Specific Cardiolipin Binding Interferes with Labeling of Sulfhydryl Residues in the Adenosine Diphosphate/Adenosine Triphosphate Carrier Protein from Beef Heart Mitochondria†

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ABSTRACT: The interaction of cardiolipin with the isolated ADP/ATP carrier protein from beef heart mitochondria has been studied by means of the unmasking of a single cysteinyl residue, Cys56, which accompanies the conformational transition of the protein [Leblanc, P., & Clauser, H. (1972) *FEBS Lett. 23*, 107−113]. The unmasking was monitored by using the static fluorescence of the sulfhydryl reagent *N*-(1-pyrenyl)maleimide (PYM). The rate of PYM binding that was observed after initiation of the conformational transition by ADP was drastically reduced in the presence of cardiolipin (CL). Phospholipids other than CL were much less effective. It can be shown that the conformational transition and the binding reaction are both affected by CL, although to varying extents. An enhancement of the rate of the ADP-dependent PYM binding was observed upon digestion of the protein bound phospholipid by phospholipase A_2. The phospholipase treatment also led to an increased ADP-independent PYM binding, thus indicating that the ADP control of the carrier transition was gradually lost. The ADP control could be fully restored through the addition of CL, provided that the phospholipase incubation had been terminated after approximately 1 h. These results will be discussed in relation to an earlier report of tight cardiolipin binding [Beyer, K., & Klingenberg, M. (1985) *Biochemistry 24*, 3821−3826] and to current structural models of the ADP/ATP carrier protein.

The ADP/ATP carrier (AAC)[1] is a particularly well-studied mitochondrial transport protein owing to its high concentration in the inner membrane, to its unusual stability in the presence of transport inhibitors, and to the availability of techniques for the reconstitution of transport activity (Klingenberg, 1985). Two distinct conformational states, corresponding to the cytosolic (c) and matrix (m) locations of the single nucleotide binding site, have been identified (Klingenberg et al., 1983). Transport inhibitors are available, namely, carboxyatractyloside (CAT) and bongkrecic acid (BKA), which specifically bind to the carrier in the c-state and in the m-state. A c-state inhibitor that binds more weakly than CAT is the decarboxylation product atractyloside (ATR), which, in contrast to CAT, is competitive with ADP or ATP (Klingenberg, 1985).

Various cysteinyl residues can be alkylated in the AAC protein depending on the hydrophobicity of the reagent. Two sulfhydryl residues become accessible to *N*-ethylmaleimide (NEM) through the addition of ADP (Leblanc & Clauser, 1972) which has been correlated with the c−m transition of the carrier (Klingenberg, 1974; Aquila et al., 1982a; Aquila & Klingenberg, 1982). NEM binds almost exclusively to Cys56 (Boulay & Vignais, 1984), while the more hydrophilic eosinylmaleimide reacts preferentially with Cys159 (Majima et al., 1993). Recently, cross-linking with copper−*o*-phenanthroline has shown that Cys56 protrudes into the matrix space in the m-state but not in the c-state of the protein (Majima et al., 1995).

In an earlier study, it has been shown that six molecules of cardiolipin (CL) are tightly bound to the detergent-solubilized AAC protein from beef heart mitochondria (Beyer & Klingenberg, 1985). This tightly bound phospholipid does not undergo exchange with exogeneously added cardiolipin on a time scale of hours (Schlame et al., 1991). More recently, it was demonstrated that a site directed mutation (C73S) in a yeast AAC (AAC2 from *Saccharomyces cerevisiae*) leads to partial loss of the tight CL binding and, at the same time, to an absolute requirement for the transport activity of CL (Hoffmann et al., 1994).

Here, we are making use of the unmasking of Cys56 during the nucleotide-induced conformational change for an investigation of phospholipid binding in the membrane−protein interface. The c−m rearrangement was followed in the detergent-solubilized carrier using the fluorescent dye *N*-(1-pyrenyl)maleimide (PYM). As could be demonstrated by competition and by limited proteolysis of the protein, this compound reacts with the same cysteinyl residues as NEM. Cardiolipin (CL) strongly affects the binding rate of PYM, while uncharged and negatively charged lipids other than CL are much less potent. The regulatory role of CL will be discussed with respect to a kinetic scheme which comprises the displacement of ATR by ADP, the conformational rearrangement, and the covalent labeling reaction.

