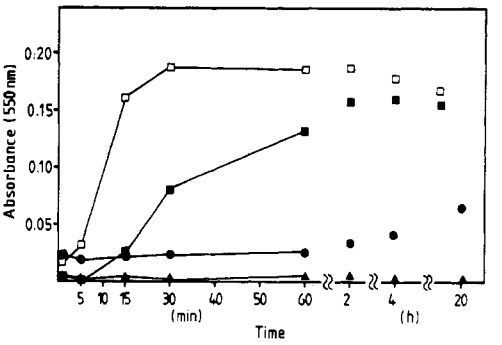

FIGURE 9: Precipitation of the AAC protein in the presence of CL as determined by the change in sample turbidity. The AAC protein (0.7 mg/mL in 0.23% w/v of Triton X-100) was incubated with CL in a 1-cm cuvette. The optical density was measured at 550 nm. Molar ratios of CL/protein were 3.1 (▲), 6.3 (■), 12.6 (□), and 25.1 (●).

enzymatic attack from the aqueous phase. One way to test this assumption was by measuring the kinetics of spin reduction after addition of ascorbate to Triton-solubilized PASL and to the PASL–protein complex (100 mM ascorbate, corresponding to a 1000-fold excess over the spin-label) as shown in Figure 7. The spin reduction was measured continuously by recording the center peak line height at constant field strength (Hartsel et al., 1987). It should be noted that in the protein-containing sample the center peak is a superposition of signal components from protein-bound phospholipid and phospholipid in detergent micelles (about 50% each in the experiment shown in Figure 7). The time dependence of the signal reduction was a single exponential both with and without the AAC protein (Figure 7). The spin reduction rate as obtained from Figure 7 is about 12% slower in the protein-containing sample, which is within the limits of experimental error. Thus, it must be concluded that the protein-bound phospholipids are not strongly shielded by the protein surface so as to prevent nitroxyl reduction by ascorbate.

In another experiment, the cleavage of CLSL or PASL by phospholipase A_2 was followed in the ESR spectra of protein–phospholipid complexes (Figure 8). Phospholipase cleavage of the protein-bound phospholipid results in the decrease of the immobilized spectral component in favor of the mobile component. Thus, in the protein-containing samples, the cleavage reaction was tentatively evaluated by measuring the amplitudes of the low-field narrow line and of the center peak. Even without protein, the signal amplitude increases slightly upon phospholipase treatment of CLSL solubilized in Triton X-100, indicating that enzymatic splitting of the spin-labeled phospholipid is accompanied by some narrowing of the ESR lines. Unfortunately, without protein the increase in signal amplitude is rather small and cannot be readily compared with the changes observed with protein due to the unknown differences in line width.

The normalized changes in first-derivative peak to peak amplitudes of the low-field and center-field signals are nearly identical as shown in Figure 8. It may be inferred that after 60 min of enzyme reaction about 85% of the phospholipid has been cleaved by the phospholipase. Although this experiment provides only a qualitative estimate of the enzyme kinetics due to the complicated superposition of different signal components of different line shape, it again allows the important conclusion that the protein-bound phospholipid is easily accessible to the rather small phospholipase molecule (molecular weight 14000).

Aggregation of Solubilized AAC Protein in the Presence of Charged Phospholipids. As mentioned above, the solubilized AAC protein is prone to aggregation and precipitation upon addition of the negatively charged phospholipid PA or CL. The effect has been studied in more detail in order to gain further insight into the interaction of the carrier protein with these phospholipids. The protein aggregation was studied qualitatively by measuring the turbidity of the solution after addition of CL as shown in Figure 9. The change in optical density suggests that the rate of CL-induced aggregation depends on the CL/protein ratio. Surprisingly, the turbidity increases with maximal rate at an intermediate lipid/protein ratio (12/1 mol/mol), whereas at a still higher ratio (25/1 mol/mol) the change is rather slow (Figure 9).

These findings have been confirmed by ESR spectroscopy

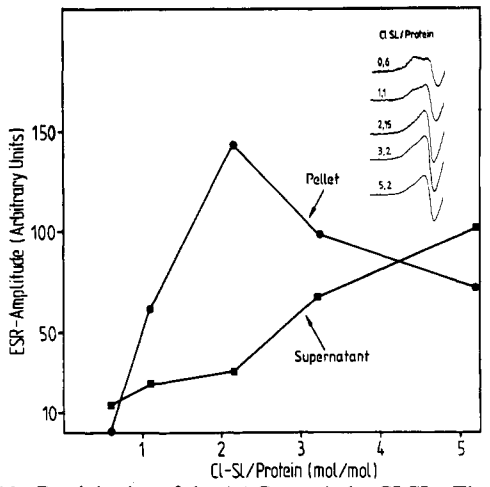

FIGURE 10: Precipitation of the AAC protein by CLSL. The protein (5.3 mg/mL in 1.6% w/v of Triton X-100) was incubated with increasing amounts of CLSL for about 20 h at 4 °C. The aggregated lipid–protein complexes were centrifuged in glass capillaries, and signal amplitudes were determined in the pellets and supernatants. The low-field region of the supernatant spectra is shown in the insert.

