The Reconstituted ADP/ATP Carrier Can Mediate H⁺ Transport by Free Fatty Acids, Which Is Further Stimulated by Mersalyl

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Nickolay Brustovetsky* and Martin Klingenberg

From the Institute of Physical Biochemistry, University of Munich, Goethestrasse 33, 80336 Munich, Federal Republic of Germany

In a reconstituted system, the participation of the ATP/ADP carrier (AAC) in the free fatty acid (FFA)-induced proton transport was demonstrated (i) by direct measuring of the proton transport through the membranes of AAC proteoliposomes and (ii) by monitoring of the transmembrane potential Δψ in AAC-cytochrome-c oxidase (COX)-coreconstituted proteoliposomes. FFA increased the initial rate of proton transport in AAC proteoliposomes and decreased Δψ in AAC-COX proteoliposomes. Inhibitors of AAC suppressed the effects of FFA. Without AAC or with inactive AAC, FFA cannot maintain proton leakage through the membrane. In these cases, even a small increase of Δψ was induced by FFA. These results demonstrate for the first time that FFA can facilitate the transfer of protons through the inner mitochondrial membrane thus accelerating the FFA-cycling efficiency of the AAC-mediated protonophoric action of FFA. Mersalyl also sensitized the protonophoric action of the FFA against nucleotides so that even guanine nucleotides, which are inactive in transport, become inhibitory. The effect of mersalyl is rationalized in terms of a specific interaction with cytochrome 159 being attracted as anion by surrounding positive charges. This might open a gate similarly as suggested for eosin 5-maleimide interaction (Majima, E., Koike, H., Hong, Y.-M., Shinohara, Y., and Terada, H. (1993) J. Biol. Chem. 268, 22181-22187) and, thus, transform the AAC into unidirectional transport mode.

It is generally recognized that the uncoupling action of FFA1 is connected with their ability to increase proton permeability of the inner mitochondrial membrane (for review, see Ref. 1). FFA cross the membrane in undissociated form, transferring protons, and return through the membrane as anion (2, 3). In the experiments with planar bilayer membrane, however, FFA have only a low protonophoric activity. It was shown that this is due to the limiting permeability of FFA anions (3, 4). From this point of view, the relatively high uncoupling action of FFA in mitochondria remains unclear.

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To whom correspondence should be addressed. Tel.: 089-5996-415. Fax: 089-5996-415.

*The abbreviations used are: FFA, free fatty acids; CCCP, carbonyl cyanide m-chlorophenylhydrazone; AAC, ADP/ATP carrier; COX, cytochrome-c oxidase; Δψ, transmembrane potential; TPP⁺, tetraphenylphosphonium cation; CAT, carboxyatractyloside; ATR, atractyloside; BKA, bongkrekic acid; NEM, N-ethylmaleimide; BSA, bovine serum albumin, free from fatty acids; C₃-E₈, octaethylene glycol monododecyl ether; Tricine, N-[2-hydroxy-1,1,1-thioglyceylmethyl]glycine.

Progress in this field was recently achieved by the discovery of the participation of AAC in the FFA-induced uncoupling of oxidative phosphorylation (5, 6). It was suggested that AAC can facilitate the transfer of FFA anion through the inner mitochondrial membrane, thus accelerating the FFA cycling (6, 7). It was shown that inhibitors of AAC, carboxyatractyloside, bongkrekic acid, pyridoxal 5-phosphate, and palmityl-CoA suppressed, at least partly, FFA effect on the respiratory rate and on the Δψ of mitochondria. The substrates of AAC, ADP, and ATP can also inhibit the uncoupling action of the FFA, but with smaller efficiency (5, 6). In this paper, we have shown for the first time that the FFA-mediated H⁺ transport can be stimulated also in the proteoliposomes containing isolated and functionally intact AAC.