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[1] Abbreviations: AAC, ADP/ATP carrier protein; ATR, atractyloside; BHM, beef heart mitochondria; BKA, bongkrecic acid; CAT, carboxyatractyloside; CL, cardiolipin; ESR, electron spin resonance; MOPS, 2-(*N*-morpholino)ethanesulfonic acid; NEM, *N*-ethylmaleimide; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PYM, *N*-(1-pyrenyl)-maleimide.

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MATERIALS AND METHODS

Chemicals and Enzymes. The inhibitors ATR and CAT were obtained from Sigma and from Boehringer, Mannheim. BKA was a generous gift from Professor W. Berends (Delft). PYM and NEM were from Sigma. Phospholipids were purchased from Sigma or from Avanti Polar Lipids, Inc., Alabaster, AL. The lipids were checked for purity by thin-layer chromatography. Phospholipase A_2 from pig pancreas was from Sigma and the crude venom of *Trimesurus flavoviridis*, containing phospholipase A_2, was obtained from Boehringer, Mannheim. Dimethyl-CL was essentially prepared as described previously (*Organic Syntheses*, 1963). Briefly, dry bovine heart CL was converted into the free acid by 0.1 N HCl and treated in $CHCl_3/CH_3OH$ solution with an excess of diazomethane at 0 °C. The solvents were removed by a stream of nitrogen and the product was dried in vacuo. The product was used without further purification. Monolyso-CL was prepared by cleavage with the *T. flavoviridis* venom as described by Okuyama and Nojima (1965) and purified by preparative thin-layer chromatography.

Sample Preparation. The ATR-loaded ADP/ATP carrier protein was isolated from beef heart mitochondria with Triton X-100 in a buffer with 100 mM Na_2SO_4, 10 mM MOPS, and 0.05 mM EDTA at pH 7.2, essentially as described previously by Aquila and Klingenberg (1982). The ADP/ATP carrier in the CAT-loaded form was prepared as described by Riccio et al. (1975a,b). Protein fractions of the ATR−carrier complex from the final column (Ultrogel AcA 34, LKB) containing 40−70 μmol of lipid phosphorus/mg of protein were collected for binding experiments. Earlier protein fractions with less than 30 μmol of phosphorus/mg of protein were discarded as the carrier was found to be less stable at low phospholipid/protein ratios, in contrast to the CAT−AAC complex (Beyer & Klingenberg, 1985; Drees & Beyer, 1988). The Triton X-100 concentration was 0.2−0.4% (w/v) and the total Triton/phospholipid ratio was typically >30 (mol/mol).

Fluorescence Labeling of the Isolated Carrier. In a typical experiment, the ATR−protein complex was diluted with 100 mM NaCl, 0.05 mM NaN_3, and 10 mM MOPS, pH 7.2, to a final concentration of 0.05 mg/mL. Protein rearrangement and sulfhydryl alkylation was initiated by the sequential rapid addition of 5 μL of PYM in absolute ethanol (0.12 mg/mL) and of 5 μL of ADP (2.4 mM) with a spatula to 200 μL of the protein solution in a 5-mm × 5-mm quartz cell. The fluorescence of PYM was excited at 340 nm and the emitted light was measured at 375 nm using a Perkin-Elmer MPF-44 fluorescence spectrophotometer equipped with an XBO 150 W/1 xenon lamp. The excitation and emission slits were set at 4.0 and 5.5 nm, respectively.

Phospholipase Treatment. Pig pancreas phospholipase A_2 was added from an enzyme suspension (approximately 7000 units/mL) as indicated in the figure legends. Phospholipid cleavage was initiated by addition of $CaCl_2$ from a stock solution at a final concentration of 1 mM. The cleavage reaction was stopped by addition of a 2-fold excess of EDTA. For NMR experiments, the enzyme and $CaCl_2$ were directly added to the NMR tube.

Phosphorus NMR Spectroscopy. NMR spectra were acquired using a Varian VXR 400 spectrometer operating at a phosphorus frequency of 161 MHz. Spectra were obtained at 10 °C with $^{31}P-\{^1H\}$ broadband proton decoupling. Chemical shifts were referenced to 85% phosphoric acid.

