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Review

The ADP and ATP transport in mitochondria and its carrier

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ABSTRACT

Different from some more specialised short reviews, here a general although not encyclopaedic survey of the function, metabolic role, structure and mechanism of the ADP/ATP transport in mitochondria is presented. The obvious need for an "old fashioned" review comes from the gateway role in metabolism of the ATP transfer to the cytosol from mitochondria. Amidst the labours, 40 or more years ago, of unravelling the role of mitochondrial compartments and of the two membranes, the sequence of steps of how ATP arrives in the cytosol became a major issue. When the dust settled, a picture emerged where ATP is exported across the inner membrane in a 1:1 exchange against ADP and where the selection of ATP versus ADP is controlled by the high membrane potential at the inner membrane, thus uplifting the free energy of ATP in the cytosol over the mitochondrial matrix. Thus the disparate energy and redox states of the two major compartments are bridged by two membrane potential responsive carriers to enable their symbiosis in the eukaryotic cell. The advance to the molecular level by studying the binding of nucleotides and inhibitors was facilitated by the high level of carrier (AAC) binding sites in the mitochondrial membrane. A striking flexibility of nucleotide binding uncovered the reorientation of carrier sites between outer and inner face, assisted by the side specific high affinity inhibitors. The evidence of a single carrier site versus separate sites for substrate and inhibitors was expounded. In an ideal setting principles of transport catalysis were elucidated. The isolation of intact AAC as a first for any transporter enabled the reconstitution of transport for unravelling, independently of mitochondrial complications, the factors controlling the ADP/ATP exchange. Electrical currents measured with the reconstituted AAC demonstrated electrogenic translocation and charge shift of reorienting carrier sites. Aberrant or vital para-functions of AAC in basal uncoupling and in the mitochondrial pore transition were demonstrated in mitochondria and by patch clamp with reconstituted AAC. The first amino acid sequence of AAC and of any eukaryotic carrier furnished a 6-transmembrane helix folding model, and was the basis for mapping the structure by access studies with various probes, and for demonstrating the strong conformation changes demanded by the reorientation mechanism. Mutations served to elucidate the function of residues, including the particular sensitivity of ATP versus ADP transport to deletion of critical positive charge in AAC. After resisting for decades, at last the atomic crystal structure of the stabilised CAT–AAC complex emerged supporting the predicted principle fold of the AAC but showing unexpected features relevant to mechanism. Being a snapshot of an extreme abortive "c-state" the actual mechanism still remains a conjecture.

Contents

Abbreviations: AAC, ADP/ATP carrier; BHM, bovine heart mitochondria; SMP, submitochondrial particles; ATR, atractyloside; CAT, carboxyatractyloside; BKA, bongkrekic acid; AdN, adenine nucleotides; T, ATP; D, ADP; PP_i, pyrophosphate; MCF, mitochondrial carrier family; TMH, transmembrane helix; C-loop, cytosol loop; M-loop, matrix loop; ITF, induced transition fit; SBCGP, single binding center gated pore; EAM, eosine maleimide; HTS, hydroxylapatite; PL, phospholipid; CL, cardiolipin; FA, fatty acid; LAPAO, lauroyl propyl aminoxide; LDAO, lauroyl diamine oxide; PEG, polyethylene glycol; PheG, phenylglyoxal; Fo, formycin; CrK, creatine kinase; CrP, creatine phosphate; CyP, cyclophilin; CsA, cyclosporin; HFS, hyperfine splitting; VDAC, voltage dependent anion channel; NEM, *N*-ethyl maleimide; DTNB, 2,2′ dinitro dithiodibenzoate; DAN, 1,5 dimethylamino-naphtoyl; N, naphtoyl