Recently it was shown that treatment of reconstituted AAC by mersalyl causes reversible switching from obligate counter-exchange to uniport with simultaneous loss of the substrate specificity (8). Mersalyl might be expected also to accelerate the transport of FFA anions by AAC and, thus, to increase the FFA-uncoupling activity. The mersalyl effects on FFA uncoupling cannot be studied on mitochondria because of the abundance of mercurial-sensitive enzymes (9-17). Therefore, we studied the FFA effects under the influence of mersalyl on the AAC-COX proteoliposomes where we found enhanced uncoupling effects of FFA. Moreover, the sensitivity of the FFA effects toward the various ligands of AAC was strongly increased. Preliminary results have been published previously (18).

MATERIALS AND METHODS

Chemicals—C₃-E₈ was from Fluka or U. S. Biochemical Corp.; TPP-Br was from Fluka. Sephadex G-75 and Sephacryl S-500 were from Pharmacia Biotech Inc.; carboxyatractyloside and nucleotides were from Boehringer Mannheim. [¹⁴C]ADP was from Amersham Corp., and Dowex 1-X8 was from Bio-Rad. Phosphatidylcholine purified from turkey egg yolk, cardiolipin, and Amberlite XAD-4 were from Sigma. Hydroxylapatite and ['H]CAT were prepared as described previously (19-20).

Isolation of AAC—Mitochondria were prepared from beef hearts as described by Blair (21). The isolation of AAC from the bovine heart mitochondria were performed as described by Gawaz et al. (22) for AAC from yeast mitochondria.

Isolation of COX—COX was isolated from bovine heart mitochondria by the method of Fowler et al. (23) as described by Errede et al. (24) but without of the cholate-ammonium sulfate fractionation. A final protein concentration of the isolated COX preparation was 5.9 mg/ml. The concentration of cytochromes (α+α') was determined spectrophotometrically using an extinction coefficient of 16.5 m⁴·cm⁻¹ for the absorbance of the sodium dithionite-reduced enzyme at 605 - 630 nm (25). The cytochrome (α+α') concentration was 5.0 nmol/mg of protein.

Reconstitution of AAC—The reconstitution of bovine heart AAC in proteoliposomes essentially followed a procedure described earlier for AAC from yeast mitochondria (22). A mixture of 40 mg of turkey egg yolk phosphatidylcholine and 1.5 mg of cardiolipin (3.6% w/w) was thoroughly dispersed in a homogenizer with a solution containing 11% C₃-E₈ (w/v), 60 mM Na₂SO₄, 1 mM EGTA, 275 mM Tricine-OH, pH 6.0. In some experiments we used only phosphatidylcholine without cardiolipin for reconstitution. For the ADP-exchange experiments, 100 mM ADP was added to the mixture, and the Tricine concentration was 275 mM.
lowered to 175 mM, 450 pM of the solubilized lipids were mixed with 1.5 ml of the mitochondrial extract from mitochondria at a final detergent ratio about of 0.015 protein/ phospholipid. This gave a final concentration of 5% C12E5, 13.5 mM Na2SO4, 0.6 mM EGTA, 63.5 mM Tricine-OH, pH 8.0, about 20 mg/ml phospholipid, and, in the nucleotide-exchange experiments, 23 mM ADP. 

Proteoliposomes were prepared by gradual removal of detergent with a hydrophobic ion exchange material, Amberlite XAD-4. The total amount of Amberlite (2.0 g) yielded a detergent/Amberlite ratio of 50 mg/g beads. For the removal of the external solutes, the proteoliposome suspension was passed through Sephadex G-75 column (30 x 1 cm) pre-equilibrated with 100 mM sucrose, 30 mM Na2SO4, 1 mM Tricine-OH, pH 7.5, 1 mM EDTA. The samples were eluted with 50 mM NaCl. The amount of [3H]ADP taken up was measured in the eluted fractions. All others conditions are described under "Materials and Methods."