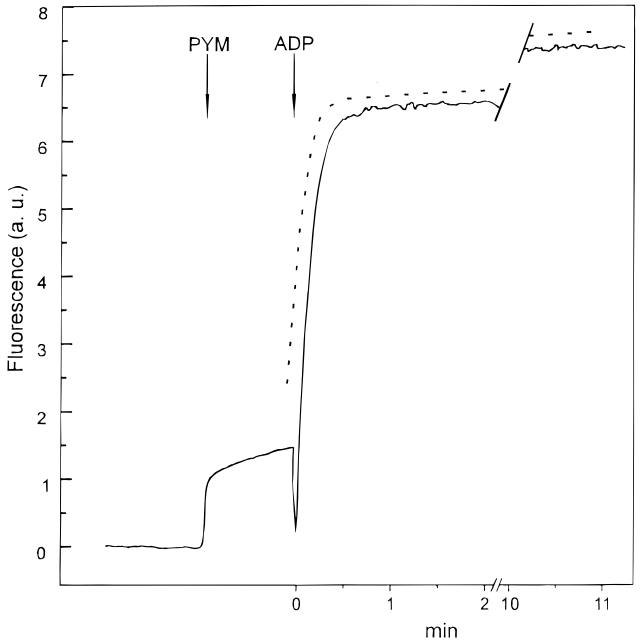

FIGURE 1: Unmasking of cysteinyl residues in the AAC protein upon c−m rearrangement as detected by static PYM fluorescence. The rearrangement was initiated by the addition of ADP. Protein concentration, 0.05 mg/mL. ADP and PYM were added at 80- and 14-fold molar excess, respectively. Dashed curve: nonlinear least-squares fit according to eq 4 using the standard Marquardt method. The fitted parameter values were $a_0 = -0.2 \pm 3.9$, $a_1 = 2.1 \pm 0.8$, $a_2 = 112.6 \pm 3.6$, and $k = 10.7 \pm 0.6$. The variance χ^2 of the data set was 4.06.

RESULTS

Conformational Rearrangement and PYM Binding. The AAC protein was isolated from beef heart mitochondria as an ATR−AAC complex in order to stabilize the protein in the c-conformation during membrane solubilization and protein purification (Klingenberg, 1985). The transition from the c- into the m-conformation was initiated by an addition of ADP, which competes with the binding of ATR. The fluorescence of the thiol reagent PYM was used to monitor the transition, taking advantage of the unmasking of sulfhydryl residues (Leblanc & Clauser, 1972; Aquila et al., 1982a; Aquila & Klingenberg, 1982). The fluorenscence level reached after a few minutes signifies alkylation of the reactive cysteinyl residues and completion of the c → m rearrangement (Figure 1). At the same time, the carrier is irreversibly trapped in the m-conformation (Aquila et al., 1982a; Aquila & Klingenberg, 1982).

The sequence of additions shown in Figure 1 was chosen in order to alkylate any reactive sulfhydryl residue which may be present *before* starting the c−m rearrangement by ADP. There is indeed a rapid, though small, increase in fluorescence immediately after the addition of PYM, probably as a result of partial protein denaturation. The extent of this ADP-independent rapid PYM binding was somewhat variable among different protein fractions. Notably, in early column fractions containing less than 20 mol of phospholipid

phosphorus/mg of protein, the ADP-independent PYM binding sometimes amounted to more than 30% of the total PYM binding obtained after the addition of ADP.

The overall process involves a sequence of consecutive reactions, namely, the displacement of carrier-bound ATR by ADP (eq 1), the rearrangement of the protein from the c- into the m-state (eq 2), and the covalent maleimide binding (eq 3):

$$\mathrm{ATR{\cdot}AAC_c} + \mathrm{ADP} \leftrightarrow \mathrm{ADP{\cdot}AAC_c^*} + \mathrm{ATR} \quad (1)$$

$$\mathrm{AAC_c^*} \overset{\mathrm{ADP}}{\leftrightarrow} \mathrm{AAC_m^*} \quad (2)$$

$$\mathrm{AAC_m^*} + 2\mathrm{PYM} \rightarrow \mathrm{PYM_2{-}AAC} \quad (3)$$

In the above scheme, the c- and m-conformations are denoted by AAC_c and AAC_m, respectively, and the asterisk accounts for the fact that the carrier protein is in a labile, transport-competent form after the displacement of ATR by ADP. Assuming the reversibility of eqs 1 and 2 and taking into account that ADP has been added in 80-fold molar excess (eq 1), the total rate is expected to follow an exponential rate law. Observation of the fluorescence over more than 30 min, however, showed that the PYM fluorescence slowly increases further in an apparently linear fashion rather than approaching a maximum level. Thus, the time dependence of the increase in fluorescence was fitted to an appropriate kinetic model:

$$F = a_0 + a_1 t + a_2(1 - \mathrm{e}^{-k^* t}) \quad (4)$$

where a_0 accounts for the fluorescence observed without ADP addition, a_2 for the initial exponential, and a_1 for the linear fluorescence increase observed after completion of the initial rapid reaction. The determination of k will be useful for an assessment of the initial rate of PYM binding. A four-parameter least-squares fit to the fluorescence data according to eq 4 is shown in Figure 4.