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1. Introduction

In eukaryotic cells the ADP/ATP transport through the inner mitochondrial membrane occupies a central metabolic role as a critical step in the supply of ATP from mitochondria to the cytosol. The transport works as an ADP versus ATP exchange across the inner mitochondrial membrane and is catalysed by the ADP/ATP carrier (AAC). To cope with this role the AAC is equipped with several outstanding features to be elucidated in this review. For this reason the ADP/ATP transport and the underlying carrier have been a

paradigm not only for the mitochondrial carrier family but also for understanding the mechanism of transport through biomembranes in general. Among several reasons, there are at least two arguments to support this stand of the AAC. First, the central role of the AAC in oxidative metabolism requires high expression levels in most eucaryotic cells, exceeding other transporters; second, the nature of the transported substrates, being adenine nucleotides is outstanding in their central and multiple roles in metabolism and regulation. In all cells adenine nucleotide occurs at high concentrations for coping with their universe of functions. Being large and highly charged substrates their transport through biomembranes requires a carrier with highly efficient catalytic qualities.

In this review the salient developments of the ADP/ATP transport and its carrier will be elaborated. Among the topics to be covered are transport, regulation, carrier mechanism, structure/function relationship, and the physiological implications. The important area of AAC import into mitochondria, the regulation of expression, and the rare clinical aberrations of AAC are not reviewed to keep the size of the review manageable. Attention should be drawn to previous reviews on the ADP/ATP transport system [1–9]. In addition, a number of reviews on the mitochondrial carrier family include ADP/ATP transport [1,10–15].

2. The early history

As the power stations of eukaryotic cells, mitochondria deliver energy in the form of ATP to the cytosol. Although much effort was devoted to elucidate the system of oxidative phoshorylation, it remained unclear up to the early 1960ies of how mitochondria receive substrates for oxidation and how they ship ATP to the cytosol. For many, the latter question seemed to be non-existent, holding that ADP and P_i are directly converted to cytosolic ATP somewhere at the inner mitochondrial membrane. The seemingly inconspicuous problem gained first attention when isolated mitochondria were found to contain high amounts of adenine nucleotides [16–18] with an unclear role in oxidative phosphorylation. Only after it was accepted that the inner membrane presents an osmotic barrier, were the "bound" nucleotides form a "soluble" pool within the matrix space, interacting with the ATP synthase. In this "endogenous" pool of AMP, ADP and ATP, the degree of phosphorylation could be varied by uncouplers, substrates and oligomycin, indicating their participation in oxidative phosphorylation [19–23]. Some of these nucleotides were thought to be tightly bound to the ATP synthase and involved as intermediate receptors and donors in the phosphate transfer to external ATP. More specifically it was asked, whether these "bound" nucleotides were on the main pathway involved in the synthesis of external ATP or bypassed (see Chapter 5 "Micro-compartmentation"). If yes, different views were expressed on how the internally generated ATP was linked to the external ATP [22,24]. For example a *trans*-phosphorylation ("mesomerase") was proposed to transfer γ-phosphate across the membrane from internal ATP to external ADP [24]. Only by introducing rapid separation and quenching techniques and by using ^{14}C instead of ^{32}P labelling, was the physical transport of external ^{14}C-ADP, etc. into mitochondria unequivocally shown and the exchange against the endogenous counterparts established [20,25,26]. Thus the recognition of an active endogenous nucleotide pool and of a nucleotide transport was inter-dependent.

In this context the historical role of atractyloside (ATR) is to be clarified. Being known as an inhibitor of oxidative phosphorylation [27,28], it was shown to prevent "binding" of ATP to mitochondria [22,23,29–31]. ATR was concluded to bind and thus to block the ATP synthesis site [30,32]. Only after the ADP/ATP transport was established [20,26], was ATR identified as an inhibitor of the transport, rather than of ATP synthesis [26,33]. The first "Bari-Meeting" in 1965 with its fruitful discussions contributed to elucidate these problems and to resolve the divergent views in the following years [34]. It is important to stress this point since until today the central position of the mitochondrial pool in ATP synthesis is occasionally not appreciated by assuming a "micro-compartmentation" of ATP leading to unsubstantiated conclusions about the metabolic organisation of the ATP export (see Chapter 5).

