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REVIEW ARTICLE

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Exploring the proton transport mechanism of the mitochondrial ADP/ATP carrier: FA-cycling hypothesis and beyond

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Abstract

The mitochondrial ADP/ATP carrier (AAC, ANT), a member of the SLC25 family of solute carriers, plays a critical role in transporting purine nucleotides (ATP and ADP) as well as protons across the inner mitochondrial membrane. However, the precise mechanism and physiological significance of proton transport by ADP/ATP carrier remain unclear. Notably, the presence of uncouplers—such as long-chain fatty acids (FA) or artificial compounds like dinitrophenol (DNP)—is essential for this process. We explore two potential mechanisms that describe ADP/ATP carrier as either (i) a proton carrier that functions in the presence of FA or DNP, or (ii) an anion transporter (FA^- or DNP). In the latter case, the proton is translocated by the neutral form of FA, which carries it from the matrix to the intermembrane space (FA-cycling hypothesis). Our recent results support this hypothesis. We describe a four-step mechanism for the "sliding" of the FA anion from the matrix to the mitochondrial intermembrane space and discuss a possible generalization of this mechanism to other SLC25 carriers.

KEYWORDS
bilayer lipid membranes, MD simulations, membrane proteins, mitochondrial transporter, reconstituted protein, uncoupling protein

1 | INTRODUCTION

In mitochondria, oxidative phosphorylation is responsible for the production of adenosine triphosphate (ATP) through the phosphorylation of adenosine diphosphate (ADP). Protons are transported from the mitochondrial matrix to the intermembrane space by the respiratory chain complexes embedded in the inner mitochondrial membrane, generating a transmembrane proton motive force (Δp). The proton gradient drives protons back into the matrix via the F_1/F_0-ATP synthase, transferring the potential energy of the gradient to synthesize more accessible chemical compound ATP from ADP and inorganic phosphate.

One of the important player in this process is the ADP/ATP carrier (AAC, also referred as adenine nucleotide translocase, ANT in literature), which exchanges ADP from the intermembrane space against ATP from the mitochondrial matrix to provide chemical energy to the cell and maintain ATP production in mitochondria. Interestingly, in addition to its role in substrate transport, the ADP/ATP carrier has also been implicated in proton transport.

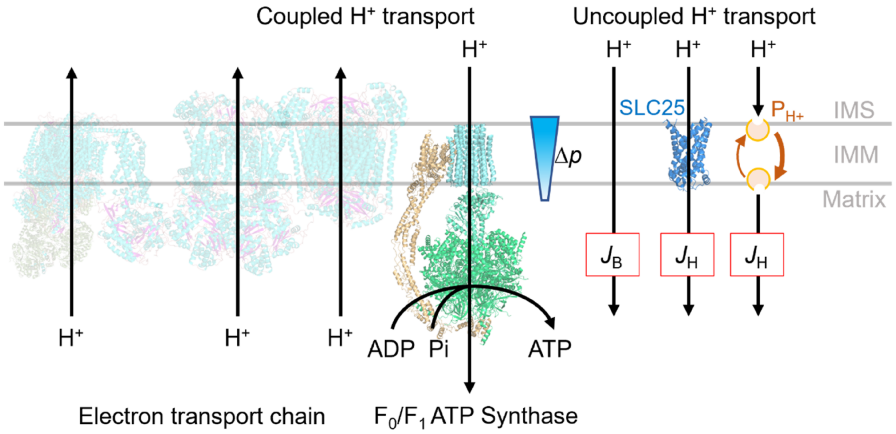

FIGURE 1 Proton (H^+) transport across the inner mitochondrial membrane (IMM) in mitochondria is divided into coupled and uncoupled H^+ transport. H^+ are pumped from the matrix to the intermembrane space (IMS) through the electron transport chain, establishing an H^+ gradient that contributes to the proton motive force Δp. H^+ can then pass through the F_0/F_1 ATP synthase (green), which couples the dissipation of $\Delta\Phi_H$ to ATP production. Uncoupled H^+ transport dissipates Δp without ATP production as a result of unregulated, basal H^+ transport J_B and H^+ transport J_H mediated by SLC25 carriers (blue) or protonophores P_{H+}.

In this review, we aim to explore the significance of the proton leak across the mitochondrial inner membrane, the role of uncoupling proteins including UCP1 as a classical proton transporter, and the involvement of the ADP/ATP carrier in (i) basal proton leak, which was reported to be influenced, among other factors, by the abundance of the ADP/ATP carrier in the IMM; (ii) proton movement coupled to the ADP/ATP exchange; and (iii) FA-activated proton transport, which is facilitated and accelerated by the ADP/ATP carrier. We will also discuss the proposed mechanisms underlying proton transport. For clarity, the term "ADP/ATP carrier" will be used consistently throughout this review, although we will retain the original terms (AAC or ANT) when referring to specific studies.

2 | PROTON LEAK IN MITOCHONDRIA

2.1 | Origin of proton leak

The dissipation of the transmembrane proton gradient ($\Delta\Phi_H{}^+$) is usually coupled to ATP production in mitochondria. Protons can also return to the mitochondrial matrix through alternative pathways, leading to uncoupling and potentially reducing the efficiency of ATP synthesis. The pathways the proton takes across the inner mitochondria membrane are still under investigation. Both phospholipids and proteins appear to contribute to proton conductance (Figure 1). Two mechanisms are commonly discussed: (1) inhibitor-insensitive basal proton conductance (J_B) and (2) protein-mediated inhibitor-sensitive proton leak (J_H) (Brand 1990; Nicholls 2021).

The first mechanism is present in mitochondria from all tissues studied, may contribute to metabolic rate, and remains insensitive to all known activators or inhibitors. Potential factors influencing this basal conductance could result from physicochemical changes in the membrane, which include: (i) variations in phospholipid composition and fatty acid (FA) composition and levels (Brookes et al. 1998); (ii) lipid modifications, surface area and membrane bending (Jovanovic et al. 2019; Jovanovic et al. 2022; Pohl and Jovanovic 2019); and (iii) membrane potential (Rupprecht et al. 2010). Mitochondrial carriers constitute a large fraction of the IMM and could modulate the structure of the IMM, thereby increasing H^+ permeability. Basal H^+ leak was proposed to be modulated by the abundance of the ADP/ATP carrier in different tissues (Brand et al. 2005). Several members of the SLC25 family, including uncoupling proteins (UCP) and ADP/ATP carriers (AAC, ANT), as well as artificial uncouplers, such as DNP, CCCP (carbonyl cyanide-m-chlorophenylhydrazone), and FCCP (carbonyl cyanide-4-(trifluormethoxy)-phenylhydrazone), have been proposed as major contributors to J_H (Figure 1).