Boulay et al. were able to identify Cys56 as the preferential binding site of the sulfhydryl reagent NEM (Boulay & Vignais, 1984). Labeling of Cys56 is much faster (on a time scale of minutes) than NEM binding to the remaining three cysteinyl residues (Cys128, -159, and -256), as shown more recently by Majima et al. (1993). The question of whether PYM binds to the same residue as NEM was addressed by a competition experiment (Figure 2). Preincubation with NEM and ADP *before* PYM addition leads to an increasing suppression of the PYM fluorescence (depending on the preincubation time), as shown by the spectrofluorometer traces in Figure 2. Complete suppression of PYM binding will occur after 5 min of preincubation, indicating that NEM and PYM compete for the same binding site. Likewise, NEM terminates the fluorescence increase when added *after* PYM and ADP (not shown).

This result was confirmed by cleavage of the PYM-labeled AAC protein by CNBr (experiment not shown) according to Babel et al. (1981). The label was found exclusively in the large N-terminal fragment ($M_r \approx 22\,000$), which contains three cysteinyl residues, including Cys56 (Aquila et al., 1982b). Thus, similar to NEM (Boulay & Vignais, 1984; Majima et al., 1993), PYM seems to react much faster with Cys56 than with the single cysteinyl residue (Cys256) in the smaller C-terminal CNBr fragments (Majima et al., 1993, 1994).

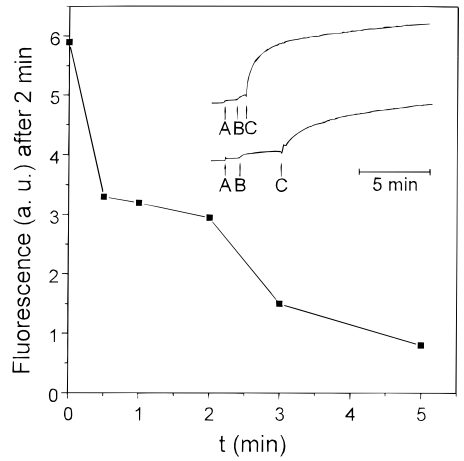

FIGURE 2: Competition between NEM and PYM. The AAC protein (0.05 mg/mL) was preincubated with ADP, and NEM was added after 1 min. PYM was added after the time t as indicated in the abscissa, i.e., ADP−1 min−NEM−t−PYM. The fluorescence was read 2 min after PYM addition. Inset: Representative spectrofluorometer traces. (A) ADP; (B) NEM; (C) PYM.

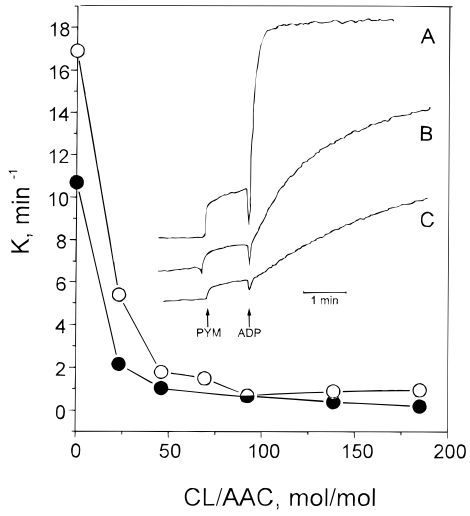

FIGURE 3: CL dependence of the initial PYM binding rate (cf. eq 4). Sequence of additions: (●) CL−5 min−PYM−1 min−ADP; (○) ADP + BKA−1 min−CL−5 min−PYM. BKA was added in 50-fold molar excess. Other additions took place as outlined in Figure 1. Before addition of CL, there was a total ratio of phospholipid phosphorus/protein of 64 (mol/mol) in the protein preparation. Inset: Spectrofluorometer traces obtained without BKA preincubation. (A) without CL; (B) molar ratio of added CL/protein, 46; (C) molar ratio of added CL/protein, 139.