For [3H]CAT binding measurements to 60 ml of the proteoliposomal suspension, 45 mM ADP and 5.3 mM [3H]CAT was added; in another aliquot (also 60 ml) 45 mM ADP and 5.8 mM CAT was added, and then, after a 10-min preincubation, 5.3 mM [3H]CAT was added. Both aliquots of the proteoliposomes were incubated overnight at 4 °C. Afterwards, free CAT was separated from the proteoliposomes by small Sephacryl S-500 column (2 x 0.4 cm), pre-equilibrated with 100 mM sucrose, 30 mM Na2SO4, 1 mM Tricine-OH, pH 7.5, 1 mM EDTA. The samples were eluted by the same solution and amount of [3H]CAT taken up was measured in 10 eluted fractions (100 µl each). The difference in the [3H]CAT binding between CAT-preincubated and unpreincubated proteoliposomes was calculated. Quantitative determinations of Δψ were performed accordingly Kamo et al. (27). The Δψ generation was initiated by addition of 16.7 mM ascorbate and 6.7 µM cytochrome c to the proteoliposomes. The final concentration of TPP+ was 1.0 µM. The medium for this measurements contained 170 mM mannitol, 30 mM Tris-H2SO4, pH 7.2, 0.5 mM EGTA, temperature of medium 30 °C.

**TABLE I**

| Content of proteoliposomal membranes | [3H]CAT binding | Δψ |
|--------------------------------------|-----------------|----|
| COX                                  | nMol/mg protein | mV |
| AAC                                  |                 |    |
| Cardiolipin                          |                 |    |
|                                      | +               | 160|
|                                      | +               | 100|
|                                      | +               | 615|
|                                      | +               | 615|

**RESULTS**

The Reconstituted AAC Retains Ability to Participate in the FFA-induced Proton Transport—H+ efflux from the proteoliposomes with reconstituted AAC was measured with a pH electrode in the absence and in the presence of CAT or BSA as shown in Fig. 2. H+ transport is initiated by the addition of valinomycin, which induces the influx of the external K+ so that the H+ efflux is driven by a K+ diffusion potential. A significant initial rate of proton efflux was obtained even in the absence of exogenous FFA. Addition of BSA to the proteoliposomes...
diminished the rate of H⁺ transport that shows FFA-dependent nature of the H⁺ efflux (Fig. 2). The significant inhibition of H⁺ efflux by CAT indicates that reconstituted AAC retains the ability to participate in the proton transport. The dependence on the concentration of exogenous oleate is shown in Fig. 3A. The absolute extent of CAT inhibition remained almost the same, while the relative portion of CAT inhibition became smaller. The CAT-inhibited portion of proton transport is increased strongly with temperature (Fig. 3B). This high temperature dependence correlates with the high temperature dependence of ADP/ATP exchange in mitochondria (33). These experiments support a participation of AAC in the FFA-induced H⁺ transport. At the same time, the CAT-insensitive H⁺ transport in this system was quite high. This activity might be due to formation of the ionic pairs between FFA anions and K⁺-valinomycin. The ability of K⁺-valinomycin to form a complex with organic anions is well known (for review, see Ref. 2). As a result, the rate that the FFA anions transfer through the membrane could be increased as proposed for hydrophobic anions and artificial uncouplers (29, 34). This agrees with the recent observation that K⁺-valinomycin results in an acceleration of transmembrane ΔpH decay in the presence of oleate (35).

To overcome this problem, we performed coreconstitution of AAC together with COX as Δψ generator instead of K⁺-valinomycin system. The main characteristics of these proteoliposomes are shown in Fig. 1 and in Table I. In our experiments, the absence of cardiolipin in the membrane strongly diminished nucleotide exchange activity of AAC and Δψ-generating capability of COX. It coincides with earlier observations (36–38). At the same time, the lack of cardiolipin virtually did not affect [¹⁴C]CAT binding that reflects an incorporation of AAC into the membrane. Except the experiments with proteoliposomes, which did not contain cardiolipin, the coreconstitution was only considered to be successful when both the [¹⁴C]ADP-ADP exchange activity and the COX-generated protonomotive force were determined to be sufficiently high. Direct measurements of the passive proton transport in this system were not possible because of active H⁺ extrusion by COX and also because of the necessity to use K⁺-valinomycin to generate noticeable ΔpH (39). Therefore, we measured the distribution of the lipophilic cation TPP⁺ between proteoliposomes and external medium for estimating of Δψ (28, 29).