2.2 | Physiological and therapeutical role of the mitochondrial proton leak

Although proton leak might initially appear wasteful from an energy production perspective, it plays a vital role in maintaining cellular homeostasis and offers significant therapeutic potential. The primary physiological function of proton leak is to regulate the mitochondrial membrane potential ($\Delta\Phi_m$). A high membrane potential increases the risk of electron leakage from the electron transport chain (ETC), which promotes the formation of reactive oxygen species (ROS). Proton leak reduces $\Delta\Phi_m$, thereby decreasing ROS production and protecting cells from oxidative stress. This mechanism acts as a natural defense against oxidative damage, which can impair mitochondrial function and lead to cell injury.

In addition to its protective role, proton leak is essential for non-shivering thermogenesis, a process mediated by uncoupling protein 1 (UCP1) (see section 1.4). This heat production is vital for maintaining body temperature in mammals, particularly in cold environments.

Modulating mitochondrial proton leak holds promise for the treatment of various conditions, particularly

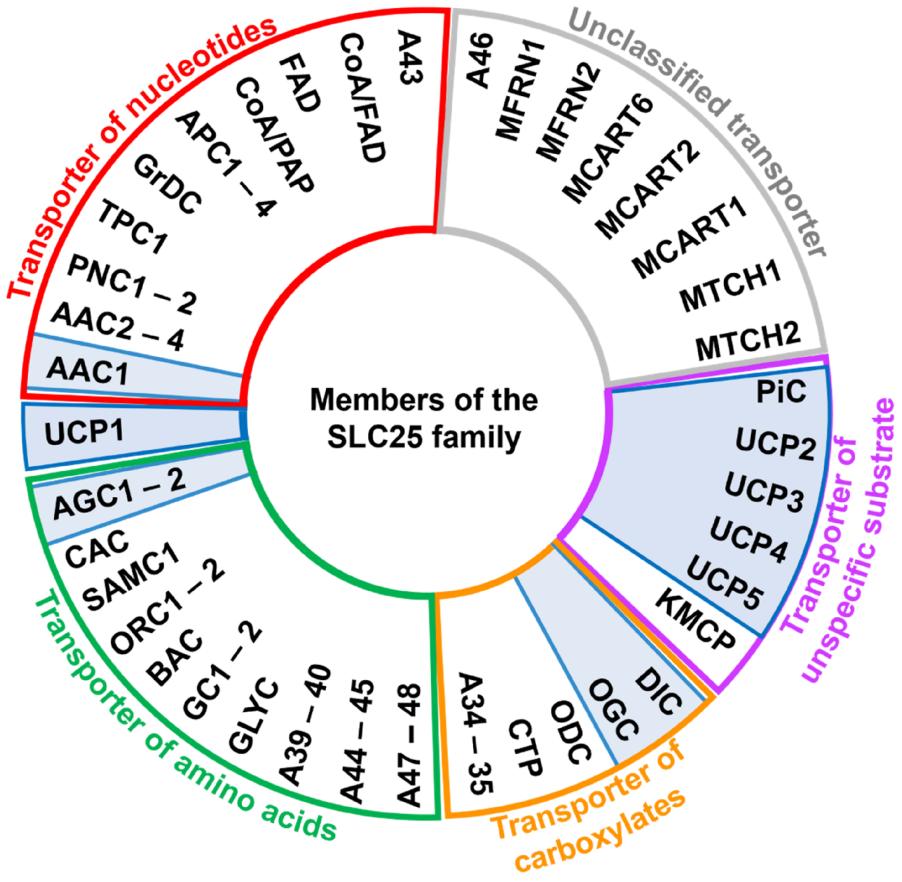

FIGURE 2 The 53 members of the SLC25 superfamily of mitochondrial carriers are classified into different subgroups based on the primary substrate they transport. These include nucleotides (red), amino acids (green), and carboxylates (orange). Carriers that transport different types of substrates are highlighted in pink, while unclassified carriers are shown in gray. Carriers involved in regulated H^+ transport (J_H) are marked with a blue background. Scheme adapted and modified from Palmieri (2014).

those related to oxidative stress and energy metabolism disorders. These include oxidative stress-related diseases and metabolic disorders, such as neurodegenerative disorders, cardiovascular diseases, cancer, obesity, diabetes and aging (Cadenas 2018; Divakaruni and Brand 2011; Perry et al. 2015). By enhancing proton leak and reducing ROS production, therapies that mildly uncouple mitochondrial respiration may offer protection against oxidative damage. However, such interventions must be carefully controlled to avoid disrupting cellular energy homeostasis, as seen in cases of DNP overdose (Grundlingh et al. 2011).

2.3 | Mitochondrial SLC25 superfamily

The solute carrier superfamily 25 (SLC25) is the largest family of membrane transporters located in the inner mitochondrial membrane (Kunji et al. 2020; Palmieri 2014). All relevant substrates for mitochondrial function are transported into the mitochondrial matrix through the different members of this superfamily. The family is classically divided into different smaller groups based on the type of substrate they transport—nucleotides, carboxylates, and amino acids (Figure 2). An evolutionary analysis of all SLC carriers classified the SLC25 family members into 10 subgroups, each originating from distinct genetic lineages (Ferrada and Superti-Furga 2022).

Although several carriers were identified at genetic level, their physiological function remains unresolved. The UCP family stands out, since they cannot be included in either of the groups so far. For a long time, UCPs were believed to exclusively mediate the proton leak associated with mitochondrial uncoupling. However, different studies have now demonstrated that UCP2 and UCP3 can transport a variety of substrates, including C4 metabolites such as phosphate, aspartate, oxaloacetate, sulfate and sulfite (De Leonardis et al. 2024; Gorgoglione et al. 2019; Kreiter et al. 2023a; Lunetti et al. 2022; Raho et al. 2020; Vozza et al. 2014). These findings have led to the proposal of a dual transport function for some mitochondrial carriers (Pohl et al. 2019).

The investigation of the expression and tissue specificity of the SLC25 members is challenging. It is hampered by the high homology of the SLC25 proteins, the low specificity of commercial antibodies and the lack of appropriate positive and negative controls. Furthermore, protein expression in unrelated to the gene expression of several SLC25 proteins, due to the post-translational regulation of the mitochondrial carriers (for review, see Pohl et al. 2019).

