Cardiolipin and mitochondrial carriers

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ABSTRACT

Members of the mitochondrial carrier family interact with cardiolipin (CL) as evident from a variety of functional and structural effects. CL stabilises carrier proteins on isolation with detergents, with the Pi carrier as the prime example. CL is required for transport in reconstituted vesicles, prime examples are the Pi- and ADP/ATP carrier (AAC). CL binds to the AAC in a graded manner; 6 CL/AAC dimer bind tightly as measured on the 31P NMR time scale. 2 additional CL/dimer bind reversibly and a fast exchanging envelope of phospholipids includes CL as measured on the ESR time scale. In the crystal structure of the CAT–AAC complex 3 CL bind to the periphery of the AAC in a three-fold pseudo-symmetry. The binding of CL is implicated to contribute lowering the high transition energy barriers in the AAC. Para-functions of the AAC, as in the mitochondrial pore transition (MPT) and in cell death are linked to the CL binding of the AAC. Ca++ or oxidants can sequester or destroy AAC bound CL, rendering AAC labile, allowing pore formation and degradation. Thus AAC, by being vital for energy transfer, constitutes an Achilles heel in the eukaryotic cell. AAC together with CL is also engaged in respiratory supercomplexes. Different from AAC the similarly structured uncoupling protein (UCP1) has no tightly bound CL, but CL addition lowers affinity of the inhibitory nucleotide binding that may contribute to the physiological regulation of the uncoupling activity by ATP.

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Abbreviations: PL, phospholipid; CL, cardiolipin; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PG, phosphatidyl glycerol; PA, phosphatidic acid; AAC, ADP, ATP carrier; PiC, phosphate carrier; UCP, uncoupling protein; MCF, mitochondrial carrier family; TMH, transmembrane helix; HTP, hydroxyapatite; CAT, carboxyatractyloside; BKA, bongkrekic acid; MPT, mitochondrial transition pore; PVM, pyrene maleimide; DAN, 2′-O-[5′-dimethylaminonaphthoyl]; DANSYL, 2′-O-[5′-dimethylaminonaphthoyl]-sulfonyl

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1. Introduction

The inner membrane of mitochondria contains a family of carriers (MCF) that catalyses the passage of solutes between the intramembrane and matrix spaces [1,2]. The transport of these solutes is involved in metabolism, and in maintaining adequate levels of nucleotides, coenzymes and metal in the matrix space. Thus the carriers facilitate the metabolic interaction of mitochondria with the cytosol and the supply of cofactors in the biogenesis of mitochondria [3–5]. Given the prokaryotic origin of mitochondria the carriers can be viewed as to allow the symbiosis of the mitochondria with the host eukaryotic cell. In this perspective carrier functions have evolved to bridge between both spaces the disparate thermodynamic environments [6] that are defined by the redox potential of the NAD/NADH system and by the phosphorylation potential of the ATP/ADP/Pi system [7].

Based on genomic analysis, in S. cerevisiae 45 and in H. sapiens 43 genes code for proteins with sequence patterns characteristic for the MCF, such as 6 segments qualifying for transmembrane helices. The sequence is segmented in 3 similar domains each containing 2 THM, all arranged in a pseudo three-fold symmetry [for review see 8]. The atomic structure of the paradigm MCF member, the ADP/ATP carrier (AAC) from bovine heart (see review [9]) was recently resolved [10], essentially substantiating the three-fold symmetry with six helices surrounding a broad funnel. For stabilisation in these crystals the AAC is complexed with carboxyatractysloside (CAT), imposing an “abnormal” c-state in which the binding center is opened to the cytosolic side. This structure, being the only one available for the MCF, has served as a template for modelling the structure of other carriers and to identify the putative substrate binding sites [11,12]. It also formed the basis for molecular dynamic studies in which electrostatic steering was shown [13,14,15] to be the major factor for capturing the substrate in the binding site of the AAC.

Because the AAC is equipped with exceptional features to cope with its central role in metabolism, such as high expression levels, highly specific inhibitors, aimed against different translocation states, it was a paradigm for elucidating a carrier mechanism in general [9], and for resolving the structural elements, by starting with the first sequence then exploring the folding and finally the atomic structure. Most important in the present context is that the AAC excels in unusually strong interaction with cardiolipin (CL) [16]. Although it may be not surprising that mitochondrial carriers interact with CL, being a major lipid component of the inner mitochondrial membrane, it is the wide range and variety of interactions found with these carriers that raise special interest and will be reviewed here. First the more general protective effects of CL on the mitochondrial carrier proteins will be reviewed and this will be followed by reporting the more selective effects of CL on transport activity, and on secondary carrier functions.

2. Protective effects of cardiolipin on isolated mitochondrial carriers

On solubilisation of mitochondrial carriers, detergents replace to a large extent the membrane lipids. Without their native environment the carriers become labile and the supplementation of phospholipids especially of CL may protect and facilitate the purification of the carrier in a functionally intact state. The stabilisation of the carrier proteins is also the reason that CL addition increases the bypass fraction of hydroxyapatite thus improving the yield and the purity. The final criterion for the beneficial effect was increased transport activity as measured in the reconstituted proteoliposomes. The most detailed research on the stabilisation and activation by CL of mitochondrial carrier was performed with the phosphate carrier (PiC). Although there was general agreement on the positive role of CL, variant results were reported as to the preponderance of these three effects. Despite some discrepancies, it is clear that all the effects reflect interaction of CL with and the carrier proteins and that there may be a hierarchy of strong and specific and of weaker less specific interactions, as will be discussed below.

