The Reconstituted ADP/ATP Carrier Activity Has an Absolute Requirement for Cardiolipin as Shown in Cysteine Mutants*

(Received for publication, June 14, 1993, and in revised form, September 1, 1993)

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Although the site-directed C73S mutation in the ADP/ATP carrier (AAC) AAC2 gene from Saccharomyces cerevisiae produced a glycerol-positive strain, indicating that the mutant AAC is active, on isolation and reconstitution in egg yolk phosphatidyicholine, the C73S AAC had no transport activity, whereas the wild-type AAC was fully active. Only on addition of cardiolipin was an exchange activity with the C73S AAC obtained. The AACs isolated from the other cysteine mutants did not (C244S) or only marginally (C271S) require cardiolipin for transport on reconstitution. [3H]Carboxyatractylate binding as a measure of incorporated AAC molecules was unchanged on addition of cardiolipin in all mutants, indicating that cardiolipin does not increase the incorporation of the AAC. It also shows that cardiolipin is required only for translocation and not for binding. The activity of the C73S mutant AAC shows half-saturation with cardiolipin at 2% by weight or at 1.15 mol % in the phosphatidylicholine vesicles. Other acidic phospholipids tested such as phosphatidylserine and phosphatidic acid did not affect. Among various cardiolipin derivatives, the selectivity for cardiolipin is high. Only monolyso cardiolipin still retains 12% activity. After removal of the bulk of phospholipid, the content of bound phospholipids was assayed by 31P NMR. By unmasking with SDS, in the wild-type AAC and in the C73S AAC, 6.4 mol and only 1.8 and 2.9 mol of bound cardiolipin/mol of AAC dimer are found, respectively. Presumably, on isolation, cardiolipin is lost from the more labile C73S mutant AAC. Although the absolute requirement for cardiolipin is unique for the C73S AAC, it is concluded that in this mutant, the unmasking of the cardiolipin requirement demonstrates a general cardiolipin requirement of the wild-type AAC and of AACs from other sources.

Cardiolipin (CL) is distinguished from other phospholipids by having two negative charges and carrying four acyl groups. These negative charges make it a good candidate for phospholipid-protein interaction in mitochondria. Therefore, it is no surprise that extensive literature exists on the role of CL in mitochondria. The involvement of CL has been studied on different levels: (a) activation of enzymatic activity, (b) interaction and binding with specific proteins, and (c) the general role in energy transduction. Among the proteins most frequently cited for CL interaction are the mitochondrial carriers. CL addition proved to be extremely useful for the isolation and separation of several mitochondrial carriers, especially in the work of the group of Palmieri (for review, see Krämer and Palmieri (1989, 1992)). Specific binding of CL to mitochondrial carriers has so far only been reported for the ADP/ATP carrier (AAC) (Beyer and Klingenberg, 1985). With 31P NMR, tight binding of 6 molecules of CL to the isolated AAC dimer was determined by unmasking the 31P-labeled signal of CL after denaturation with SDS. Thus, the AAC binds more CL than reported so far for any other mitochondrial protein. Since the removal of CL required denaturation, it was not possible to demonstrate a requirement of CL for AAC transport activity. Previously, on reconstitution of the bovine AAC, CL was shown to enhance transport activity (Krämer and Klingenberg, 1986; Brandolin et al., 1980). However, no specific requirement for CL addition was observed. It could be argued that this requirement was met by the tightly bound CL. Also on reconstitution, Pi and citrate transport was claimed to be increased specifically by CL (Kadenbach et al., 1982). However, this could not be confirmed in later work (Mende et al., 1983; Wohlrab et al., 1984), and the effects were suggested to be due to improved incorporation rather than activation per se (Krämer and Palmieri, 1989). During studies of structure-function relationships of the AAC from Saccharomyces cerevisiae by site-directed mutants (Klingenberg et al., 1992), an unexpected requirement for CL in the reconstitution of the AAC from cysteine mutants was observed. The requirement for CL is absolute and highly specific. Evidence is presented that suggests that the dependence on CL addition is due to a loss of bound CL on the isolation of this mutant AAC. Thus, the unmasking of the requirement for CL in this C73S mutant AAC allowed us to demonstrate that CL is an activator of a mitochondrial carrier. This conclusion may apply not only to this mutant, but also to the wild-type AAC from yeast and to AACs from other cells.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidylcholine from turkey egg yolk (Sigma) was partially purified. CL from bovine heart, phosphatidylglycerol, phosphatidylserine, and phosphatidic acid were obtained from Sigma (Munich, Germany). Monolysosy-CL was from Avanti Polar Lipids, Inc. (Birmingham, AL). The CL derivatives, such as acyl-CL, were prepared by acylation of CL using the acylanhydrides (oleoylanhydride, myristoylanhydride, and acetylacylanhydride) as described by Knirsch et al. (1989). Dilysosy-CL was generated by phospholipase A2 from pig pancreas (Boehringer Mannheim). C12:0E2 (octaethylen glycol monododecyl ether) was obtained from Fluka.