The action of palmitate and oleate on Δψ generated by COX in proteoliposomes without AAC is shown in Fig. 4. Instead of an expected decrease, the FFA addition resulted in an increase of Δψ. A similar result was obtained earlier in another laboratory (6) and was explained as a partial conversion of ΔH into Δψ. Indeed, immediately after the addition, the FFA, in their undissociated form, carry some protons on distribution across the membrane (35) that can result in a dissipation of ΔpH. The
\[ \Delta \psi \text{, increased after addition of FFA, was stable and did not decrease within the time of the experiment. Low concentration of } \text{CCCP and inhibition of } \text{COX by } \text{NaN}_2 \text{, very effectively decreased } \Delta \psi \text{. In all of these cases, inhibitors of AAC had no effect on } \Delta \psi. \]

The following experiments were performed with the AAC-COX-coreconstituted proteoliposomes. In this case, the addition of oleate caused distinct decrease of \( \Delta \psi \) (Fig. 5, A and C) in contrast to the increase of \( \Delta \psi \) in the COX proteoliposomes (Fig. 4). CAT and BKA restored \( \Delta \psi \); afterwards, BSA had a negligible effect (Fig. 5A). Similar results were obtained in these and all of the following experiments using palmitate (not shown). It is interesting, that ATR, a less effective inhibitor of AAC, could not increase \( \Delta \psi \) (Fig. 5C). Surprisingly, CAT (added after ATR) was ineffective, and only BKA retained the ability to restore \( \Delta \psi \). Olate failed to decrease \( \Delta \psi \) if added after CAT and BKA (Fig. 5B). In the same experiments, low concentration of CCCP effectively lowered \( \Delta \psi \). In the proteoliposomes without cardiolin, which had AAC with very low functional activity (Fig. 1), oleate did not decrease but even increased \( \Delta \psi \) (Fig. 5D) as in the case of COX-proteoliposomes (Fig. 4). CAT and BKA added after oleate had very small effects in these experiments. As shown in Fig. 6, incubation of the AAC-COX proteoliposomes with higher concentration of oleate stronger decreased the initial level of \( \Delta \psi \), which could be also restored by CAT and BKA. It should be noted that CAT and BKA increased \( \Delta \psi \) even in the absence of exogenous FFA (Fig. 6A). The obtained results clearly indicate that, in the reconstituted system, the protonophoric action of FFA is mediated by AAC. This assertion agrees with the conclusions that were made from the mitochondrial studies (5–7). The obtained results also stressed an importance of the functional activity of the reconstituted AAC for its participation in the FFA protonophoric action.