2.1. The phosphate carrier

As first reported for the phosphate carrier (PiC), addition of CL during the solubilisation and isolation by Triton X100/114 greatly improved the transport activity after reconstitution [17,18]. The protective effect of CL was most pronounced at higher (>3%) Triton concentration whereas Triton alone largely inactivates the PiC. If CL was included in the extracts, a high transport activity was obtained on reconstitution into vesicles, obviously by restituting CL that had been removed by the high detergent concentration. It was first thought [18] that the inactivation is irreversible since CL added at a later stage to the lipids for the reconstitution of the proteoliposomes did not activate transport. In subsequent work a reversibility of PiC inactivation by detergent was found [19] by showing that CL could reactivate the PiC also when added after the solubilisation to the Triton extracts, but again not, when CL is integrated with the liposome forming lipids. At variance, in other work CL addition was claimed to increase the yield of PiC extracted [20] from mitochondria rather than to protect the PiC. The yield increase by CL addition to the detergent extract was found to be at the stage of the hydroxyapatite (HTP) column, where CL greatly promoted the amount of PiC protein eluted [21]. Without CL addition, the eluted fraction from the HTP column contained little PiC and on subsequent addition of CL the major amount of PiC was eluted [21]. These results showed that without CL the PiC in the Triton X 100 extract adsorbs to the HTP and that CL by binding to the PiC maintains or reverts the protein into a non-adsorbing conformation. There was a general agreement that these effects on stabilisation and purification yield were highly specific for CL and could not be achieved with other anionic lipids. Both results, the reactivation of soluble PiC [19] and the desorption from HTP by CL [21] can be viewed as demonstrating the reversibility of the PiC inactivation by these detergents. Why in the reactivation work [19] without CL the PiC is not adsorbed at HTP at variance to the desorption results [21] is not clear. Different types of HTP, and/or the detergent and salt concentrations are probable factors. The purity of the PiC, i.e. the relative proportion of the PiC to other proteins in the eluted fraction could be improved by CL addition under conditions where other co-eluting membrane proteins with similar size, such as porin and AAC, were retained or retarded on the HTP [21].

An ambivalent problem is the increase of the PiC transport activity by CL. In the first work with reconstituted liposomes an activation of transport by CL was shown [17,18]. Subsequently the same group showed that the PiC cannot be reactivated, when CL is added at a later stage to the liposome lipids [19]. From this work it became clear that for activation CL has to be applied to the solubilised carrier and thus dispersed in the detergent micelles. It seems that endogenous CL together with PiC can be carried over from the soluble state into the liposomes. When PiC is solubilised at lower detergent concentration, when PiC is nearly inactive, activity depends entirely on CL addition. The amount of CL required for full activity increases on CL addition. The amount of CL required for full activity increases on the detergent present. Also for desorbing PiC from HTP sufficient CL must be added to the detergent buffer to reconstitute a fully active PiC [21].

By showing that CL addition strongly enhanced the amount of extracted PiC protein [20] it was concluded that the increase of transport activity by CL was due to an increase of the amount PiC and not due to an increase of the specific transport activity. This seemed to be supported by experiments in which various proportions of acidic phospholipids such as phosphatidic acid (PA) and CL are incorporated
into liposomes [22]. Maximum transport rates were observed with about 8% PA and replacement of PA by CL strongly decreased the rate. These results agree with the previous reports [19] that unless CL is admixed to the soluble PiC it will be inactive. In contrast PA seems not to have this requirement. In other work a clear dependence of reconstituted PiC transport on CL incorporated into liposomes was reported [23,24]. Further CL, dispersed without detergent, on incubation of up to 3 h with proteoliposomes was able to fully activate transport. Antibiotics adriamycin, doxorubicin, B-dauromycin, that form adducts with CL, abolished reversibly the activation by CL. The different response to CL may be partially explained by the prior exposure in these experiments of the PiC to CL added during the extraction. Thus PiC is in a pre-active or reversibly inactivated state, whereas PiC extracted without CL seems in an irreversibly inactive state unable to respond to the CL included in the liposomes.

In conclusion apparently there may exist two levels of interaction of CL with PiC: A first stage binding that is highly specific for CL and maintains the carrier in a “ready” state. A second stage less specific binding, where CL can be replaced by other anionic phospholipids, and that brings the carrier into the active state. This attempt of unifying the seemingly contradictory results has to await further evidence and would be supported by analogy to the direct molecular evidence of a two stage interaction of CL with the AAC, reviewed below.

2.2. Carboxylate carriers

The stabilisation of detergent solubilised carriers by CL has been observed with several other members of the MCF and used for the isolation of functionally “intact” carriers from mitochondria (see review [4]). Featuring similar molecular weights and structures their separation and purification were a challenge in which CL played a major role. The judicious fine tuning of CL addition combined with variations of the conditions of how the extracts were passed through HTP and celite columns enabled the purification of these carriers. The isolation of the tricarboxylate carrier from liver mitochondria profited early from the CL interference [25] but it took some time to elucidate the intricacies for separating the tricarboxylate carrier [26]. Isolation of the dicarboxylate carrier from liver mitochondria also involved the use of CL for increasing the yield [27,28–30]. Similarly, the carrier for oxoglutarate isolated from heart [31] and from liver mitochondria [29] depended on the addition of CL for their isolation.