The S. cerevisiae strains and the construction of the mutants were as described previously (Lawson et al., 1990; Gawaz et al., 1990). The yeast strains were grown as described (Gawaz et al., 1990). The isolation of the mitochondria followed essentially the described procedure.

Reconstitution—Reconstitution and measurement of exchange activity and of [3H]Carboxyatractylate binding were performed as elsewhere described.

* This work was supported by NATO Travel Grant RG074487 and a grant from the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: CL cardiolipin; AAC, ADP/ATP carrier; CAT, carboxyatractylate; ATR, atractylate.
RESULTS

To explore the structure-function relationship, site-directed mutagenesis was applied to the ADP/ATP carrier (AAC2) in S. cerevisiae (Gawaz et al., 1990; Klingenberg et al., 1992). The dependence on fermentable sources of yeast mutant cells was an important indicator for an impairment of AAC activity by the mutation. Of particular interest was the role of Cys-73, which could be classified as an essential cysteine since alkylation of the homolog Cys-66 in the bovine heart AAC inhibited transport (Boulay and Vignais, 1984). However, the C73S mutation in yeast AAC2 resulted in a mutant that was able to grow on nonfermentable carbon sources, such as glycerol, indicating that the AAC was still functional (Klingenberg et al., 1992). The reconstitution of the isolated carrier from this mutant, however, produced no activity under the same conditions in which the wild-type AAC was fully active. This discrepancy from what one would expect from the growth conditions of the whole mutant cells was finally resolved when it was discovered that CL addition to the liposomes activated the mutant AAC. A crucial experiment is reproduced in Fig. 1, where the ADP/ATP exchange activity is given for both the wild-type and mutant AACs. Whereas the wild-type AAC was fully active and not further activated by CL, the C73S mutant AAC was completely inactivated unless CL was admixed to the phospholipid. Also, the 2 other cysteines (Cys-244 and Cys-271) in AAC2 were changed to serine. As shown in Table I, the isolated AACs from these mutants were quite active even without CL. Addition of CL stimulated the exchange rate 1.5-fold only in the C271S AAC. No dependence on CL of reconstituted AAC2 from any of the other mutants or revertants has been observed so far.

Table I also indicates the amount of AAC molecules incorporated into the vesicles as determined by [3H]CAT binding. In all these cysteine mutants, [3H]CAT binding was retained in the proteoliposomes nearly to the same extent as in the wild-type AAC. Upon admixture of 8% CL, binding was partially decreased. This effect is unexplained, but may be due to partial aggregation of the CL-containing proteoliposomes that do not pass the column. At any rate, the data show that the activation by CL is not due to an increase in the incorporation of AAC. Furthermore, it can be concluded that the transport-inactive AAC still retains its binding capacity for CAT.

The dependence of the exchange rate on the content of added CL is shown in Fig. 2. The uptake rate is virtually zero without CL addition and increases linearly up to 4% CL. Maximum activation is reached at 8–10% CL. At 15 and 20% CL, the uptake rate is diminished. The content of CL required for half-maximum activation is 2.0% by weight, which corresponds to 1.14 mol % bulk PC.

Fig. 2 also shows the dependence on CL content of [3H]CAT binding to the proteoliposomes. Within the considerable inaccuracy of these measurements, there is no increase in binding related to CL content. Instead, at CL content exceeding 8% by weight, [3H]CAT binding decreases. It seems that higher amounts of CL decrease the incorporation of AAC into the liposomes, and this causes a diminished transport activity when the activating effect is overcompensated by the decrease in AAC incorporation.

The requirement for CL addition provides a unique opportunity to study the specificity of the activating effect with respect to the other acidic phospholipids and to variations in the CL structure (Table II). Acidic phospholipids such as phosphatidylserine and phosphatidic acid did not activate the exchange activity to a marked extent. A number of CL derivatives and analogs were tested to elucidate their structural requirement for the activating function. CL (monolysocardiolipin) with one acyl chain removed still retained 12% of the original activity. Loss of both acyl groups from the β-positions (dilysocardiolipin) resulted in a completely inactive product. Substitution with acyl groups at the β-position of the central glycerol also inactivated CL to a large extent. With myristoyl-CL and oleoyl-CL, 10 and 8% activity was still retained, respectively. No activity was seen with acetyl-CL. Surprisingly, neutralization of the two negative charges by esterification in dimethyl-CL still produced ~9% of the original activity. This would be in line with the finding that the CL analog dioleoylphosphatidylglycerol, which contains only one negative charge, still produces 15% activity. In conclusion, small modifications in the CL structure strongly diminish the ability to activate the AAC exchange. The right configurations of both the acyl groups and the charges are important.