**Mersalyl Increases the FFA Effects and Modifies the AAC Sensitivity to Various Ligands**—Taking into account the recently discovered effect of mersalyl on the transport properties of reconstituted AAC (8), we have examined the effect of mersalyl on the FFA-induced increase of proton permeability in AAC-COX proteoliposomes. In the presence of mersalyl, the initial level of \( \Delta \psi \) was lower (Fig. 7, A and B); also, the \( \Delta \psi \) decrease by oleate was stronger. Mersalyl did not decrease \( \Delta \psi \) in proteoliposomes containing only COX. The effects of CAT and BKA were also more pronounced under mersalyl. Dithioerythritol prevented all of these mersalyl effects. In this case, the action of oleate and CAT was even weaker than without mersalyl treatment (Fig. 7C). Mersalyl also increased the effects of CAT and BKA in the absence of exogenous FFA (Fig. 8, A and B). Simultaneous preincubation of the AAC-COX proteoliposomes with mersalyl and oleate further lowered the initial level of \( \Delta \psi \) and increased the AAC inhibitors effects (Fig. 8C, compare with Fig. 6B). Under these conditions, the extent of the inhibition by CAT and BKA was affected by mersalyl also; the influence of CAT was stronger than that of BKA (Fig. 8, C and D). Obviously with mersalyl the c-state population of AAC is relatively enhanced as seen by the relative CAT and BKA responses (Figs. 7 and 8). BSA, added together with mersalyl and oleate in the preincubation medium, prevented their effect (data not shown). It is interesting that with mersalyl, ATR (which could not increase \( \Delta \psi \) without mersalyl preincubation (Fig. 5B)) noticeably enhanced \( \Delta \psi \) (Fig. 8E). Correspondingly, CAT, added after ATR, had only a small effect, and BKA had almost the same effect as without ATR. But the total effect of ATR and CAT was smaller than that of CAT alone (Fig. 8, C and E). Obviously, mersalyl treatment of the AAC enhances the sensitivity toward ATR so that it can block access of CAT. It should be also stressed that the alkylating SH-reagent, NEM (2 mm), virtually did not influence the initial level of \( \Delta \psi \) and only slightly decreased the oleate, CAT, and BKA effects (data not shown).

In contrast to other authors (8, 40), we were able to avoid in our experiments the appearance of nonspecific cationic permeability under mersalyl treatment of the proteoliposomes. Thus, with a K + -selective electrode, we observed no difference in the K + efflux rate in mersalyl-treated and untreated proteoliposomes (data not shown). In spite of a difference in the initial level of \( \Delta \psi \) between untreated and mersalyl-treated proteoliposomes, the latter could produce quite high and stable \( \Delta \psi \). The immunosuppressant cyclosporin A, which is an inhibitor of the nonspecific permeability pathway of the inner mitochondrial membrane (41, 42), had no effect. Whereas CAT favors opening of cyclosporin A-sensitive nonspecific pore of the inner mitochondrial membrane (43–46), in our experiments it decreased the membrane permeability of AAC and AAC-COX proteoliposomes.

It is known from mitochondrial studies that ADP can, at least partly, replace CAT in the inhibition of the uncoupling action of the FFA; ATP is much less effective, and GDP is ineffective (6). In our experiments, ADP failed to increase \( \Delta \psi \) of the mersalyl-untreated proteoliposomes. But with mersalyl and oleate, ADP distinctly increased the initial level of \( \Delta \psi \) (Fig. 9, A and B). Surprisingly, ADP completely prevented the CAT effect in these conditions. Moreover, under the influence of ADP, the effect of BKA proved to be significantly bigger (Fig. 9B). Similar effects were obtained with ATP (data not shown). It is also intriguing, that under these conditions, even GDP and GTP had a similar, but somewhat smaller, influence on the membrane proton permeability and on the effects of CAT and BKA (Fig. 9, C and D). Probably, mersalyl treatment sensitizes the AAC toward guanine nucleotides, since without mersalyl treatment these nucleotides had no influence.

**DISCUSSION**

Studies with planar bilayer membrane show that the proton permeability of the phospholipid membrane is determined mainly by FFA and is increased under action of transmembrane potential ~80 mV (3, 4). The increase of the conductivity of the planar bilayer membrane by FFA remains, however, low as
FIG. 5. The action of oleate addition and AAC inhibitors on Δψ of the AAC-COX proteoliposomes. The dependence from the presence of cardiolipin in the membrane. Traces A and B differ by the order of the additions of oleate and of AAC inhibitors; trace C, ATR was added before CAT and BKA; trace D, proteoliposomes without cardiolipin. Additions were as follows: oleate, 16 μM; CAT, 0.5 μM; BKA, 3 μM; ATR, 10 μM; BSA, 0.1%; CCCP, 10 nM. The conditions of the experiments are described in the legend to Fig. 4. The concentration of phospholipids in the vessel was about 10 mg/ml.