Phospholipid Dependence of the PYM Binding Rate. It has been demonstrated earlier by ^{31}P NMR spectroscopy that there are approximately six tightly bound CL molecules in the solubilized AAC protein from beef heart (Beyer & Klingenberg, 1985). A remarkable dependence of the PYM binding rate on the presence of *added* CL was observed here as shown in Figure 3. Beef heart CL was added from a stock solution in Triton X-100 and the detergent concentration was held constant during the titration by appropriate dilution of the stock solution. Variation of the detergent concentration by ±15% had little effect on the rate of the fluorescence increase. The rate constant k (eq 4) decreases considerably upon titration of the protein with CL. It can be also recognized (spectrofluorometer traces in Figure 3) that CL

Table 1: Effect of Various Phospholipids on the Initial PYM Binding Rate[a]

phospholipid added	rate constant k[b]
control[c]	100.0
dimethyl-CL	113.7
monolyso-CL	97.1
egg PC	82.5
egg PE	63.1
egg PG[d]	54.5
dioleyl-PG	40.2
egg PA[d]	32.0
dioleyl-PA	28.7
E. coli CL	1.3
beef heart CL	0.8

[a] The ATR−AAC complex (0.09 mg/mL) was preincubated for 30 min at 0 °C with 75 mol of phospholipids/mol of AAC protein. The total phospholipid/protein ratio before phospholipid addition was 69 mol/mol. The Triton X-100 concentration was kept constant at 0.3% (w/v). [b] Percent of control value (k = 9.8 min^{-1}). Errors were less than 10% in three consecutive experiments. [c] Without phospholipid addition. [d] Prepared from egg PC.

has the additional effect of suppressing the ADP-independent PYM fluorescence. Similar results were obtained when CL was dried from a chloroform/methanol solution and the deposits were solubilized in the Triton-containing protein solution (not shown).

Preincubation of the protein with ADP and with the transport inhibitor BKA can be employed to allocate the steps in eqs 1−3 that are affected by CL. Simultaneous addition of ADP and BKA results in the transition of the carrier into the m-conformation. Noncovalent BKA binding stabilizes the m-state while it does not interfere with the unmasking of Cys56 (Erdelt et al., 1972; Klingenberg et al., 1983; Munding et al., 1987; Majima et al., 1993). It may be expected that the labeling rate would become CL-independent after the rearrangement into the m-state if CL only affected the ATR displacement and the conformational transition, i.e., eqs 1 and 2. The results shown in Figure 3 (open symbols), however, indicate that the CL effect must be attributed primarily to competition with the maleimide binding rather than to the ADP/ATR exchange or to the conformational transition of the carrier. The former steps may only be involved at CL/AAC molar ratios <50. Without CL addition, ADP + BKA preincubation indeed led to an enhancement of the rate constant k by approximately 30% (Figure 3). It may be further noted that, after preincubation with CL and ADP yet without BKA, the binding rates were similar to those obtained after preincubation with CL alone, indicating that in the presence of CL the inhibitor displacement is not rate-limiting (experiment not shown).

An important question is whether the lipid effect on the PYM binding rate is *specific* to CL. Other phospholipids, notably those with net negative headgroup charges or lipids which are structurally related to CL, may be suspected of interacting with the AAC protein. This problem was addressed by comparing the rate of fluorescence increase after preincubation with a number of charged and zwitterionic phospholipids. At a molar ratio of added lipid to AAC protein of 75/1, the addition of CL results in a reduction of the normalized binding rate by >98% of the control value (Table 1). Such charged lipids as PG, PA, and monolyso-CL, which may be considered as fragments of the CL molecule, are much less effective as compared to the control without phospholipid addition. Even a slight enhancement

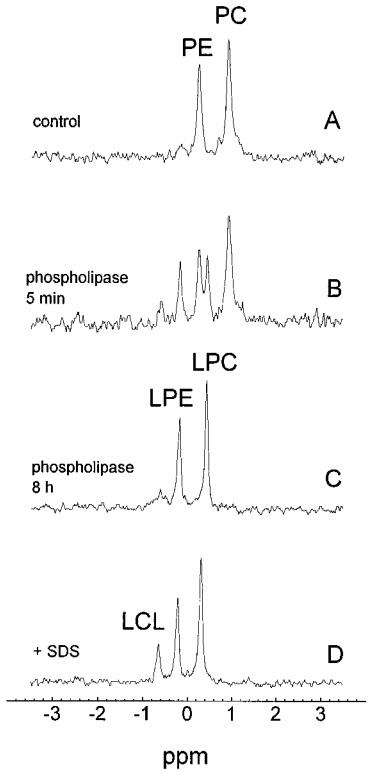

FIGURE 4: Phosphorus NMR spectra showing the phospholipase A_2 cleavage of phospholipids in a sample of the detergent-solubilized CAT−AAC complex. (A) Control spectrum. Protein concentration 3.3 mg/mL. (B) Spectrum acquired 5 min after the addition of approximately 70 units of pig pancreas phospholipase A_2. (C) Spectrum acquired 8 h after enzyme addition. (D) After addition of 1% (w/v) sodium dodecyl sulfate. Temperature, 10 °C.