The amount of incorporated AAC was determined by [3H]CAT binding. Measurement of ADP/ATP exchange activity was at 15 °C. The proteoliposomes were reconstituted with and without 8% CL by weight, and all experimental details are described under "Experimental Procedures."

**Table I**

<table>
<thead>
<tr>
<th>Mutant AAC</th>
<th>Exchange rate</th>
<th>[3H]CAT binding</th>
<th>Molecular activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol ADP / g protein / min</td>
<td>μmol / g protein</td>
<td>min⁻¹</td>
</tr>
<tr>
<td>C73S</td>
<td>5</td>
<td>3.6</td>
<td>1.5</td>
</tr>
<tr>
<td>C944S</td>
<td>902</td>
<td>6.3</td>
<td>143</td>
</tr>
<tr>
<td>C271S</td>
<td>420</td>
<td>3.1</td>
<td>135</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1190</td>
<td>6.8</td>
<td>175</td>
</tr>
</tbody>
</table>

**Cardiolipin Requirement of ADP/ATP Carrier**

1941

![Graph](image1.png)

**Fig. 1. Influence of cardiolipin on ADP exchange transport of reconstituted wild-type and C73S mutant AACs.** Shown is the time course of [14C]ADP uptake into ADP-loaded proteoliposomes. Proteoliposomes were prepared either with or without 8% CL added to egg yolk phosphatidylcholine. The exact conditions are described under "Experimental Procedures."
Klingenberg, 1985). The sucrose contains Cl2ES to prevent protein carried over from the mitochondria in the AAC preparation. For this purpose, a major portion of the unbound phospholipids were removed by sucrose gradient centrifugation.

Yeasts. Only free or very loosely bound phospholipids will precipitate from the bulk of the protein fractions.

The amount of phosphatidylethanolamine and from the C73S mutant was determined by NMR spectroscopy. The major signals are seen, which can be identified as CL, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylinositol (PI). The amount of phosphatidylinositol is typically high in yeast mitochondria. After denaturation by SDS, the CL signal increases markedly, whereas the signals for other phospholipids show only small increases. There is also a marked unexplained downfield shift, particularly pronounced for phosphatidylethanolamine. Several NMR spectra for assaying the phospholipid contents were taken of different sucrose gradient-purified AAC preparations of C73S mutants as well as preparations of the wild-type AAC.

The phospholipid content evaluated from the 31P NMR spectra in the wild-type and C73S mutant AAC was determined chemically to 3.4 mol. The sum \( \Sigma \) of phosphatidylcholine (PC) + phosphatidylethanolamine (PE) + phosphatidylinositol (PI) + CL as measured by 31P NMR gives 3.2 mol of PI. The sample volume was 2.7 ml containing 0.5 ml of D2O and 3.08 mg of protein. The probe was measured before and after addition of 1% SDS as powder. The 31P NMR spectra were taken at 61.9 MHz at 10 °C with 20,000 scans, 1.5-s delay, 1.5-s acquisition times, 90 pulses at 25 °C, line broadening of 3 Hz, and proton decoupling.

**Table II**

<table>
<thead>
<tr>
<th>Phospholipid addition</th>
<th>Exchange rate (V)</th>
<th>V/VCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>600</td>
<td>1.0</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>20</td>
<td>0.034</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>5</td>
<td>0.01</td>
</tr>
<tr>
<td>Myristoyl-CL</td>
<td>20</td>
<td>0.03</td>
</tr>
<tr>
<td>Oleoyl-CL</td>
<td>30</td>
<td>0.05</td>
</tr>
<tr>
<td>Acetyl-CL</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dicyclo-CL</td>
<td>36</td>
<td>0.06</td>
</tr>
<tr>
<td>Diphosphatidylglycerol</td>
<td>25</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*Phosphatidylglycerol.

Content of Cardiolipin in Isolated AAC—The amount of phospholipids bound to the isolated AAC from wild-type yeast and from the C73S mutant was determined by 31P NMR. For this purpose, a major portion of the sucrose gradient centrifugation was removed by sucrose gradient centrifugation. An appropriate sucrose gradient had been developed earlier (Beyer and Klingenberg, 1985). The sucrose contains C12E8 to prevent protein aggregation and to allow segregation of the mixed detergent/phospholipid micelles from the heavier protein/detergent micelles. The separation is not complete, but as shown in Fig. 3, the major portion of the phospholipid is segregated from the bulk of the protein fractions.