FIG. 6. An increase of Δψ of the AAC-COX proteoliposomes under the action of CAT and BKA in the absence of exogenous FFA. The effect of the preincubation of the proteoliposomes with increasing concentrations of oleate. Trace A, without oleate; trace B, proteoliposomes were preincubated 5 min at 30 °C with 16 μM oleate; trace C, proteoliposomes were preincubated 5 min at 30 °C with 32 μM oleate. Additions were as follows: CAT, 0.5 μM; BKA, 3.0 μM. The conditions of the experiments are described in the legend to Fig. 4. The concentration of phospholipids in the vessel was about 10 mg/ml. In these experiments, 0.5 μM CAT resulted in maximal effect so that the next addition of CAT did not give further increase of Δψ.

compared with mitochondria (5, 6). Similar results were observed with pure phospholipid liposomes and COX proteoliposomes (47, 48). Recently, a higher protonophoric effect of FFA on COX proteoliposomes was obtained with higher concentrations of oleate (49). In our experiments (Fig. 4) as well as in other laboratories in COX proteoliposomes and in proteoliposomes prepared with detergent extract of liver mitochondria, no evidence was found for FFA in a concentration low enough (8–16 μM) to maintain proton leakage (39, 50).

The transfer of protons by FFA is limited by the low permeability of the FFA anions (3, 51). In contrast to the model systems, in mitochondria FFA can quite effectively uncouple oxidative phosphorylation (for review see Ref. 1), probably due to interaction with some proteins (for example, with AAC (5, 6, 52–54), perhaps, by facilitating FFA anion transfer (7)). However, it is difficult to directly demonstrate the transfer of the hydrophobic FFA anions through AAC so that the question remains still open.

The reconstituted system with isolated components as used in the present report permits us to pinpoint the participation of specific components in the studied process. With the isolated and reconstituted AAC (in particular together with COX) good evidence for the participation of AAC in the FFA-induced H+ transport is provided by the use of specific AAC inhibitors, CAT...
and BKA. Both counteract the Δψ depressing effect of FFA (Figs. 5 and 6). The stepwise inhibition by CAT and BKA can be interpreted in terms of the population of the incorporated AAC in the c- and m-state. With CAT the molecules in the c-state and with BKA, those in the m-state are inhibited.

The weaker inhibitor of AAC, ATR, could not inhibit the Δψ-depressing effect of FFA (Fig. 5C). Interestingly, CAT added after ATR also lost the capability to restore Δψ. It should be noted that a similar result was observed with isolated mitochondria (6, 53). ATR failed to block the interaction of AAC with FFA, but strongly interfered with the interaction of CAT with the enzyme. CAT can replace bound ATR (33), but, obviously, the displacement of ATR required more time.

In our work, only the CAT- and BKA-sensitive effects were regarded due to specific AAC interaction. This has to be differentiated from an unspecified H⁺ permeability of lipid membrane that is increased by the incorporation of integral membrane proteins (55). Recently, a coreconstitution of an AAC extract with bacteriorhodopsin into proteoliposomes was reported and shown to have high H⁺ permeability (40), especially under mersalyl action. However, this H⁺ permeability was insensitive to CAT as well as to FFA. One of the most important prerequisites of the successful work with reconstituted AAC was to be assured that the incorporated AAC was still functionally active as assayed by the adenine nucleotide exchange capability (Fig. 1). Omission of cardiolipin from the reconstitution causes loss of both adenine nucleotide exchange (Fig. 1) and FFA-induced AAC-mediated H⁺ transport (Fig. 5B). Unfortunately, in the above mentioned work (40), no assay as to whether the incorporated AAC was still active was given. Therefore the functional participation of AAC in the FFA-induced H⁺ transport in these experiments remains doubtful.

Recently it was shown that mercurials induce in AAC an unidirectional anion transport with less substrate specificity.
posomes preincubated with oleate. In the vessel was about 10 mg/ml.