of the rate constant was observed in the presence of dimethylcardiolipin (denoted by dimethyl-CL in Table 1), which has no headgroup charge at all. Thus, it can be concluded that CL indeed interacts in a specific manner with the AAC protein. Obviously both the diphosphatidylglycerol structure and the two discrete negative headgroup charges of CL are necessary for this lipid−protein interaction.

Phospholipase Cleavage of Protein-Bound CL. The tightly protein-bound CL fraction that has been identified previously by ^{31}P NMR spectroscopy (Beyer & Klingenberg, 1985) can be cleaved by phospholipase A_2, in spite of its association with the protein surface (Figure 4). The AAC protein, isolated as CAT−protein complex from CAT-preloaded mitochondria (Riccio et al., 1975a,b), was incubated with phospholipase A_2 in the NMR tube. ^{31}P NMR spectra were recorded during the incubation time of several hours. The signals of the residual uncharged lipids, namely, PC and PE, gradually disappear in favor of the respective signals of lyso-PC and lyso-PE. The lyso-CL produced by the phospholipase treatment remains loosely bound to the protein as can be concluded from the broad signal centered at −0.6 ppm. Addition of 1% (w/v) sodium dodecyl sulfate eventually results in drastic line narrowing, indicating that lyso-CL has been displaced from the protein binding sites.

This result prompted us to study the effect of phospholipase A_2 treatment on the sulfhydryl unmasking as seen by the rate of PYM binding. The most noticeable consequence of phospholipase treatment is a partial loss of the ADP control of the labeling reaction as shown in Figure 5A−C. At the same time, the ADP-independent labeling increases with phospholipase incubation. The ADP dependence of the

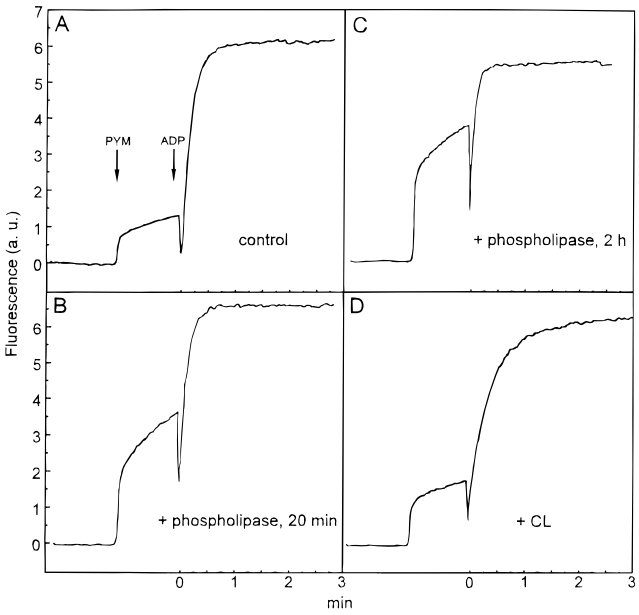

FIGURE 5: Effect of phospholipase treatment and CL replenishment on the ADP control of PYM binding. (A) Control experiment without phospholipase addition. Protein concentration 0.05 mg/mL, total phospholipid/protein ratio 64 (mol/mol) before CL addition. (B, C) The ATR−AAC complex (1 mL) was incubated at 4 °C with 20 units of phospholipase A_2 for 20 min (B) or for 2 h (C). (D) Preincubation with CL for 5 min after 20 min of phospholipase treatment; CL (added)/protein, 0.2 μmol/mg. The fluorescence increase elicited by ADP is slower here than in panel A, since the amount of CL added represents approximately 4 times the CL present in the control experiment.

labeling reaction returns, however, when CL is added after the enzyme treatment (Figure 5D).

There are three observations that must be noted, in addition to the results shown in Figure 5. First, after prolonged phospholipase treatment ($\gg$2 h), CL replenishment did not restore the ADP control, indicating that CL digestion eventually leads to denaturation of the carrier. Second, addition of CAT after termination of the cleavage reaction by EDTA had almost no effect on the ADP-independent increase in fluorescence. Thus, after partial digestion of the tightly bound CL, CAT alone is unable to reestablish the original c-state conformation. Third, among the phospholipids other than CL, only egg PA (prepared from egg PC) had a marginal effect on the ADP control, again indicating that the lipid−protein interaction is highly selective for CL.