Fig. 4 shows a 31P NMR spectrum of the signals of the phospholipids associated with the isolated C73S mutant AAC from yeast. Only free or very loosely bound phospholipids will present fairly sharp signals, like those shown in Fig. 4. After scanning the spectrum of the native preparation, SDS was added to denature the AAC and to release bound phospholipids. Four major signals are seen, which can be identified as CL, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylinositol (PI). The amount of phosphatidylinositol is typically high in yeast mitochondria. After denaturation by SDS, the CL signal increases markedly, whereas the signals for other phospholipids show only small increases. There is also a marked unexplained downfield shift, particularly pronounced for phosphatidylethanolamine. Several NMR spectra for assaying the phospholipid contents were taken of different sucrose gradient-purified AAC preparations of C73S mutants as well as preparations of the wild-type AAC.

The phospholipid content evaluated from the 31P NMR spectra in the wild-type and C73S mutant AACs is given in Table III from experiments with two different isolates of the C73S AAC. Both in the wild-type and C73S mutant AACs, the content of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol is not changed or even somewhat decreased by addition of SDS. This decrease is not well understood. It can be concluded that none of these three phospholipids is tightly bound to the wild-type or C73S AAC.
The amount of CL bound to the wild-type AAC is nearly the same as that previously reported for the bovine heart AAC (Beyer and Klingenberg, 1985). In both the bovine and yeast wild-type AAC isolates, no CL is required for activation of ADP exchange transport. As compared to these two AACs, the isolated C73S AAC is deficient by >60% bound CL. It is concluded that this defect is correlated to the dependence on the addition of CL on reconstitution.

**DISCUSSION**

The striking occurrence of CL in the inner mitochondrial membrane had a strongly suggestive influence on the assumption that CL is specifically required for the function of the mitochondrial membrane-bound proteins. Most widely studied is the activation of cytochrome oxidase by CL (Yu et al., 1975; Robinson et al., 1980; Robinson, 1982; Fry and Green, 1980; Abramovitch et al., 1990). With CL addition, the oxidase activity was enhanced; however, no absolute requirement for CL was observed, even after complete exchange of bound CL (Abramovitch et al., 1990). A specific CL binding requirement associated with function has so far been shown only for cytochrome oxidase (see Powell et al. (1987)). In the ATP synthase complex, some tightly bound CL is also bound without necessarily being important for function in the reconstituted system (Robinson, 1982; Eble et al., 1990). The isolated ubiquinol-cytochrome reductase complex can be fully activated with neutral phospholipids, but it also specifically binds CL as shown by $^{31}$P NMR (Hayer-Hard et al., 1992).

A CL requirement for the activity of mitochondrial carriers has been reported in several cases (for review, see Krämer and Palmieri (1989)). For the reconstituted phosphate carrier, the CL requirement seemed to be well established with a 50% stimulation by CL (Kadenbach et al., 1982; Mende et al., 1983). Also, citrate transport was reported to be stimulated by CL (Stipani et al., 1980). However, in all these cases, the requirement for CL was not absolute or could not be confirmed in later works. This is particularly well documented for the phosphate carrier activity (Mende et al., 1983; Wohlrab et al., 1984; Stappen and Krämer, 1993). In our first reports on the reconstitution of the AAC from bovine heart, it was shown that addition of acidic phospholipids including CL was required for high activity. Although highest activity was observed with CL, phosphatidylserine and, as shown in later studies, even cholesterol provided high translocation activity (Krämer, 1982).

**TABLE III**

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Wild-type</th>
<th>C73S mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-SDS</td>
<td>+SDS Δ</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mol phospholipid/mol AAC dimer</td>
<td>mol phospholipid/mol AAC dimer</td>
</tr>
<tr>
<td>PC$^{a}$</td>
<td>27.0 22.0</td>
<td>-4.0 30.5 33.0</td>
</tr>
<tr>
<td>PE</td>
<td>17.5 19.6</td>
<td>-2.5 23.5 21.0</td>
</tr>
<tr>
<td>PI</td>
<td>9.1 11.1</td>
<td>1.1 23.5 21.0</td>
</tr>
<tr>
<td>CL</td>
<td>6.1 12.5</td>
<td>6.4 8.3 11.2</td>
</tr>
</tbody>
</table>

*PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.*

The amount of CL bound to the wild-type AAC is nearly the same as that previously reported for the bovine heart AAC (Beyer and Klingenberg, 1985). In both the bovine and yeast wild-type AAC isolates, no CL is required for activation of ADP exchange transport. As compared to these two AACs, the isolated C73S AAC is deficient by >60% bound CL. It is concluded that this defect is correlated to the dependence on the addition of CL on reconstitution.