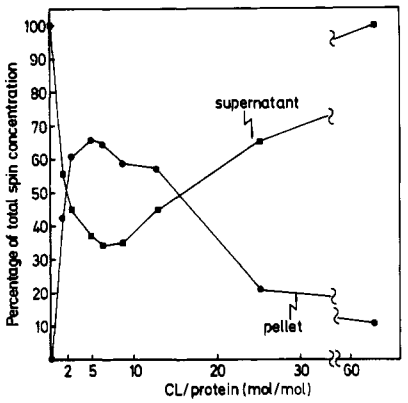

FIGURE 11: Precipitation and resolubilization of the AAC protein tagged with the spin-labeled inhibitor CATSL. The CATSL–AAC–complex was prepared as described by Munding et al. (1983). Protein and Triton concentrations were adjusted to 4.0 mg/mL and to 1.3% w/v, respectively. Aliquots of this solution were incubated with CL so as to obtain the different CL/protein ratios as indicated in the abscissa. The samples were incubated for 4 h at 0 °C followed by centrifugation at 25000*g*. The center-field amplitude was measured at 0 °C in the pellets and supernatants.

as shown in Figures 10 and 11. In one approach (Figure 10), aliquots of the protein solution were added to carefully dried films of CLSL. After incubation for 24 h at 4 °C, the samples were centrifuged at 2500*g* in glass capillaries. The supernatants were transferred into fresh capillaries, and ESR spectra were run with both pellets and supernatants. The spin densities in the pellet and supernatant fractions were evaluated in terms of the center peak amplitude rather than by double integration of the total spectra. This procedure proved to be advantageous for low-intensity spectra, since it circumvents the uncertainties due to base-line distortions. It must be noted, however, that direct comparison of signal amplitudes in terms of spin densities in the pellets and supernatants is not possible since the pellets are "pointlike" samples containing much less water than the diluted supernatants which results in an enhanced signal intensity. A maximum error of about 15% will also be introduced by the different contributions of the broadened central peak originating from the immobilized, protein-bound component.

Upon addition of increasing amounts of CLSL, the signal intensity detected in the pellet fractions runs through a maximum at an approximate CLSL/protein ratio of 2 mol/mol (Figure 10), in qualitative agreement with the changes in sample turbidity (Figure 9). The decreasing spin density in the pellets at ratios >2 mol/mol corresponds to an enhanced increase in signal amplitude in the supernatant samples. These observations suggest that the precipitated phospholipid–protein complex resolubilizes upon addition of excess cardiolipin.

The separation of solubilized CLSL and aggregated CLSL–protein complexes also shows up in the low-field part of the supernatant spectra (see insert in Figure 10). At a CLSL/protein ratio of 2.15 mol/mol (corresponding to the maximum spin density in the pellet fraction), the CLSL spectrum in the supernatant exhibits almost exclusively the mobile component, whereas at higher and also at lower CLSL concentrations the spectra reveal two superimposed components.

When the tightly binding spin-labeled inhibitor CATSL (Munding et al., 1983) was used, the protein aggregation could be followed more directly. Binding of this molecule to the protein results in an ESR line shape which indicates very strong immobilization. Thus, CATSL affords an additional advantage, since protein denaturation can be immediatedly detected in the spectrum.

The protein was labeled by CATSL as described under Materials and Methods, and protein precipitation was induced by addition of CL. Again, supernatants and pellets were separated by centrifugation of the turbid samples in glass capillaries. In this case, the total spin density in the samples and also the spectral line shape remained constant. Identical line shapes, indicating spin-label immobilization beyond the rigid limit, were found in all supernatant and pellet samples. Thus, after allowing for the enhanced ESR sensitivity in the pellets, the signal amplitudes could be evaluated in terms of the fractions of solubilized and precipitated protein, respectively. The combination of the corrected amplitudes always gave the same total signal intensity within ±10%.