3. ADP/ATP carrier

3.1. Activation by CL

The AAC affords at least two “technical” advantages over the other mitochondrial carriers for a more incisive investigation of the carrier — CL interaction at the molecular level: the high content in mitochondria and thus the availability of sizable amounts of purified AAC protein, and the strong stabilisation of the structure by the inhibitor ligand carboxyatractyloside (CAT) thus preventing protein unfolding and degradation [32,33]. No stabilisation by added CL was necessary for the isolation of the bovine heart CAT–AAC complex. However, little was done to explore the stabilisation of the unliganded AAC by CL, since most of the AAC reconstitution work was performed [34–36] before the use of CL to stabilise the carriers. In the first exploration of the conditions for reconstituting transport of isolated and purified AAC, that represented also the first reconstitution of any purified carrier, variations of the lipid composition played an important role [36,37]. Egg yolk PC turned out to be the basic “mortal” for tight vesicles, much superior to the previously used asolectin in reconstitution with undefined membrane protein preparations. The ADP–ATP exchange transport was stimulated by acidic phospholipids [37,38] with the efficiency sequence of CL > PS > PA. CL was the most effective but not exclusive activator among the acidic lipids. Transport was also possible without acidic lipids, when PE was present in high proportion, although in mitochondria the content of PE is low [37]. Surprisingly, cholesterol that is mostly absent from the inner membrane of mitochondria, was also an effective stimulant [39]. Notably, the activation of the reconstituted AAC was side specific as shown by changing the surface potential and determining the orientation of the AAC in the proteoliposomes [40].

On reconstituting a mutant of the yAAC2 (C73S) from S. cerevisiae, an absolute and highly specific dependence on CL was noted [41]. wt-yAAC2 did not require any CL admixed to the liposomes whereas C73S-yAAC2 was completely inactive without CL. Other acidic PL could not replace CL, only monoloyo-CL activated transport to 12% of the rate with CL. Parallel 31P NMR measurements of the isolated AAC preparations revealed that the content of tightly bound CL, as measured by the difference of the CL signal strength before and after SDS addition (see also chapter 3.2), is strongly decreased in the mutant C73S-y AAC2 as compared to wt-yAAC2 (2.2 versus 6.4 mol CL/mol AAC dimer). Obviously in C73S-yAAC2 the content of tightly bound CL has reached a level on isolation or in the mitochondria below the threshold for sustaining transport activity. In another case, starting with recombinant AAC from Neurospora crassa expressed in inclusion bodies in E. coli the threshold requirement for CL is clearly demonstrated [42]. In this case, the nAAC isolated from inclusion bodies does not carry any endogenous CL. On reconstitution an absolute dependence on CL is observed with a sigmoid dependence on the CL content. Only above a threshold of 4% CL transport becomes strongly activated reaching a maximum around 10%.

3.2. Binding of CL to AAC

The AAC–CAT complex of bovine bAAC1 as isolated first by using Triton X100 [32,43]. Hydrodynamic and chromatographic analysis showed [44], that the AAC-Triton X100 micelle consisted of 150 mol Triton X100/mol dimer AAC and 16 mol PL-phosphate/mol dimer. Most surprising was the finding of tightly bound CL in the isolated AAC based on 31P NMR measurements of the PL composition were performed parallel to a chromatographic analysis [16]. In the unfractionated AAC preparation, 31P signals characteristic for PE and PC were found with the noted absence for CL. In contrast, by chromatography of the lipid extracts the composition of PC/PE/CL was measured to be similar to the bulk lipid of the inner membrane, with CL amounting to 20% of the total PL. After separating the protein containing micelles from the “free” micelles by sucrose density centrifugation, PE and PC were largely found to be associated with the floating “free” micelle fraction, whereas CL was confined to the AAC fraction. By 31P NMR in the free micelles distinct signals for PE and PC were measured [16] with narrow bandwidth indicating a high flexibility. In the protein micelles, only after increasing sensitivity 6 times, comparative size 31P NMR signals became visible, corresponding to traces of “mobile” PE and PC and, in a broad band, corresponding to CL. The mobility of CL calculated from the bandwidth of about 30 Hz corresponds to the tumbling time of the AAC micelle, indicating immobility of the CL within the micelle.

The tight binding of CL is further revealed by the response of the 31P signals to SDS addition, as shown in Fig. 1. Even after 4 days exposure to SDS no “sharp” CL signal appears. Only on heating the sample a narrow band 31P signal is observed, reflecting the release of CL. By “titrating” the AAC–H-CAT preparation with increasing amounts of SDS, 3H-CAT was found to be released earlier than CL from the AAC, indicating a higher sensitivity of the binding sites for CAT than those for CL to the SDS induced structure change. The absence of CL from the “free” micelles indicates that most of the CL is contained in the protein fractions removed by HTP and sepharose gel chromatography, suggesting that most of the CL is protein bound. The AAC can be estimated to bind tightly 10% and loosely another 4% of the
The amount of CL binding as evaluated from the $^{31}$P signal of the CL released by SDS was 6 mol CL bound/AAC dimer [16]. Thus the amount of CL bound to the AAC exceeded by far that reported to other mitochondrial components (see review [45]). The unusual binding strength was further corroborated by showing [46] that $^{14}$C-CL did not exchange with the endogenous bound CL on an hour scale. Hydrogenation of the bound CL did not loosen the binding as shown by $^{31}$P NMR [46], indicating that a specific acyl chain is not required.