Although highest activity was observed with CL, the binding center of the AAC can assume the ground state where it can accept CAT (Klingenberg, 1976). Therefore, it is proposed that the role of CL is in facilitating the transition between the translational states (for review, see Klingenberg (1991)).

Why is the C73S mutant AAC dependent on CL addition after isolation and reconstitution? The other two cysteine mutant AACs, C244S and C271S, only marginally require CL on reconstitution. Other AAC mutants investigated so far, whether they produce an active or a largely inactive AAC, are not further activated by addition of CL in the reconstitution system.

The most persuasive explanation for the requirement for CL of the C73S mutant AAC is a loss of CL on isolation. When still resident in the yeast cell with inner mitochondrial membrane CL, the AAC is obviously active and permits growth on glycerol. This explanation also implies that the wild-type AAC and even AACs from other plant and animal cells require the presence or binding of CL for activation. However, on isolation and reconstitution, usually the AAC retains its bound CL and thus does not require further CL addition. In fact, the bovine heart AAC, which has generally no requirement for CL on reconstitution, contains 6 molecules of tightly bound CL/AAC dimer, as shown by $^{31}$P NMR analysis (Beyer and Klingenberg, 1985). The same type of NMR analysis shows here that the wild-type AAC dimer contains 6 molecules of tightly bound CL, whereas the C73S AAC after its isolation contains only 1.7-2.9 molecules of tightly bound CL. This suggests that the loss of CL in this mutant AAC causes the requirement for CL supplementation. NMR analysis can only be performed on the more stable CAT-AAC complex since the ATR-AAC complex does not survive its native state during the stress of sucrose gradient and prolonged NMR measurements. Probably, the amount of CL bound is still lower in the ATR-AAC complex, which has to be used in the reconstitution if one assumes that the liability of the AAC structure is responsible for the CL loss.

This leads to the question of why the C73S mutation causes the apparent loss of bound CL on isolation. Here, the instability of this mutant AAC as compared to the wild-type AAC seems to be the most likely cause. In an assay of the protein stability, the isolated mutant AACs were compared with the wild-type AAC with and without partial protection by binding with ATR. The C73S AAC not liganded with ATR was irreversibly inactivated much faster on solubilization and isolation than the free wild-type AAC. Although for protection during the reconstitution, the AAC is loaded with ATR, subtle conformation changes even in the still native ATR-AAC complex might cause the loss of bound CL. Possibly even in the mitochondrial membrane, CL is bound less tightly to the mutant than to the wild-type AAC. In conclusion, these results seem to indicate that the tight binding...
of CL to AAC is important for activity. Subtle structural change due to the replacement of cysteine 73 by serine apparently loosens CL binding.

In former studies on the bovine heart AAC, it has been shown that CL is bound so tightly that it cannot exchange with \([^{14}C]\)CL (Schlame et al., 1991). However, by spin labeling CL, the additional binding of 2 molecules of CL has been measured (Drees and Beyer, 1988). This binding was tighter than that of other spin-labeled phospholipids. To what extent this second type of less tight, yet specific binding of CL also contributes to the activity is not yet clear. These data show that the CL-binding sites in the AAC responsible for transport activity are quite specific since slight modifications of the CL structure drastically decrease their potency. In our studies of the distribution of the numerous lysine residues in the bovine heart AAC, we showed that a number of lysines became accessible to pyridoxal phosphate only after the phospholipid membrane had been stripped off (Bogner et al., 1986). We assumed that some of these cationic lysines associate with the acidic head groups in the membrane. The unusually high number of lysines in the AAC would clearly provide an abundance of CL-binding sites and support the exceptionally high amount of CL bound and required for function in the AAC.

Is the absolute requirement for CL for the reconstitution of the C73S AAC also evidence for a general CL requirement of the AAC in mitochondria? From the above reasoning, one might conclude that it is. The unmasking of the CL requirement in this mutant may be only circumstantial, but we may regard it as a fortunate paradigm that allows us to suggest that the AAC in general requires CL binding. This may be true even for other members of the mitochondrial carrier family. It can be expected that further AAC mutants will be obtained with an unmasked CL requirement. This may eventually help to understand which amino acids are involved in the CL-AAC interaction.

REFERENCES