2.4 | The proteins of the UCP subfamily

UCP1, the best studied uncoupling protein, is only expressed in brown adipose tissue (BAT) and is involved in non-shivering thermogenesis by dissipating the proton motive force as heat under cold acclimating conditions (for reviews, see Nedergaard and Cannon 2018; Nicholls 2023; Ricquier 2017). Long-chain fatty acids or dinitrophenol are necessary to assist UCP1-mediated uncoupling (Beck et al. 2007; Bertholet et al. 2022; Zuna et al. 2021). It was also proposed that coenzyme Q is an essential co-factor for UCP1 function, but its involvement was not confirmed by several experimental approaches including model systems, isolated mitoplast and isolated yeast mitochondria (Esteves et al. 2004; Fedorenko et al. 2012; Jaburek and Garlid 2003). Other proteins of the UCP family (UCP2-UCP5) were also shown to transport protons in the presence of fatty acids (FAs) in different experimental setups including biomimetic systems (Beck et al. 2007; Hoang et al. 2012; Macher et al. 2018; Urbankova et al. 2003). This transport function was initially associated with ROS regulation (for review, see Krauss et al. 2005) and is currently under debate (Bouillaud et al. 2016; Nedergaard and Cannon 2003; Pohl et al. 2019). The proton turnover rates of these proteins in the presence of FAs are similar to that of UCP1 under thermoneutral conditions ($k = 5–14$ H^+/s; Beck et al. 2007; Macher et al. 2018; Urbankova et al. 2003), putting forward the idea about their general ability to transport protons. The physiologically relevant function of UCP1 originates from its massive (10–20 times compared to baseline levels; Cannon and Nedergaard 2004) upregulation during cold acclimation. Since UCP2-UCP4 expression levels are generally lower (Rupprecht et al. 2012), their protonophoric function is not as physiologically apparent as for UCP1. Interestingly, UCP5 was not found at the protein level under physiological conditions or inflammation in case the evaluated antibodies were used (Rupprecht et al. 2012; Smorodchenko et al. 2017).

Arguments against the proton-transporting function, especially that for UCP2, miss strong scientific rigor. The absence of two specific histidine residues in UCP2 (H145, H147), which are claimed to be critical for UCP1's function (Bienengraeber et al. 1998), was erroneously proposed to impair UCP2's protonophoric function. Based on the phylogenetical analysis, Keipert et al. (2024) proposed that UCP2 lack two crucial amino acids potentially involved in the proton transport, mediated by UCP1 (F289 and Q100) and cannot transport protons. Both statements contradict the published results, showing UCP2-mediated proton transport in the presence of fatty acids (Beck et al. 2007; Rupprecht et al. 2010; Zackova et al. 2003). The group of Kirichok reported the absence of UCP2-mediated proton transport in the control patch clamp experiments on mitoplasts from brain tissue. Usage of the evaluated antibodies, however, demonstrated the lack of UCP2 expression in brain tissue, except for microglia (Maes et al. 2023), which belongs to immune tissue.

2.5 | Other proton-transporting proteins

Besides UCPs, further mitochondrial carriers with clear substrate transporting function were reported and identified to mediate FA-activated uncoupling in mitochondria. Protonophoric function was shown for the ADP/ATP carrier (Andreyev et al. 1988), oxoglutarate carrier (Yu et al. 2001; Zuna et al. 2024), aspartate/glutamate carrier (Samartsev et al. 1997), phosphate carrier (Samartsev et al. 2011), and dicarboxylate carrier (Wieckowski and Wojtczak 1997). While the involvement of AAC1 and OGC was also tested in biomimetic systems (Kreiter et al. 2021; Zuna et al. 2024), the others have not been further evaluated (Brustovetsky et al. 1989).

3 | THE ADP/ATP CARRIER

The ADP/ATP carrier is nuclear-encoded and is the most abundant membrane protein in mitochondria (Heldt et al. 1965; Pfaff et al. 1965; Vignais 1976). The activities of ADP/ATP carrier and F_1/F_0-ATP synthase are putatively tightly coupled. Usually, ATP is transported outside the mitochondria, but if ATP production by the respiratory chain is inhibited, the function of ADP/ATP carrier is reversed, and ATP is transported into the mitochondrial matrix. Since humans metabolize the equivalent of its mass of ATP daily, ADP/ATP carrier dysfunction induces various clinical disorders, such as mitochondrial myopathies, progressive external ophthalmoplegia, neurodegenerative disorders, retinitis pigmentosa, etc. and can affect different tissues at various levels (for review, see Clemencon et al. 2013). Additionally, AAC1 has been proposed to have an important role in the mitochondrial permeability transition pore function and in apoptosis (Karch and Molkentin 2014). Increased levels of ANT mRNA and protein have been found in cold-acclimated birds (Roussel et al. 2000; Talbot et al. 2004; Toyomizu et al. 2002), implying its involvement in non-shivering thermogenesis, normally attributed to avian UCP (Emre et al. 2007).

3.1 | Isoforms and expression of ADP/ATP carrier

In humans, four isoforms of ADP/ATP carrier are present with distinct tissue expression. AAC1 is primarily