The resulting distribution of the carrier protein among the pellet and supernatant samples with increasing CL concentration is shown in Figure 11. Apparently, about 70% of the protein is strongly aggregated when the ratio of CL/protein is in the range of 2–6 mol/mol. Further addition of CL obviously leads to resolubilization of the phospholipid–protein complex in good agreement with the result obtained by stepwise addition of CLSL (see above).

It must be noted that the phospholipid-induced protein aggregation is strongly dependent on the detergent concentration. At the detergent/protein ratio typically obtained after protein purification (about 1000 mol/mol), CL failed to induce protein aggregation. Conversely, addition of Triton to the aggregated lipid–protein complex leads to complete resolubilization of the aggregates. Thus, the detergent/protein molar ratio was adjusted to 350 mol/mol, which is about 2 times the detergent binding of the AAC protein as determined by ultracentrifugation (Hackenberg & Klingenberg, 1980).

DISCUSSION

Exchange Equilibria in Micelles. It is shown in the present paper that certain aspects of lipid–protein interactions may be studied in the detergent-solubilized AAC protein by the spin-label technique. ESR measurements in these solutions exhibit similar two-component spectra as have been frequently observed in reconstituted membranes after addition of suitable spin-labeled lipids [see, e.g., Marsh (1985)].

The lipid exchange equilibria involved in lipid–protein interaction in the micellar system and in bilayer membranes are probably quite different. In a bilayer membrane, it is usually assumed that lateral diffusion within the bilayer plane de-

termines the on-rate of the transient phospholipid contact with integral membrane proteins whereas the off-rate may depend on the particular (e.g., electrostatic) lipid–protein interaction (Devaux & Seigneuret, 1985). In protein–detergent–phospholipid mixed micelles, the phospholipid and detergent molecules are likely to be in an exchange equilibrium, assuming a one by one exchange:

$$PL + D \rightleftarrows PD + L$$

(P, protein; L, phospholipid; D, detergent).

Also, the intermicellar phospholipid exchange may contribute to the mean residence time of a phospholipid molecule on the protein surface. The rate of intermicellar phospholipid exchange in a micellar phospholipid–Triton X-100 mixture has, to our knowledge, not been determined so far. However, the kinetics of micelle formation have been extensively studied [for a review, see Wennerström and Lindman (1979)]. The mean dissociation rate constant of Triton micelles was evaluated on the basis of the theory of Aniansson and Wall (1974), by Hermann and Kahlweit (1980), using the temperature jump method. In the process

$$M_1 + M_{s-1} \underset{k^-}{\overset{k^+}{\rightleftarrows}} M_s$$

(M_1, monomeric Triton; M_s, Triton micelles of aggregation number s) the constant k^- was found to be 8.4×10^5 s^{-1} at 10 °C. Assuming that this rate constant applies to Triton-solubilized phospholipids, it can be estimated that the mean lifetime of a phospholipid in a mixed micelle containing 100 detergent molecules per phospholipid is about 120 μs. This estimate clearly yields only a lower limit for the intramicellar residence time of phospholipids, since the critical micelle concentration in phospholipids is about 5 orders of magnitude lower than in Triton X-100 (Tanford, 1980). Under the experimental conditions given in Figure 2 (5.3 mg of protein/mL; 1.57% w/v of Triton X-100; phospholipid to protein ratio of 3.2 mol/mol) and allowing for the known Triton binding to the AAC protein of about 158 mol/mol (Hackenberg & Klingenberg, 1980), the detergent to phospholipid ratio in those mixed micelles which do not contain a protein molecule can be calculated to be larger than 110 mol/mol. These considerations lead us to conclude that intermicellar exchange is too slow on the ESR time scale to contribute significantly to the line broadening of the fluid spectral component in the mixed micellar protein solution. Thus, at CLSL/protein molar ratios >2, the line broadening becomes negligible, and spectra of the phospholipid spin-label in Triton alone may be subtracted (cf. Figure 2C) without recurrence to empirical adjustments of temperature or sample viscosity to match the exchange-broadened mobile component in the presence of protein (Ryba et al., 1987). Even at lower molar ratios, the small exchange broadening obviously has only little effect on the evaluation of the bound vs free ratios in Figure 3.