Therefore, it might be expected that mercurials will facilitate an interaction of FFA anions and enhance FFA anions transport by AAC, which would increase FFA-mediated $H^+$ transport. In our experiments, indeed, mersalyl treatment of the reconstituted AAC caused a distinct increase of the protonophoric action of FFA (Figs. 7 and 8). At the same time, another SH reagent, NEM, which failed to switch the AAC to uniport (8), did not enhance the effect of FFA. In another interpretation, the interaction of mersalyl is viewed to improve the accessibility of FFA to the AAC intrinsic channel. In this case, FFA could play a role as $H^+$ supplier as proposed for the interaction of FFA with uncoupling protein (56, 57) and provide a $H^+$ uniport through AAC without FFA cycling. Actually, now we unfortunately cannot discriminate between these two proposed mechanisms of the interaction of AAC and FFA. It is not excluded that these mechanisms can exist simultaneously and occur without mersalyl treatment also. At any rate, the interaction of mersalyl with accessible SH groups of the AAC seems to improve the cooperation of FFA and AAC in the $H^+$ transport.

ADP and ATP, only hardly influenced the $\Delta \psi$ of proteoliposomes without mersalyl treatment. This is in contrast to experiments with isolated mitochondria where a small but distinct recoupling effect of ADP was found (5, 6, 53). The difference consists in the inability of ADP or ATP to enter into AAC-COX proteoliposomes because their transport is tightly coupled with exchange. It seems that nucleotides must enter into the vesicles and interact with AAC from the inside to affect the FFA-induced AAC-mediated $H^+$ transport.

The mersalyl treatment sensitized the AAC toward various ligands. Now both ATR and ADP (or ATP) are able to inhibit the effect of FFA (Fig. 8E and 9F). More strikingly, the nucleotides abolished the CAT sensitivity, whereas that of BKA is strongly enhanced (Fig. 9B). This agrees with the assumption that the effect of CAT and BKA reflects the population of the AAC in the c- and m-states. With mersalyl, ADP can enter into the proteoliposomes without exchange and transform the AAC mainly into the m-state in which only BKA is still effective. Part of the AAC molecules are apparently blocked by ADP also on the c-state.

Mersalyl sensitized the AAC even toward extremely poor ligands, such as guanine nucleotides, so that they now possess an effect similar to that of adenosine nucleotides (Fig. 9, C and D). However, the interaction between AAC and guanine nucleotides remains weaker than with ADP or ATP. Therefore, in the presence of GDP and GTP, the total response with CAT and BKA is still larger, and the redistribution of AAC in the m-state is less complete than with ADP or ATP. The sensitization by mersalyl toward ATR underlines the generality of the changes (Fig. 8E). One contributing factor to the stronger effect of weak ligands can also be the higher $H^+$ flux rate under the influence of mersalyl.

The effects of mersalyl should be due to interaction with cysteines. Recently, it was proposed, based on the interaction of SH reagents in particular with Cys-159, that the second repeat domain is involved in the transport of adenosine nucleotides (58). Cys-159 is located near a cluster of net positive charge (59, 60), which suggested to function as a gate for transport (58). The adenosine nucleotide binding site was previously localized by photaffinity labeling near this cluster (61, 62). Due to surrounding positive charges, the negatively charged eosin 5-maleimide readily interacts with Cys-159 (58), whereas electron-neutral NEM interacts much more slowly. Similarly in our experiments, NEM almost had no effect. Extending this model, we propose that, also, the mersalyl anion might be attracted to this positively charged site and, thus, open the gate and react with Cys-159. Since mersalyl anion is more compact than eosin 5-maleimide, it may reach this site even from the cytosol side when it interacts with the AAC in mitochondria and proteoliposomes (8, 63). The changes by mersalyl of the proposed gate could explain the induction of unidirectional transport, and the decrease of substrate specificity of AAC (8). Thus, the strong effects of mersalyl on the FFA-induced AAC-mediated $H^+$ transport as shown in this work might be rationalized in accordance with a specific interaction of mersalyl with Cys-159.

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