The high binding of CL can be rationalised in view of the large excess of 18 positive charges in the AAC [47,48]. A collar of lysines has been suggested to face the lipid bilayer at the height of the membrane surface [49]. In conjunction with Ca$^{2+}$ induced pore openings of the AAC (see Chapter 4.1) it was proposed [50] that the CL molecules were bound at the “collar” lysines such that their headgroups face the matrix side. The high resolution 2.2 Å crystal structure of the bovine heart AAC–CAT complex strikingly confirmed the specific binding of CL to the protein [10,51]. In the first crystal structure 2 CL bound/monomer AAC [10] and in a second crystal structure 3 CL bound/monomer were clearly discerned [51] (Fig. 2). In agreement with the predictions the phosphate groups of all CL are directed to the matrix side.

On the basis of $^{31}$P NMR line broadening, only very low dissociation rates corresponding to tight binding can be determined. A much shorter time scale is covered by ESR measurements using spin labelled (SL)-phospholipids thus permitting a more differentiating evaluation of binding strength of PL to the AAC [52]. By titrating the solubilised AAC with SL–CL, a binding of 2 molecules CL/dimer AAC was found. SL-phosphatidic acid (PA) bound with nearly same affinity, whereas the neutral SL–PC and SL–PE bound only weakly. The importance of ionic forces is revealed by the sensitivity of this binding to increased salt concentration. Cleavage by phospholipase A2 and reduction of the spin label by ascorbate suggested that SL–CL is bound at the outer perimeter of the protein [52]. Lipid binding employing SL–PL was also studied on AAC reincorporated into PL-membranes [53]. The on- and off-rates in the exchange of the SL–PL with the first shell lipids were determined. All the acidic SL–PL including SL–CL had similar slow off rates of about $5 \times 10^6$ s$^{-1}$. The number of molecules in the first shell was estimated at 50 PL/AAC dimer, pointing out a larger protein — lipid surface than calculated for a rotational ellipsoid, possibly reflecting a “rough”

![Image](image_url)

**Fig. 1.** Release of tightly bound CL from bovine heart AAC by SDS and increased temperature. $^{31}$P NMR scan (2000 transients). (A) Solubilised and purified AAC in Triton X 100. (B) On addition of 1% SDS after 4 days at 4°C. (C) After additional heating for 30 s. From [36].

![Image](image_url)

**Fig. 2.** Ribbon diagram of the bovine AAC1–CAT complex. The binding of 3 CL molecules at the periphery of the protein (CDL 800, 801, 802) is shown. A. View from the c-side. B. View in the membrane plane. C. View from the m-side. From [59].

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protein surface. These exchangeable shell lipids surround the AAC in addition to the tightly bound non-exchangeable CL.

3.3. The AAC in CL deficient mutant cells

The importance of CL for the AAC was further elucidated in crd1 cells from S. cerevisiae that are deficient in the CL synthase, containing mitochondria lacking in CL [54]. On non-fermentable source the CL deficient cells grew slowly and are temperature sensitive. The cells produced large amounts of the acidic phosphatidyl glyceride (PG) which apparently could partially replace CL needed by the various mitochondrial components such as AAC, cytochrome oxidase, cytochrome c reductase etc. In isolated mitochondria the rate of oxidative phosphorylation was reduced in the mutant to 1/4 of that of wt. It remained fully sensitive to the inhibitors of the AAC, BKA and CAT. Surprisingly the amount of yAAC2 in the mutant mitochondria as measured by 3H CAT binding was the same as in wt. Also the phosphorylation remained fully sensitive to the inhibitors of the AAC, BKA and CAT. As a consequence of the reduced oxidative phosphorylation, the respiration of the mutant was also decreased compared to the wt. This decrease was fully restored on addition of CL to the isolated mitochondria. The oxidation of NADH and succinate in isolated mitochondria was shown to be similarly affected by the CL deficiency (personal communication, F. Palmieri, C.L. Pierri). All this argues for a preferential or exclusive binding of CL to the mitochondrial carriers at the matrix side.

On reconstitution of the isolated yAAC2, the ADP–ADP exchange required more CL to be added to the phospholipids with yAAC2 from the mutant than from the wt cells [54]. Exchange activity was virtually zero with yAAC2 originating from the CL deficient yeast. The stimulation by CL reached about half of the rate of the wt. Apparently on isolation of the yAAC2 from the CL deficient cells, insufficient endogenous PG was carried over into the reconstitution.