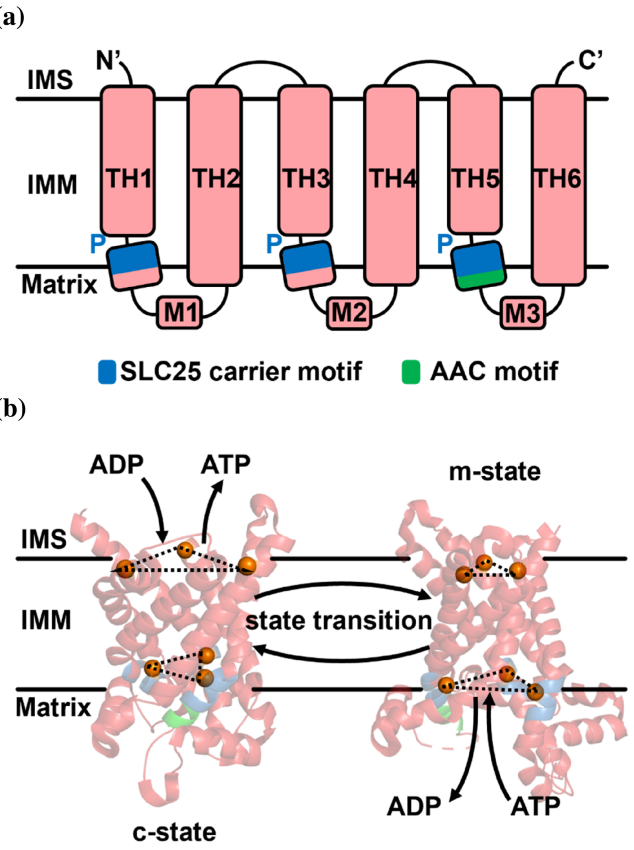

FIGURE 3 (a) The topology of AAC1 shows six transmembrane helices TH1-TH6 and three matrix helices M1-3, illustrating the proposed secondary structure shared by all SLC25 members. The SLC25 carrier motif Px[D/E]xx[K/R] (in blue) is present in TH1, 3 and 5, with proline (P) as the helix-breaking residue. The AAC motif, RRRMMM (in green), is located in TH5. (b) The structures of AAC1 highlight the structure–function relationship underlying the alternating access mechanism of AAC1-mediated ADP/ATP exchange. The PDB accession number for bovine AAC1 in the c-state is 1OKC and for *Thermothelomyces thermophilus* AAC1 in the m-state is 6GCI. Orange spheres represent the opening and closing of the cytosolic and matrix salt bridge network during the state transition.

attributed to the post differentiated tissues such as the heart and skeletal muscle (Levy et al. 2000). AAC2 is expressed in proliferating tissues (Chevrollier et al. 2011; Stepien et al. 1992) while AAC3 is reported to be ubiquitously expressed and comprises up to 10% of the total mitochondrial protein in wild-type flies (Brand et al. 2005). In contrast, ANT4 is almost exclusively found in testis (Brower et al. 2007). The reliability of the ADP/ATP carrier expression pattern in tissues at the protein level is under question, because of the very high homology of the proteins and low specificity of the commercial antibodies.

3.2 | The structure of ADP/ATP carrier

AAC1 (ANT1) was the first mitochondrial carrier to be identified and is the best studied member of the SLC25 family to date. AAC1-mediated ADP/ATP exchange is sensitive to two highly specific inhibitors, carboxyatractyloside (CATR) and bongkrekic acid (BKA), which were important tools to extensively study the structure and function of the protein. CATR is a heteroglucoside produced by the thistle *Atractylis gummifera*. It is not permeable and binds only from the cytosolic side to AAC1. BKA is a polyunsaturated tricarboxylic acid and is secreted by the bacterium *Pseudomonas cocovenenans*. Upon protonation, BKA permeates through the membrane and binds to AAC1 from the matrix side. K_d values for both, CATR and BKA, are in the nanomolar range (for review, see Clemencon et al. 2013).

The AAC1 monomer has pseudo-threefold symmetry, forming six transmembrane helices (Figure 3a). Between each odd-numbered helix (H1, H3, H5) and even-numbered helix (H2, H4, H6) are additional helices (M1, M2, M3) on the matrix side of the protein. The mitochondrial carrier motif "Px[D/E]xx[K/R]" is found in the odd-numbered transmembrane helices and contains the helix breaking proline residues. The consensus sequence "RRRMMM" of AACs is located in H5.

Bovine AAC1 in complex with CATR was the first mitochondrial carrier whose structure was resolved by crystallography with a resolution of 2.2 Å (Figure 3b, left) (Pebay-Peyroula et al. 2003). Residues 2 to 293 of the protein were resolved in combination with 82 molecules of water, one CATR and four lipids. The six transmembrane helices are tilted relative to the orthogonal direction of the membrane and to each other and form a cavity, open toward the intermembrane space, with a maximal diameter of 20 Å and a depth of 30 Å. CATR is bound in the cavity by strong electrostatic interactions with arginine (R) 79, lysine (K) 91, R187 and R234, which lock the protein in this orientation. The proline residues induce a kink that facilitates the closing of the cavity toward the matrix side by salt-bridge networks.

Recently, the structure of AAC1 in complex with BKA was solved (Ruprecht et al. 2019) and resembles the long-awaited structure of AAC1 in the m-state (Figure 3b, right). The matrix salt-bridge network is disrupted, while the cytosolic network is closed, and helices H1-2, H3-4, and H5-6 move apart to open the central binding site to the matrix side. Electrostatic interactions of the three carboxyl residues of BKA with K22, R79, and R187 (K30, R88, and R197 in the resolved structure of the fungi homolog of AAC1) tightly bind BKA in the cavity of AAC1 and lock the protein in the m-state (Ruprecht et al. 2019).

Molecular dynamics (MD) simulations were performed in the presence and absence of cardiolipin (CDL) for both, c- and m-states of the AAC1 protein. It was found that the presence of cardiolipin does not significantly affect the c-state conformational flexibility in

contrast to the m-state, which is significantly stabilized by CDL (Škulj et al. 2020). The principal component analysis was performed for the two states of AAC1 protein, together with MD simulation snapshots of the c-state and m-state with and without CDL molecule, respectively.

3.3 | The mechanism of nucleotide transport by AAC1

Two kinetic models have been proposed to explain the AAC1-mediated ADP/ATP exchange. In the sequential model, originally proposed for the aspartate/glutamate carrier, AAC1 forms homodimers that allow the simultaneous ADP and ATP exchange through coordinated conformational changes of the AAC1 dimer (Dierks et al. 1988; Palmieri et al. 1993). In contrast, the alternating access mechanism, also known as the ping-pong model, proposes that the substrate binds from one side, opening AAC1 to the opposite side where the counter-substrate can then bind (Klingenberg 1989). Detailed studies by the Kunji's group, together with the structure of AAC1 in the c-state and m-state, have verified and elaborated the ping-pong model for the AAC1-mediated ADP/ATP exchange (Figure 3b) (for review, see Ruprecht and Kunji 2019; Ruprecht and Kunji 2021). The water-filled cavity of AAC1 is open to the c-side (c-state), allowing the binding of ADP to the monomeric AAC1. The ADP binding induces slight rotations of the transmembrane helices, which close the cavity to the c-side by a salt-bridge network (occluded state). Further rotations eventually break the matrix salt-bridge network to open the cavity to the matrix side (m-state) and the substrate dissociates away from AAC1. Transport of ATP to the c-side is established by the process—binding of ATP induces the rotation of the transmembrane helices to close the matrix salt-bridge network (occluded state), disrupt the c-side salt bridge and open the cavity to the c-side. In the absence of ADP or ATP, AAC1 cannot undergo the conformational changes. While the resolved structures of AAC1 in complex with CATR and BKA clearly support the ping-pong model, the snapshot does not allow an unambiguous reconstruction of the transport cycle. Structures of apo AAC1 as well as with bound ADP or ATP in the occluded state and in the c- or m-state are missing. Thus, the mechanism of AAC1-mediated ADP/ATP exchange is not fully understood yet.