Identification of Lipid Binding Sites. The finding of two CLSL binding sites in the solubilized AAC may be contrasted to the earlier observation of six tightly bound CL molecules per protein dimer as obtained by ^{31}P NMR spectroscopy (Beyer & Klingenberg, 1985). The extraneously added CLSL may partially displace "endogenous" phospholipids, or, alternatively, it may bind in addition to the tightly bound CL of mitochondrial origin. Exchange of CL for added CLSL on the protein surface cannot be ruled out on the basis of the present experiments. However, the finding that CLSL is almost completely bound when the molar ratio of protein/CLSL $\gg 1$ argues against displacement of the tightly bound CL for added CLSL. This assumption is also corroborated by the observation that an excess of the detergent Triton X-100 results in the partial release of bound CLSL. It has been demonstrated by ^{31}P NMR (Beyer & Klingenberg, 1985) that the binding of endogenous CL is not affected by excess Triton. Thus, it must be assumed that the spin-labeled phospholipids are less tightly bound on the protein surface than the endogenous cardiolipin detected by the NMR method. This notion is in line with the finding that the fatty acid ester bond or the nitroxide moiety is rapidly attacked by phospholipase A_2 and by ascorbate, respectively.

Specificity of Phospholipid/Protein Interaction. In reconstituted membranes, spin-label ESR studies revealed a selectivity between different phospholipids for the association with a number of integral membrane proteins. Determination of the proportion of immobilized and mobile lipids led to the conclusion that the different extent of lipid–protein interaction resides in the dissociation constants rather than in the number of binding sites for the different phospholipid classes. Relative dissociation constants (with respect to PC) could be deduced provided that the intramembranous concentration of spin-labeled molecules was low (Brophy et al., 1984; Marsh, 1985).

The reasoning underlying these calculations does not apply to the micellar system. First, in the detergent-solubilized protein, mainly detergent molecules rather than phospholipids in a host membrane compete for "binding sites" on the protein surface. Second, in the membrane reconstitution experiments, an average dissociation constant and a number of binding sites were determined by variation of the total lipid/protein ratio.

In the present experiments, it was necessary to keep the total detergent/protein ratio and the detergent concentration strictly constant. The lipid binding was determined by the Scatchard equation in analogy to binding equilibria in water-soluble enzymes:

$$b/c_{prot} = n - K_d b/c_{prot} f$$

where K_d is the dissociation constant of the lipid–protein complex, b and f are the bound and free ligand concentrations, respectively, and c_{prot} is the protein concentration. It is important to note that the apparent dissociation constant calculated from the Scatchard plot (see Figure 3) refers to the total volume of the aqueous mixed micellar solution and will therefore depend on dilution with aqueous buffer. A true dissociation constant, which would be independent of dilution and detergent concentration, requires a knowledge of the effective volume of the micelle phase and also of the activity coefficients of phospholipids solubilized in the micellar core. Thus, only an effective dissociation constant could be determined, since these data are not readily available. Also no attempt was made to determine a relative dissociation constant with respect to some neutral phospholipid, e.g., PC, since the weakness of the PC–protein interaction results in large experimental scatter which prevents the determination of a K_d value for these lipids. It may be argued, however, that there is little association selectivity for the neutral phospholipids with respect to the nonionic detergent and also that these phospholipids and the detergent molecules have access to the same number of low-affinity "binding sites".

Lipid-Induced Protein Aggregation. It can be assumed that the CL-induced aggregation of the solubilized protein also ensues from the strong interaction of the charged phospholipids with the protein surface. The precipitation of the protein is not simply caused by exceeding the solubility threshold of CL. This is shown unequivocally by the resolubilization of the aggregates when additional CL is added. Rather, the aggregation and resolubilization may be a consequence of the delicate balance between hydrophobic and ionic interactions among the protein molecules. Thus, protein precipitation may

be prevented even at reduced detergent concentration by electrostatic repulsion due to the large number of 18 uncompensated positive charges in the protein sequence (Aquila et al., 1982) which are most probably located near the protein surface.

Binding of CL molecules to the solubilized protein is likely to neutralize a corresponding number of positive charges on the protein surface, thereby leading to the formation of protein aggregates. The lipid–protein complex may again assume a negative net charge upon adsorption of more than two CL molecules, each of which confers two negatively charged phosphodiester residues onto the protein surface. The ensuing electrostatic repulsion may then lead to resolubilization of the protein aggregates.

The importance of electrostatic protein–protein interaction has been demonstrated by computing the spatial distribution of the electrostatic potential in electron-transfer proteins with known crystal structure (Matthew et al., 1983). The dependence of the electron-transfer rate on ionic strength was attributed to electrostatic interactions between the transfer proteins. Thus, it is tempting to speculate that the interaction of charged lipids with membrane proteins may also regulate protein aggregation and protein dispersion in biological membranes.

ACKNOWLEDGMENTS

We thank Renate Lafuntal and Lieselotte Schmidt-Erhard for excellent technical assistance.

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