3.4. CL and structure/function relations in AAC

CL binding is visualised to contribute to the electrostatic network that is geared to propagate the high interaction energies of ADP3− and ATP4− binding into the large conformation changes during the translocation. With ATP4− and ADP3−, the AAC transports large and highly charged solutes requiring large rearrangements within the carrier structure. According to the “induced transition fit” (ITF) [55,56] the high activation energy barriers of large configuration changes must be compensated by the interaction energies between substrate and protein reached in the transition state. These requirements are satisfied in the AAC by a high share of electrostatic interactions between substrate and AAC on the one side and within the protein on the other side. The extraordinarily high and tight binding of 3 CL/AAC monomer may play a role in matching the large interaction energies. The additional ionic bonds could markedly increase the interaction energy for facilitating the necessary large rearrangements in the transition between the c- and m-states. In the AAC–CL3 complex the additional 6 negative charges are more than balanced by the high amount of 18 uncompensated basic residues/AAC monomer [47]. In comparison, UCP1, which lacks tight binding of CL, has an excess of only 9 basic residues [57]. Correspondingly, the substrates, H+ or fatty acid anions are small and carry only one charge. In the AAC the excess of positive charges is distributed such that more charges are at the matrix than at the cytosolic half (11:7). Simulating the structure of the other MCF members from the AAC structure, also in most other carriers the positive charge excess is larger in the matrix than in the cytosol half (personal communication, F. Palmieri, C.L. Pierri). All this argues for a preferential or exclusive binding of CL to the mitochondrial carriers at the matrix side.

Binding of CL was originally envisaged to involve a “collar” of lysines at the matrix side [49,50]. The crystal structure vindicated the tight binding of 3 CL/monomer, and provides a detailed picture of CL interactions with the AAC [10,51]. As shown in Fig. 2 in each of the 3 repeat domains one CL binds to the uneven numbered kinked transmembrane helices (TMH). The acyl chains are close to aromatic residues that surround the intrahelical prolines responsible for the kinks. During the translocation, going from the c-state to the m-state, the cavity initially opened to the c-side has to be closed by the c-gate and the m-gate becomes open [58]. This process is envisaged to involve pivoting of the uneven numbered TMH around the prolines, such that the m-section of the helix is turned outside and the c-section inside [59]. Concomitantly the even numbered TMH are twisted [60]. These configuration changes might translate via the aromatic groups into position changes of the adjacent CL acyl chains. In particular the phosphate headgroups may contribute to the opening of the gates on the transitions between the c- and m-states. According to the crystal structure the CL phosphates are linked to basic residues near the N-terminal of the small matrix helices. On transition to the m-state these ion bonds may be loosened and shifted to form new ion bonds with basic residues near the C-terminal side of the matrix helices in case they are now geometrically favoured. In this context it is noteworthy, that according to 31P NMR measurements CL binds also tightly to isolated AAC brought into the m-state by binding BKA (K. Beyer unpublished), although the AAC seems to have a less rigid structure according several criteria [9].

The preferred binding also of added CL to the matrix side was demonstrated by probing the access to CS6 in the isolated AAC–ATR complex of bovine bAACh with pyrene maleimide (PYM) [61]. ADP stimulated the access to CS6 [62,63] and addition of CL, but not of other PL, inhibited the reaction of PYM. Even when AAC was fixed in the m-state by BKA, CL addition prevented the access of PYM. Treatment of the micelles with phospholipase A2 facilitated the reaction of PYM, rendering it less ADP dependent. Addition of CL restored the ADP dependency, but higher amounts of CL prevented again PYM access. The results were interpreted to reflect a direct blockage of the CS6 by excess CL. Support for this notion comes from the crystal structure where CL binds to nearby K51 and per vDW forces to I53 and I54 [59].

In the second published crystal structure dimers of the AAC can be discerned in the crystal lattice [51]. Interestingly, two CL were localised at the interface of two adjacent AAC monomers involved in linking the monomers within a dimer. The phosphate groups are connected to the matrix short helices and lysines C267 and K51 of the opposing monomers. Thus, with the hydrophobic forces of the alkyl chains CL seems to provide the glue for the dimer formation. It was proposed [59] that the two CL are also involved in the cross talk for the cooperative function of the monomers.

4. Para-functions of AAC–cardiolipin interaction

4.1. The mitochondrial pore transition (MPT)

Apart from the 1:1 exchange of ADP and ATP, the AAC can mediate an efflux of nucleotides from isolated mitochondria that is induced to varying stages by ions and prooxidants (see reviews [9,64,65]). Ca++ ions are the most prominent inducers of this permeabilisation of the inner mitochondrial membrane which has been classified as the “mitochondrial pore transition” (MPT). The criteria for involvement of the AAC were the inhibition of the MPT by ADP and by BKA and a stabilisation by CAT and atracyloside (ATR), indicating that the MPT requires the c-state of the AAC. Further support for a role of AAC in MPT came from the inhibition by SH-reagents known to stabilise the m-state [66] and by crosslinking reagents, known to stabilise the c-state [67,68]. In patch clamp studies with reconstituted AAC [50,69] large single channels (800 pS) were opened by Ca++. The channels were sustained by CAT but closed by ADP and BKA, similarly to the MPT, suggesting the involvement of AAC possibly as a central component in a larger MPT complex. As a mechanism it has been proposed that Ca++ taken up in the matrix sequesters the headgroups of the CL bound to the AAC, as illustrated in Fig. 3. [50]. Based on the high affinity of Ca++ for complexing CL [70,71], Ca++ is suggested to displace CL from the AAC, exposing the positive charges at the matrix
side of the AAC [50]. The removal of CL may destabilise the c-state structure partially by electrostatic repulsion and open the m-gate so that an open channel through AAC is formed. However by deleting all AAC isoforms in murine liver, evidence has been presented, that the AAC is not an obligatory element of the MPT [72]. Ca\(^{2+}\) inducible and cyclosporine A sensitive MTP was retained in the mutant mitochondria, delegating the AAC to a more accessory inducer role in the MPT [73]. It was also debated [74] that AAC forms the default pore in MPT and that in the mutant mitochondria another mitochondrial carrier may have replaced the AAC. A related proposal is that the pore in the MPT is formed by misfolded carrier proteins including the AAC [75].