3.4 | Electrogenic properties of ADP/ATP exchange

Although the transport of ATP and ADP is thought to be coupled to the exchange of a proton or other ion to maintain electrical balance across the membrane, direct measurement of the transmembrane currents associated with the ADP/ATP exchange is challenging. Experiments in isolated mitochondria have shown that a small fraction of the ATP transport is coupled to H^+ or K^+ co-transport (Wulf et al. 1978). The use of caged nucleotides, which bind to the ADP/ATP transporter without being transported, allowed the detection of transient currents associated with ADP/ATP exchange upon photolysis (Brustovetsky et al. 1996; Gropp et al. 1999). In our experiments we observed a small increase in total membrane conductance of ANT1-containing planar bilayer membranes when ADP and ATP were present (Kreiter et al. 2021). In a more recent study, H^+ transport coupled to the ATP/ADP exchange by AAC1 was entirely excluded (Mavridou et al. 2024). Capacitive currents measured using solid-supported electrophysiology revealed a net charge transfer of $+0.3$ for ADP transport and -0.7 for ATP transport. However, the data were normalized so that the overall charge transport of the ADP/ATP exchange was -1. This normalization disregards any potential H^+ co-transport, which could reduce the overall charge transport.

4 | PROTONOPHORIC FUNCTION OF ADP/ATP CARRIER

4.1 | History and hypotheses

The protonophoric function of the ADP/ATP carrier in the presence of palmitic acid was first observed in the late 1980s in experiments with isolated mitochondria, well before the discovery of the so-called "novel" uncoupling proteins (UCP2-UCP5) (Andreyev et al. 1988; Andreyev et al. 1989; Brustovetsky and Klingenberg 1994). In the experiments with mitochondria, uncoupling was inhibited by the addition of CATR, a specific inhibitor of the ADP/ATP carrier. Subsequent experiments with liposomes reconstituted with ADP/ATP carrier confirmed this finding. In proteoliposomes reconstituted with purified AAC1, the addition of oleic acid decreased $\Delta\Phi$ (increasing uncoupling), an effect that was reversed by CATR and BKA (Brustovetsky and Klingenberg 1994). Additionally, in BAT mitochondria from UCP1 knockout mice, FA-induced uncoupling was also inhibited by CATR (Shabalina et al. 2006). In muscle mitochondria from AAC1 knockout mice, the H^+ conductance was half that of wild-type controls (Brand et al. 2005).

Interest in the ADP/ATP carrier-mediated protonophoric function has recently been revived as a result of intensive studies in two experimental systems: (i) patched mitoplasts (Bertholet et al. 2019) and (ii) planar bilayer membranes reconstituted with purified recombinant protein (Kreiter et al. 2021). Both approaches led to consistent conclusions on the

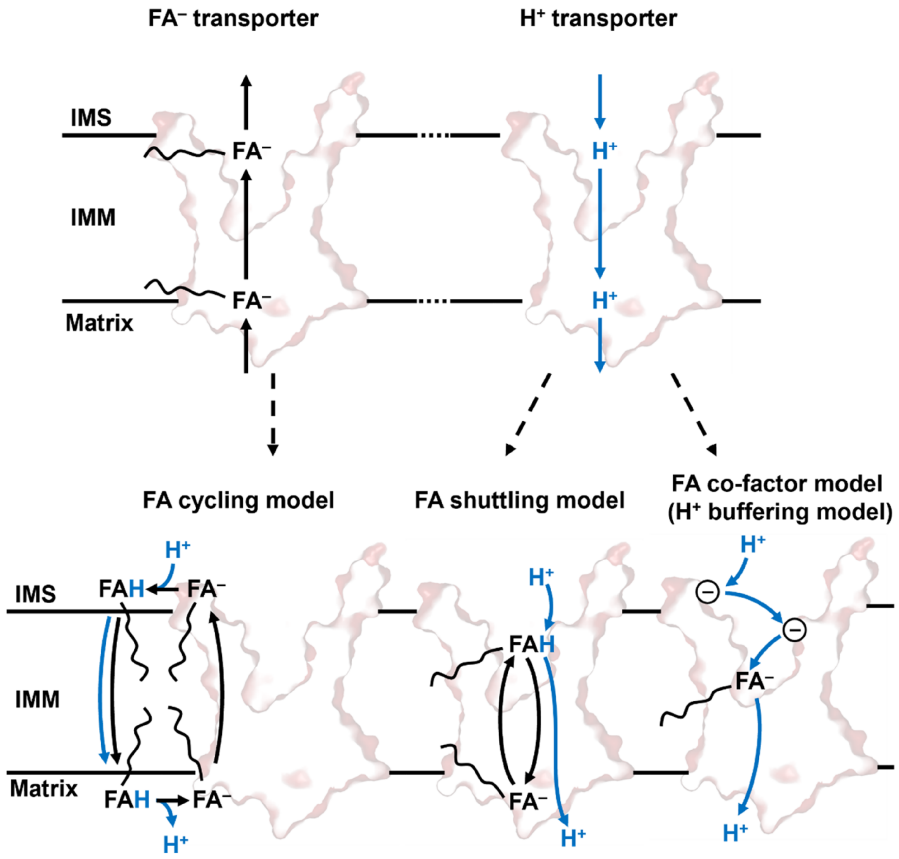

FIGURE 4 Models describing FA-activated H^+ transport mediated by AAC1/UCPs distinguish between carriers that transport FA^- (top left) and those that transport H^+ (top right). Based on these distinctions, three models have been proposed. The FA-cycling model (bottom left) suggests that H^+ is transported by the protonation/deprotonation of FA. The protonated form, FAH, can diffuse across the membrane (flip), while the protein facilitates the back transport of FA^- (flop) to complete the cycle. The FA shuttling model (bottom center) proposes that the FA^- is bound inside the protein, where it binds a H^+ and shuttles it through the center of the protein. The FA^- then returns to its original position to restart the shuttle. The FA co-factor model (bottom right), also known as the H^+ buffering model, proposes that FA^- is tightly bound within the protein structure, providing an additional negative charge to facilitate H^+ jumping through the protein via the Grotthus mechanism.

regulation of AAC-mediated uncoupling but to different suggestions for the mechanism. The discrepancies concern the critical question of whether the ADP/ATP carrier directly transports FA anion or H^+ (Figure 4).