Phospholipase digestion also leads to an enhancement of the ADP-dependent rate of maleimide binding (Figure 6). The quantitative analysis became incorrect after the phospholipase treatment due to the extremely rapid initial binding rate. Therefore, the AAC protein was first concentrated approximately 4-fold by pressure dialysis and then diluted to a final concentration of 0.05 mg/mL. This protocol, for unknown reasons, resulted in an approximately 3-fold reduction of the binding rate (cf. Figure 3) while the phospholipid sensitivity and the ADP control were not affected. Figure 6 summarizes the rate constants obtained after ADP addition for a number of phospholipase incubation times and at two different CL concentrations. It can be recognized that the addition of CL at an effective molar ratio of 60 mol/mol of the AAC protein results in a similar initial binding rate as obtained before phospholipase treatment and without CL addition.

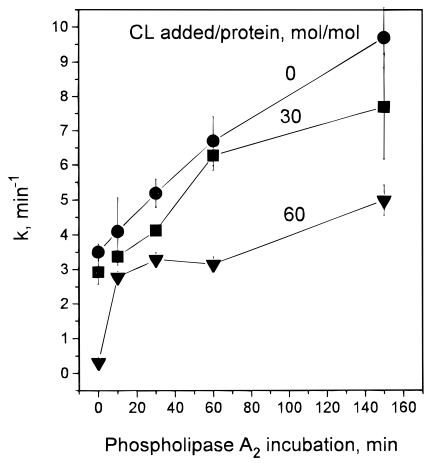

FIGURE 6: Effect of phospholipase A_2 treatment on the initial rate of PYM binding. Residual phospholipid phosphorus before CL addition, 19.8 mol/mol of AAC protein. Phospholipase, 87 units/mg of AAC protein. The phospholipase was stopped by addition of EDTA and aliquots were preincubated without CL (●) or with CL at ratios of CL/AAC protein of 30 (mol/mol) (■) and 60 (mol/mol) (▼).

DISCUSSION

The present results show that CL interferes with the alkylation of Cys56 in the mitochondrial AAC. The observation that the labeling rate in the m-state (i.e., in the presence of the inhibitor BKA; cf. Figure 3) is strongly affected by CL suggests that diffusion of the maleimide label toward the reactive sulfhydryl residues is impeded. The association of CL may be assumed to result in a partial occlusion of the surface domain that embodies Cys56. This is in line with the pronounced enhancement of the labeling rate after phospholipase treatment of the detergent−phospholipid−protein complex (Figure 6). According to ^{31}P NMR, the protein-associated CL is easily cleaved by phospholipase A_2 while the lipid headgroup remains in an immobilized state (Figure 4). The occluding effect of CL obviously involves the four acyl chains of the protein-bound lipid, indicating that the specificity of CL (cf. Table 1) cannot be attributed to the electrostatic headgroup interaction alone. Rather, it must be concluded that this lipid−protein interaction resides in the complete CL structure including the diphosphatidylglycerol headgroup and the four acyl chains of the phospholipid.

Once locked in the c-state, the carrier undergoes the c−m rearrangement only upon the addition of ADP or ATP. The progressive loss of the ADP control of sulfhydryl unmasking upon phospholipase digestion of the protein-associated CL and the return of the control after CL addition point at the importance of selective CL binding for the structural integrity of the carrier. The failure of CL replenishment to restore the ADP control after extensive phospholipase treatment indicates that the carrier protein enters a conformationally labile state when bound CL is removed. Addition of CL improves the ADP control to some extent even without phospholipase treatment (cf. Figure 3). Taken together, these observations suggest that CL binding selectively stabilizes the c-conformation of the carrier protein (cf. eq 1). It may be noted that the recently discovered single-channel conductance of the AAC was attributed to Ca^{2+} complexation of the carrier-bound CL, which results in channel opening

even in the presence of the c-state inhibitor CAT (Brustovetsky & Klingenberg, 1996).