Prooxidants are shown to enhance the sensitivity of the MPT to Ca\(^{2+}\) [65,67]. In the patch clamp system the prooxidant butylperoxide promoted pore opening by inhibiting the voltage gating, probably by oxidising the SH groups involved in gating [76,77]. This is consistent with the role of SH groups and the enhancing effect of prooxidants described in detail for the MPT [67,68,78,79]. Prooxidants and oxygen radicals may also affect the CL surrounding the AAC because of the exceptionally high degree of unsaturation of the acyl groups [46] that renders CL most vulnerable to ROS attack. Thus on treating mitochondria with Ca\(^{2+}\) and butylperoxide, [80–82] in SDS gels the protein band corresponding to the AAC was found to be shifted upward corresponding to a higher mass. It was the only clearly detectable mass increase among the mitochondrial proteins. Mostly lysines of the AAC were shown to be modified by Schiff base adducts of malondialdehyde and hexenal, produced from the oxidation of PL lipid chains. AAC was the favoured target because the aggressive aldehydes formed by the oxidants from the tightly bound CL could in situ attack the AAC. In addition the high lysine content predestined the AAC to be highly responsive to aggressive oxidant products. Notably this modification of AAC was dependent on the transport state: CAT inhibited and BKA allowed the modification, indicating that the m-state is required in which the AAC is known to be more vulnerable to trypsin [33,83].

4.2. CL and AAC in apoptosis

Multiple studies point to a role of the MPT in the early stages of the apoptotic or necrotic pathways of cell death [84–86]. Through the MPT, AAC and CL participate in triggering apoptosis, as indicated for example by the inhibition of apoptosis by the AAC inhibitor BKA that also functions as a MPT blocker [87]. However, it is still debated, whether and to what extent the MPT can be bypassed in the Bcl-2/BAX induced apoptosis [88]. Within the confines of this review on CL and carriers, it is not possible to expand on these extensively discussed questions but instead to concentrate on the role of CL and AAC in apoptosis.

Out of the apoptotic signalling proteins tBid, a 15 kDa protein generated from Bid, that is a member of the Bcl-2 family, has emerged as a key player in understanding the mechanism of mitochondria and the role of CL therein. tBid was shown (see review [89]) to enhance the permeabilisation of the outer membrane by BAX by promoting the pore forming oligomerisation of BAX and thus the release of cytochrome c, a key factor in triggering the apoptotic cascade. However, in model liposomes tBid was shown [90] not to bind to outer membrane lipids but to liposomes resembling the contact sites between outer and inner membranes that are rich in CL. Further, electron tomography of mitochondria indicated that tBid binds to the contact sites between outer and inner membranes and there to affect specifically CL [91]. The synergistic induction of cytochrome c release by tBid and BAX also depended on CL, showing that tBid requires CL for its proapoptotic action.

Concomitantly to cytochrome c release, tBid caused a macroscopic remodelling of the total inner membrane [92] where the positive curvature of the external membrane face was strongly increased and the junction pores between the intracristae to the intermembrane space are enlarged thus facilitating cytochrome c efflux. Sine CL is mainly on the matrix leaflet, it is a dilemma how tBid can interact unless it reaches the matrix by membrane instabilities at the contact sites. Bax was shown to promote transbilayer diffusion of CL [93] and thus tBid in synergism with Bax can contact CL. The negative curvature of the m-side would be consistent with tBid interactions in the model systems. These tBid effects on the membrane structure are reminiscent to membrane structure changes observed in heart mitochondria involving the AAC, as evidenced by the response to ADP [94] and to inhibitors of the AAC [95–97]. The transition from a contracted state to ATR, where the matrix surrounds the vacular intermembrane space, to a decontracted state with ADP and BKA, where the matrix formed vesicle type interstitium indicated enlargement of one membrane leaflet relative to the other. It was suggested [97] to be caused by a surface charge excess at the expanding surface that may be caused by the redistribution of anionic lipids concomitant to the transition of the AAC between the c- and m-states. The high content of AACC in heart mitochondria may dictate these overall re-configurations of the inner membrane. In electron-tomographic analysis the inner membrane changes induced by the AAC ligands were suggested [98] to be driven by the redistribution of CL, similarly as in the tBid driven changes.

In liposomes containing Ca\(^{2+}\) and CL the relation of membrane curvature to tBid induced leakage was studied [99]. On enhancing the negative curvature by addition of PE (CL:PC:PE = 1:1:1) the tBid induced leakage is markedly increased whereas in liposomes without curvature obtained by replacing CL with PG, tBid was unable to induce leakage. \(^{31}\)P NMR measurements showed that conversely tBid interaction promotes negative membrane curvature thus destabilising the lipid bilayer and inducing leakage pores [99]. Hereby tBid induces a partial hexagonal lipid phase.