Bertholet et al. (2019) suggested that FA^- likely binds within the AAC translocation pathway as a co-factor rather than as a transport substrate, which was proposed by the Klingenberg group for UCP1 as a buffering model (Figure 4, bottom right). Thus, FA^- facilitates H^+ transport by adding an additional negative charge to allow H^+ jumping through AAC1, similar to the Grotthus mechanism. Notably, this mechanism differs from the mechanism of H^+ transport through UCP1 previously proposed by the same group (Fedorenko et al. 2012). This hypothesis basically supports the fatty acid shuttling mechanism (Figure 4, bottom middle) (Klingenberg 2010). Here, the FA^- is located in the center of UCP1 and when it binds an H^+ from the IMS, the FAH complex moves through the center of UCP1 to the opposite side. After deprotonation, FA^- then slides back to the IMS side to bind another H^+. Unfortunately, no conclusive evidence has been presented to support both mechanisms in AAC1 and UCP1 (Bertholet et al. 2019).

The other model, the "fatty acid circuit hypothesis," was proposed by Skulachev's group (Skulachev 1991) and supported by Garlid et al. (2001) and our group (Beck et al. 2007). The idea is that H^+ transport is mediated by long-chain FA, which in their protonated form are able to transport protons along their H^+ gradient (flip) (Kamp and Hamilton 1992; Pohl et al. 2000). However, FA anion transport (flop) is the rate-limiting step in the FA cycle, so proteins such as AAC1 and UCP1 are required to mediate the return of FA^- to the cytosolic side of the membrane, resulting in net proton transport (Figure 4, bottom left).

4.2 | A critical view on the results obtained from patch-clamp experiments on mitoplasts

While patch-clamp experiments on mitoplasts may seem elegant, there are many pitfalls that must be addressed very carefully. For example, harsh treatment with the French press to rupture the outer mitochondrial membrane induces high shear stress and decompression that terminally deforms the inner mitochondrial membrane. Since mitochondrial function is highly dependent on intact cristae, mitoplasts may no longer be a "living system" to study mitochondrial carriers. There is no proper control of whether a correct protein is being patched. One must rely on appropriate controls, which are difficult to design (see below).

FAs, which are key to the activation of J_H, can only be added through the buffer solution. Therefore, the

amount of FA added is not representative of the actual amount in the membrane. Bertholet et al. reported that the addition of 4 μM AA from the matrix side did not induce J_H through the IMM via AAC1, whereas the addition of 2 μM AA from the cytosolic side did (Bertholet et al. 2019). However, the number of AA molecules added from the cytosolic side is several orders of magnitude higher, since the bath volume is much larger than the volume within the pipette ($n_{AA} = c_{AA} \cdot V_{bulk/pipette}$). Therefore, the claim that AA only binds from the cytosolic side needs to be carefully reconsidered.

Using the non-protonatable AA analog AA-sulfonate, it was proposed that AA binds to the central binding site of AAC1 to promote H^+ transport through the anionic pathway of AAC1 according to the FA cofactor model (Bertholet et al. 2019). However, the H^+ has to cross the salt-bridge network either on the c- or m-side, which requires extensive rearrangement of the ANT1 structure, breaking the three salt bridges at the bottom of the ANT1 cavity (Škulj et al. 2020), which costs a lot of chemical energy and is only induced by the energy released from the binding of ADP and ATP to the AAC1 cavity (Pietropaolo et al. 2016). In addition, the experimental design already rules out the FA-cycling hypothesis, in which the protonation/deprotonation of AA is a crucial step. The addition of the non-protonatable AA-sulfonate inhibited AA-activated J_H, which could be interpreted as suggesting that protonation of AA is crucial for AAC1-mediated J_H. Thus, the FA cofactor model cannot be convincingly concluded from the data.

In another study, the group investigated H^+ transport across the IMM activated by, among others, the synthetic protonophore dinitrophenol (DNP) (Bertholet et al. 2022). MD simulations and docking experiments showed that DNP binds within the central binding site and uses similar amino acids that bind ATP, ADP, and CATR. These findings are supported by the competition between DNP and ATP, in which increasing the DNP concentration results in a higher IC50 value for the inhibition of DNP-activated H^+ transport by ATP, mediated by UCP1. Similar to FA-activated H^+ transport, the authors propose H^+ translocation through the anionic pathway of AAC/UCP1. However, the isolated H^+ is not present in bulk water in this form. It exists as a solvated Eigen (H_3O^+) or Zundel cation ($H_5O_2^+$), and therefore requires the binding/unbinding to negatively charged anchors to cross the salt bridge network either on the c- or m-side. Since breaking the salt bridges costs a lot of chemical energy, extensive rearrangement of the AAC1 structure is mandatory and can only be achieved by binding of ADP/ATP. Thus, the crucial question of how exactly the solvated H^+ passes through the salt bridge network remains open. Furthermore, no structure–function analysis is performed to verify the binding sites the authors proposed for FA and DNP based on docking and MD simulations. Finally, patch-clamp recordings of mitoplasts from the heart or skeletal muscle did not show J_H when AAC1 was blocked by CATR. Since UCP3 is present in these tissues, the question arises whether the method is sensitive enough to infer the presence or absence of J_H in different tissues and its mediation by other mitochondrial carriers with lower expression than UCP1 in BAT and AAC1 in heart and skeletal muscle.

4.3 | Lessons from bilayer experiments with reconstituted ANT1

Our experimental results with ANT1 can be well described based on the FA-cycling model and are consistent with the translocation of FA^- at the protein/lipid interface (Kreiter et al. 2021).

First, we supported the observation of other groups (Andreyev et al. 1989; Bertholet et al. 2019) that AAC1 mediates proton transport only in the presence of FA (Kreiter et al. 2021; Kreiter et al. 2023b), known for UCP1-UCP3 (Garlid et al. 1996). The H^+ turnover number ($k = 14.6 \pm 2.5\ s^{-1}$) was calculated based on ANT1 concentration determined by fluorescence correlation spectroscopy (Kreiter et al. 2021). The k value depended on FA structure (acyl chain saturation and length), implying that FA transport is confined to the lipid-protein interface. Purine nucleotides (PN) and the specific inhibitors of ADP/ATP transport CATR and BKA inhibited H^+ transport, suggesting shared binding sites.