The number of CL binding sites is not directly available from the present results. An unusually tight binding of approximately 6 CL molecules/protein dimer has been previously observed by NMR spectroscopy in the detergent-solubilized protein (Beyer & Klingenberg, 1985). The interaction of *additional* negatively charged phospholipids, cardiolipin, phosphatidic acid, and phosphatidylserine, but not phosphatidylglycerol, was later shown by spin-label ESR spectroscopy (Drees & Beyer, 1988; Horvath et al., 1990). Line shape analysis revealed a saturation behavior for spin-labeled CL which is in conflict with a simple association model (Brotherus et al., 1981). These findings again led to the conclusion that there are more binding sites for CL at the protein surface than those obtained by ^{31}P NMR spectroscopy in the solubilized protein (Horvath et al., 1990). It is important to realize that the time scales pertinent to ^{31}P NMR and spin-label ESR are quite different. The tight CL association detected by ^{31}P NMR (Beyer & Klingenberg, 1985) has a lifetime of more than an hour (Schlame et al., 1991), while lipid protein interactions are observed on a microsecond scale by spin-label ESR (Drees & Beyer, 1988; Marsh, 1981).

The lipid dependence of the translocation activity has been studied by reconstitution of the bovine heart AAC into unilamellar phospholipid vesicles (Krämer & Klingenberg, 1977, 1979). The presence of negatively charged lipids resulted in an enhancement of the translocation rate in a nonsaturable manner (Krämer & Klingenberg, 1980; Krämer, 1983). It is noteworthy that the rate of PYM binding which *involves* the c−m transition (i.e., half of the translocation cycle) is reduced rather than enhanced by CL and, to a much smaller extent, by other negatively charged phospholipids. Kinetic resolution of individual steps within the route of PYM labeling, eqs 1−3, may not be feasible in the heterogeneous micellar system. Qualitatively, however, it can be concluded that the covalent maleimide binding becomes rate-limiting at CL/AAC molar ratios >50 (Figure 3), in agreement with the proposed partial occlusion of the PYM binding sites. Thus, the presently observed CL effect is not inconsistent with the phospholipid modulation of the reconstituted carrier activity (Krämer & Klingenberg, 1980; Krämer, 1983) since it does not directly account for the conformational transition of the membrane-reconstituted AAC protein.

A striking dependence of the exchange activity *in vivo* on the presence of CL was found in the AAC2 protein from yeast where certain amino acids had been replaced by site-directed mutagenesis (Klingenberg & Nelson, 1995). Specifically, the exchange of Cys73 for serine (C73S) and of Lys179 and Lys182 for isoleucine (K179I, K182I) resulted in a strict dependence of the transport activity on the presence of CL in the reconstituted system (Hoffmann et al., 1994). Phospholipids other than CL, notably acidic species, had little or no activating effect. Oxidative phosphorylation and nucleotide transport were not impaired in these mutants as they were able to grow on glycerol. This apparent contradiction was resolved when it could be shown by ^{31}P NMR that the C73S mutant AAC has a considerably lower CL binding than the wild-type carrier, indicating that CL had been lost through isolation of the protein (Hoffmann et al., 1994). Although thus far not proven experimentally, it can be assumed that the tight CL binding is also reduced in the yeast K179I and K182I mutants, a condition in agreement with the observation that CL must be replenished for full activation in reconstitution experiments (Klingenberg & Nelson, 1995).

Cys73 may be assumed to be equivalent to Cys56 in the bovine heart AAC, while Lys179 and Lys182 probably correspond to Lys162 and Lys165 in the mammalian protein. According to a structural model which incorporates the assumption of amphipathic helices, a total of six helical transmembrane domains connected by less hydrophobic loops have been postulated (Klingenberg, 1989). The lysine residues 162 and 165 are believed to be localized in a hydrophilic loop between helices 3 and 4 on the matrix side of the mitochondrial membrane. It can be assumed that these residues are involved in the electrostatic binding of the CL headgroup (Bogner et al., 1986), while additional electrostatic contacts are necessary to account for the total CL binding including the six tightly bound lipid molecules (Beyer & Klingenberg, 1985). Further mutagenesis experiments directed toward the positively charged residues in the interfacial and loop regions of the putative AAC structure will be required to identify these binding sites.

The observation that the CL binding is affected by mutagenesis of the conformationally sensitive cysteinyl residue, e.g., Cys73 in the yeast AAC2 as observed by Hoffmann et al. (1994), may then be attributed to the conformational coupling between the first loop region and the CL binding site, leading to the conclusion that CL is necessary for the stabilization of the AAC structure in its well-defined functional states. Strictly speaking, the tightly bound lipid fraction may be analogous to a prosthetic group of the protein, the removal of which eventually results in a complete loss of carrier function.

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