Another more general role of CL in tBid triggered apoptosis emerges from comparing mitochondria from wt and CL deficient cdlr yeast strains [100,101]. The inhibition of mitochondrial ATP synthesis by tBid was shown to result from the inhibition of the ADP/ATP transport

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**Fig. 3.** Scheme for interpreting the Ca\(^{2+}\) induced pore opening of the AAC and the involvement of the tightly bound CL. Ca\(^{2+}\) taken up into the matrix forms complexes with bound CL, releasing lysine groups at the matrix “collar”. The displacement of CL and the release of the positive charges at the AAC destabilises the AAC and open the m-gate in addition to the open c-gate, thus forming a pore. From [50].
The high acyl chain unsaturation of the CL bound to the AAC facilitates ablating or overexpressing the AAC\[105\]. It might be speculated, that the AAC since it is related to the ATP expression level, as shown in an independent portion that nevertheless seems to be associated with sensitivity to CAT. There exist also a large CAT insensitive and fatty acid to be caused by fatty acid cycling through the AAC as shown by the sustain this uncoupling is unclear. Part of this uncoupling is assumed binding energies of ADP3 and ATP4 and the concomitant inelasticity of the membrane caused by binding CL. 

4. CL and AAC in basal uncoupling

Basal uncoupling of mitochondria sustains parts of the basal metabolism prevailing in rest. The mechanism of the H+ “leakage" to sustain this uncoupling is unclear. Part of this uncoupling is assumed to be caused by fatty acid cycling through the AAC as shown by the sensitivity to CAT. There exist also a large CAT insensitive and fatty acid independent portion that nevertheless seems to be associated with the AAC since it is related to the ATP expression level, as shown by ablating or overexpressing the AAC [105]. It might be speculated, that the high acyl chain unsaturation of the CL bound to the AAC facilitates a slow H+ translocation through the membrane.

4.4. The AAC–CL couple as an Achilles heel

These “parafuncions" of the AAC, whether physiological, pathological or artefacts, are all consequences of its primary function that is furnishing ATP from the mitochondria to the cytosol. As was elaborated above, the unusually large virtual energy barriers of the ADP/ATP transport and the concomitant strong rearrangements are linked to the many ionic charges in the AAC protein and are further enhanced by electrostatic CL binding. These are matched by the high intrinsic binding energies of ADP3 and ATP4 in the transition state. However, these well balanced elements for an efficient ADP/ATP exchange render the AAC captive to changes in the membrane background, in particular because of the surrounding CL. They range from the more subtle redistribution of CL by tBid, the drastic withdrawal by Ca2+, to the destruction of surrounding CL by oxidants amplified by the high degree of unsaturation of the CL acyl chains and the resulting irreversible alkylation of SH and lysine groups. Thus the AAC faces a barrage of derangements that the cell may have adopted as a useful service in the MPT linked apoptosis, but that at an advanced stage leads to necrotic death. With the AAC and its interdependence to CL, the eukaryotic cell has acquired an “Achilles heel" sensitive to commonly occurring damaging factors, such as Ca2+ and oxygen radicals.

5. AAC, CL and supercomplexes

With blue native electrophoresis the existence of supercomplexes composed of the respiratory complexes III and IV were shown [106]. Further, using CL deficient yeast strains or by titrating CL with doxycyclin [107–109] a vital role of CL for the association of the complexes was demonstrated. Recently, with a new dual affinity tag AAC was found to participate in these supercomplexes [110], composed of one AAC dimer and two copies each of complex III and IV. Additional supercomplexes between AAC and other mitochondrial carriers were tentatively identified. In CL deficient mitochondria from cdr1 strain these complexes are absent or less defined. Possibly CL acts as a glue between these protein complexes by intercalating between the transmembrane sections of the components, similarly as shown in the crystal structure of (AAC)2, where 2 CL bind between the AAC monomers [51]. The (AAC)2 seems to be predestined for the anchoring role by providing the high amount of 6 tightly bound CL/dimer, out of which 4 CL may serve to connect the AAC with other proteins. The great density of AAC in the inner membrane (bovine heart 1.4, S. cerevisiae 0.45 (AAC)2/mg protein) amounts to a molar ratio (AAC)2/complex IV = 1.9 (S.c) to 2.5 (b.H.) [111,112]. Thus there is about twice as much (AAC)2 as could be recruited by the supercomplex.

It was suggested [110] that in this supercomplex the electrophotoretic ATP export profits from the adjacent H+ pumps generating a localised strong electrochemical gradient. Previously CL has been proposed [113] to function with the phosphate headgroups as a localised H+ buffer thus restricting H+ from the bulk domain. Adjacent to the respiratory H+ pumps the buffering by CL would increase the voltage component ΔΨ of the electrochemical gradient within the supercomplex. Thus besides connecting proteins, CL could locally guarantee a high ΔΨ that is needed to drive the directional ATP export versus ADP import through the AAC.

Notably, no supercomplex between AAC and ATP synthase is invoked in this recent report [110], although previously the existence of a supercomplex consisting of ATP synthase, AAC and Pic (a “synthasome") isolated from liver mitochondria was described [114]. Intuitively its existence might enhance the efficiency of newly synthesised ATP export by avoiding mixing with the matrix ATP and ADP pool. ATP would be directly received by the AAC from the adjacent synthasome. However no data on the stoichiometric composition of these detergent released fractions are given. Further, against earlier evidence [115,116], a micro-compartmentation of ATP synthesis and transport had been previously postulated [117,118] on the basis of various experimental studies (see review in [9]). However, by overcoming the former experimental problems, evidence was provided arguing against a localised cooperation of ATP synthesis and ATP [9,119].