Using the advantages of the model system, such as defined environment and the presence of only one protein under study, we investigated the influence of membrane lipid composition on the protonophoric activity of ANT1. ANT1 activity was increased in the presence of ROS-derived products such as lysophosphatidylcholines (OPC and MPC) and PE adducts (Jovanovic et al. 2022). These modified lipids decrease the bending modulus in lipid bilayers, which was demonstrated using Müller membranes without protein (Jovanovic et al. 2022). MD simulations revealed that both modified PEs and lysolipids changed the lateral pressure profile of the membrane in the same direction and by the similar amplitude. That indicated that modified PEs acted as lipid with positive intrinsic curvature. We concluded that oxidative stress decreased stored curvature elastic stress (SCES) in the lipid bilayer membrane and ANT1 sensed SCES. This is a novel regulatory mechanism for the function of mitochondrial proteins including ANT1 under oxidative stress (Jovanovic et al. 2022).

4.4 | Lessons from MD simulations (in silico approaches)

Molecular dynamics simulations revealed a large positively charged area at the protein/lipid interface that could facilitate FA anion transport across the

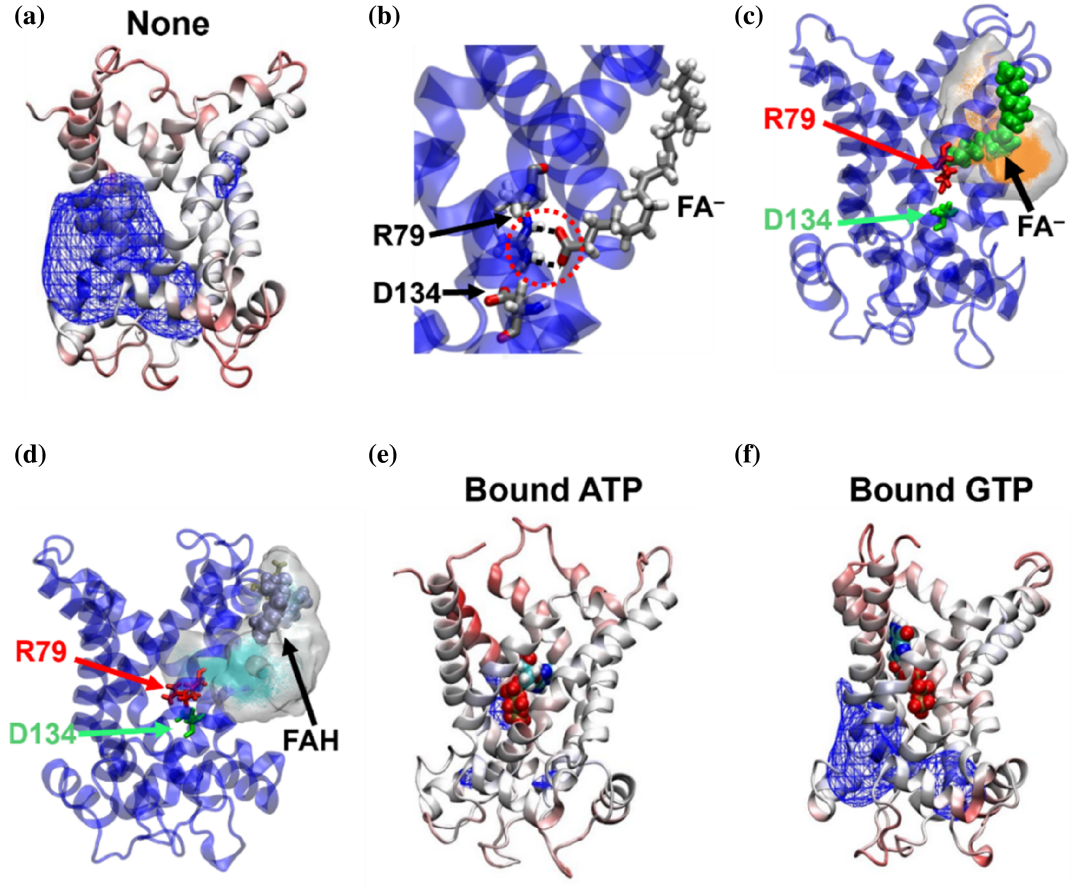

FIGURE 5 (a) Electrostatic potential of apo AAC1 calculated from MD simulations. The isosurface representing a potential of 0.9 V is shown with the wireframe. (b) Salt bridge network involving the FA anion and nearby residues R79 and D134. Probability density maps of the deprotonated (c) and protonated (d) FA around R79 of ANT1 derived from MD simulations. Electrostatic potential of AAC1 in the presence of bound ATP (e) or GTP (f). The electrostatic potentials were calculated from MD simulations. The isosurface of the 0.9 V potential is shown with the wireframe.

membrane (Figure 5a). We have shown that the head of the FA^- first binds to R59 on the matrix side of ANT1, from where it slides along the outer positively charged electrostatic potential while the FA acyl chain remains in the hydrophobic core of the bilayer. The FA^- then binds to the central cavity of ANT1, where it forms a tight salt bridge network with R79 and D134 (Figure 5b). The anionic FA remains bound to ANT1 (Figure 5c), while the protonated neutral FA is immediately released from ANT1 and dissociates into the lipid bilayer (Figure 5d). Binding of ATP (Figure 5e) or GTP (Figure 5f) lowers the electrostatic surface potential, thus hindering the sliding of FA^- from the matrix side to the central binding site of ANT1 (Kreiter et al. 2021).

4.5 | ADP/ATP carrier intensifies the protonophoric function of other non-proteinaceous uncouplers

Recently, we have shown that dinitrophenol (DNP), a well-known protonophoric substance with an unclear mechanism of action, interacts with ANT1 and UCP1-UCP3 that enhances the protonophoric function of DNP (Zuna et al. 2021). To date, protein-mediated uncoupling is known to occur only in the presence of free long-chain fatty acids. Notably, the effect of the protonophore with a structure other than FA could also be enhanced by proteins. These results are important because the protein to lipid ratio in the IMM is ~3:1, meaning that DNP effects are even more pronounced under physiological conditions. In addition, we used site-directed mutagenesis and molecular dynamics simulations to show that the same arginine (R79) as in the case of FA can be a binding site for DNP in ANT1, which brings us a step closer to understanding the molecular mechanism of DNP interaction with proteins.

5 | FA ANION SLIDING AS A NOVEL MECHANISM IN THE FA-CYCLING HYPOTHESIS

Based on the experimental findings and MD simulations, we proposed a novel mechanism by which ADP/ATP carrier facilitates the transport of the FA

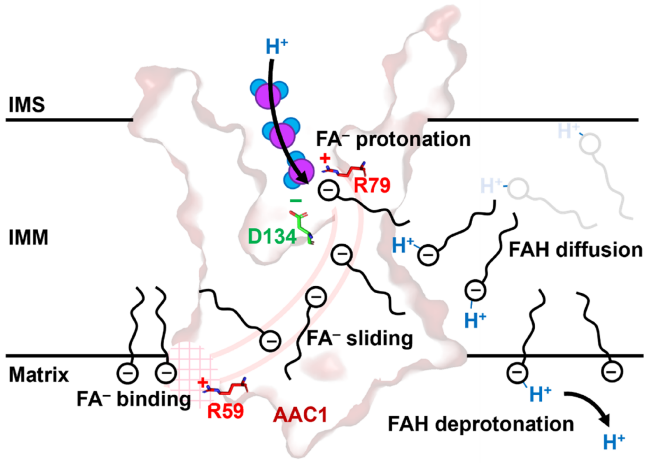

FIGURE 6 The refined mechanism of ANT1-mediated FA^- transport involves several key steps (Kreiter et al. 2023b). First, FA^- accumulates in the positively charged cloud around R59 at the ANT1-lipid interface on the matrix side. The FA^- then slides along the ANT1 surface, guided by a positively charged surface potential generated by the positively charged amino acids of ANT1, ultimately reaching R79. Upon binding to R79, FA^- can receive a H^+ from the intermembrane space (IMS) through the coordinated interaction between R79, D134 and surrounding water molecules. The protonated FAH then diffuses into the membrane moving either toward the cytosolic side, completing the classical cycling mechanism, or toward the matrix side, where it can be directly deprotonated, providing an alternative route for the H^+.

anion along the protein-lipid interface, which we entitled "FA anion sliding" (Figure 6) (Kreiter et al. 2023b). This process involves five distinct steps:

1. FA anion binding: The FA anion binds to the positively charged amino acid patch around R59, located at the protein-lipid interface on the matrix side of the membrane and including at least K48, K51, and K62.
2. FA anion sliding: The positively charged amino acids at the protein-lipid interface generate an electrostatic potential that connects R59 to R79 in the substrate binding site. This electrostatic network facilitates the movement of the FA anion along the protein-lipid interface into the central lipid bilayer.
3. FA anion protonation: The FA anion slides between helix 2 and 3 to bind to R79, which provides access to the water-filled cavity of the protein. Here, it forms an electrostatic network with R79 and D134, promoting FA protonation via surrounding water molecules. These water molecules enable the rapid H^+ transport from the intermembrane space to the FA anion without disrupting the overall charge distribution.
4. FAH diffusion: The protonated neutral FA (FAH) dissociates from R79, detaches from the protein, and diffuses rapidly and independently across the lipid bilayer (Skulj and Vazdar 2019). Notably, FAH can move not only toward the intermembrane space as proposed in the FA-cycling hypothesis, but also toward the matrix. This additional possibility for the FA choice may shorten the FA-cycling pathway, as the FA anion only needs to cross half the lipid bilayer to bind to R79, while the protonated FAH diffuses only half the distance to reach the matrix interface where it releases the proton.
5. FAH deprotonation: Once the FAH reaches the lipid-matrix interface (by flip-flop from the IMS-lipid interface or direct movement from R79 to the matrix side), it releases a proton (H^+) into the mitochondrial matrix and the FA anion can rebind to the protein, which starts the cycle again.

6 | GENERALIZATION OF THE ADP/ATP CARRIER MECHANISM FOR OTHER SLC25 MEMBERS

The amino acids that we have identified to be involved in the mechanism of FA^- transport mediated by ADP/ATP carrier are very well conserved among the members of the SLC25 superfamily, suggesting a general mechanism for transporting FA^- across the inner mitochondrial membrane (Zuna et al. 2024). For UCP2, we have demonstrated that the mutation of R60 reduces UCP2-mediated H^+ transport, similar to R59 in ANT1 (Skulj et al. 2021). Additionally, the mutation of R90 of OGC, which is the homologous amino acid of R79 in ANT1, led to the reduction of H^+ transport in the presence of FA and loosing inhibition of H^+ transport by ATP.

An important parameter to consider is the H^+ turnover number of the different mitochondrial carriers. For UCP1 and the ADP/ATP carrier, this number is 3–5 times higher than for UCP2 and UCP3. Several factors, as outlined in the FA-sliding model, may explain this difference, although further research is required. First, the pseudo threefold symmetry would, in principle, allow three pathways for the FA sliding. UCP1 and the ADP/ATP carrier may utilize two or three pathways, while other carriers may engage fewer. Second, the number of positively charged residues on the matrix side may influence the attraction and binding of FA^-, potentially being further modulated by the difference between lysine and arginine residues. Third, the exact orientation of FA^-, arginine and aspartate in the substrate binding site could influence the H^+ transfer and FA protonation, which in turn would alter the binding time of the FA in the central cavity of the carrier.

The hypothesis requires further investigation and is currently being tested for various mitochondrial carriers, including UCP3, DIC, and AGCs.

7 | CONCLUSION

Uncoupling represents a promising therapeutic target for modulating excess energy, with potential applications in treating obesity, regulating ROS production, and inducing mitochondrial dysfunction that can lead to cell death. While the well-known UCP1 is a major player in proton transport, other members of the SLC25 family also contribute to this process, in addition to their roles in substrate transport. The ADP/ATP transporter appears to be another protein with a dual function—both substrate and proton transport. Contrary to previous beliefs that fatty acids solely activate this process, recent findings suggest that the ADP/ATP transporter actually enhances the uncoupling effect of fatty acids, similar to other non-protein proton transporters such as DNP, CCCP, and FCCP. The significance of this process remains unclear, and further experimental and computational studies are needed to elucidate its role.

AUTHOR CONTRIBUTIONS

Elena E. Pohl: Writing – original draft; writing – review and editing; conceptualization; funding acquisition. **Mario Vazdar:** Writing – original draft; writing – review and editing; funding acquisition. **Jürgen Kreiter:** Writing – original draft; writing – review and editing; conceptualization; visualization.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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