6. Uncoupling protein

Uncoupling proteins (UCP) form a subfamily in MCF consisting of up to five members in mammalians [120,121]. UCP1, occurring exclusively in brown adipose tissue, is the best studied member. It catalyses H+ transfer through the inner membrane converting the electrochemical gradient generated by electron transport into heat. The H+ translocation is dependent on free fatty acids. But the mechanism of the H+ transfer is still a matter of controversy [122–124]. Essentially two models are discussed, UCP directly transports H+ [125] or UCP transports fatty acid anions and the free fatty acids return through the lipid bilayer thus indirectly catalysing H+ transport [124]. The H+ transport is inhibited by the purine nucleotides ADP, ATP, GDP, and GTP. The binding of these nucleotides and the concomitant inhibition are strongly pH dependent revealing binding with increasing pH.

On reconstituting isolated UCP1 a high H+ transport activity was measured [126,127] in proteoliposomes with only egg yolk PC as lipid component. Addition of acidic PL such as PS decreased the activity. CL addition at >10% also decreased activity. A striking effect of CL was observed at the inhibition of the H+ transport by nucleotides (G. Basset and M. Klingenberg, unpublished), as demonstrated in Fig. 4. CL strongly attenuates binding of GTP and the inhibition of H+ transport. Thus in a typical setting at pH 6.85, the Kp for 32P-GTP is increased 10 fold from 13.9 to 13.7 µM by 12% CL. This effect of CL may have great significance for the regulation of the uncoupling activity of UCP. The cytosolic concentration of the physiologically dominant regulator free (not complexed with Mg2+) ATP seems too high to permit UCP1 to assume the active state. The concentration of free ATP ranges at 100 to
300 μM which would saturate UCP1 at pH 7 with $K_D = 1 \mu M$. Because of the strong pH dependency of $\Delta \mu G_0 = \Delta \mu \Phi \approx -1.8$ [128] a shift of pH to 7.5 would increase the $K_D$ to 8 μM, which is still insufficient to relieve the ATP inhibition. A cytosolic pH increase up to 8.0 was noted on activation of thermogenesis in brown adipose tissue [129]. Since CL increases the $K_D$ about 10 fold, the $K_D$ could reach 80 μM at pH 7.5 and thus largely liberate UCP1 from ATP. With these findings, the conclusion (cf [130–132]) that nucleotides do not play a role in intracellular regulation of UCP1 because of a too high affinity, seems to be unfounded. Thus with the binding affinity decrease caused by CL, the strong well documented pH dependence of the nucleotide binding in UCP1 [133–135], gains additional significance as a major physiological regulator of uncoupling activity. These results may also at least partially explain the discrepancies reported on the GDP sensitivity of reconstituted UCPs where vesicles with and without CL have been employed [136,137].

UCP1 has been first isolated from brown adipose tissue following procedures only slightly modified from the isolation of AAC [44,138,139]. In both cases the isolated protein formed a dimer in the detergent (e.g. Triton X100) micelle. According to $^{31}P$ NMR spectra the adherent PL had proportions of PC/PE/CL = 45/48/19 Mol/UCP1 dimer, similar to that in the mitochondrial inner membrane (K. Beyer, M. Klingenberg, unpublished). On addition of SDS the increase of the CL signal, amounting to only 0.6 mol/mol dimer, was within the measuring error. Thus, in contrast to AAC, isolated UCP1 does not contain tightly bound CL, but is highly responsive to CL addition as evident in the regulation of UCP1 by nucleotides. The binding of nucleotides to UCP1 has two stages, first a loose binding without inhibition of $H^+$ transport and second a tight binding leading to inhibition [140,141]. Fluorophore (DAN-, DANSYL-) derivatives of ATP or GTP signal binding to UCP1 by their strong fluorescence increase, but $H^+$ transport was not inhibited. Only adenine- and guanine-, di- and tri-phospho nucleotides drive UCP1 into the subsequent inhibited state by changing the structure into a more tightened form as shown by increasing resistance to trypsin [140].

The transition from the initial binding to the second stage is notably slow [123]. In this setting CL seems to shift the distribution between the loose and tight binding state of UCP1 in such a manner that on decrease of nucleotide concentration the active state of UCP1 becomes favoured.

7. Conclusions

Mitochondrial solute carriers constitute a major part of the inner membrane proteins. Facing membrane lipids consisting of up to 20% CL, it is not surprising that the carriers have developed a symbiosis with CL. The carriers are specifically qualified for binding CL by their net positive charge excess that reaches a maximum in the AAC, where the tightly bound CL may have a cofactor role for facilitating transport by providing additional electrostatic energy. A wide range of effects are produced by this interaction, from activating transport, stabilising the protein and modulating inhibitor binding. Withdrawal of CL from the AAC by Ca$^{2+}$, tBid or by oxidation of CL deranges the AAC, promoting apoptotic or necrotic cell death. All this is based on binding of CL, ranging from a tight specific cofactor type binding in the AAC, additional specific binding, to the participation of CL in the surrounding lipid annulus. The dependence of the carriers, in particular of AAC, on CL imposes an Achilles heel on the eukaryotic cell.

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