This early research was intertwined with the question of the general compartmentation in mitochondria, the role of inner and outer membranes. Using silicon layer filtration, the differentiation of total volume of filtrated mitochondria or mitochondrial pellet represented by the ^3H_2O space and the ^{14}C-sucrose impermeable matrix space [25,26,34] allowed to define the inner membrane as the osmotic barrier, not freely permeable to solutes. The volume changes of the matrix space visualised by electron microscopy in response to variation of the external sucrose or KCl concentrations were in agreement with the volumetric measurements [35]. The outer osmotically inactive membrane in isolated mitochondria was shown to be freely permeable to molecules with MW <4 kDa [35]. On this background the gateway role of the AAC in phosphate transfer not only from ATP synthase but also between the different phosphate transferases known to exist in the matrix and intramembrane space was illustrated in Fig. 1.

In the early 60ies the paradigm example for solute transport through membranes was the glucose transport in erythrocytes [36,37]. The large internal space of erythrocytes permitted easy measurements of kinetics whereas transport measurements in mitochondria were a challenge due to the much smaller inner volume surrounded by a large convoluted inner membrane surface, requiring higher time resolution and micro scale analytical techniques. A further complication was the rapid metabolic conversion of the transported substrates in mitochondria. New micro-analytic anion exchange chromatography methods permitted to define the endogenous nucleotide metabolism [21]. The refinement of the original centrifugal silicon layer filtration technique [38] enabled brief exposure to the labelled solutes, rapid separation from the medium, extraction and fixation of the phosphorylation status by the bottom $HClO_4$ layer [39]. Introduction of the inhibitor stop method and the continuous development of semi and fully automated rapid mixing, rapid sampling and stopping machines permitted resolution of transport binding down to the 100 ms range [40,41].

3. The ADP/ATP transport

The terms ADP/ATP transport or ADP/ATP exchange will be generally used, when the specifications, in what combination ATP

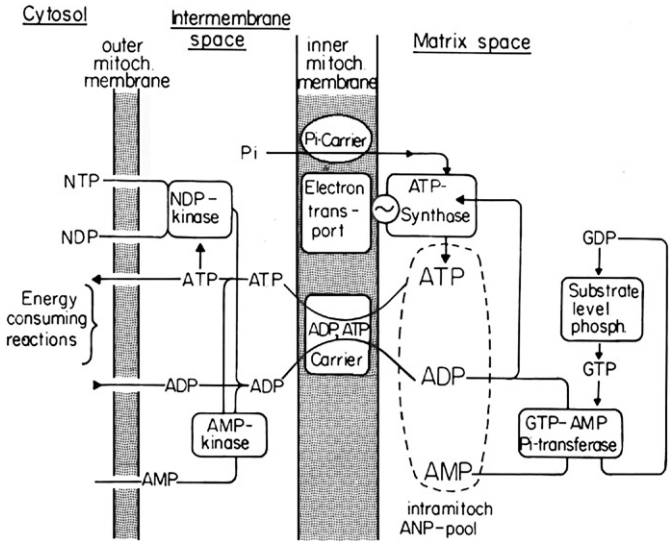

Fig. 1. The ADP/ATP carrier as a gateway, inter-relating the phosphate transfer networks in the two mitochondrial and the cytosolic compartments. The components in the phosphate transfer network correspond to liver mitochondria. From [2].

and ADP are exchanged, are not relevant. Otherwise, the four ADP_e–ATP_i etc exchange combinations are specified (see Chapter 5). The underlying transporter is termed the ADP/ATP carrier (AAC). Other terms used are "translocase", which reflects the pioneering role of the ADP/ATP transport in understanding carrier linked transport. Similarly, the *Escherichia coli* lactose transporter was early called "permease". The popular term adenine nucleotide transporter (ANT) is misleading since this term also implies transport of AMP which is explicitly excluded by the AAC.

After the first communications [20,25,26] on the discovery of the ADP/ATP exchange and early confirmatory work [33], a full characterisation of the ADP/ATP transport in mitochondria was given in two detailed key papers [42,43]. All these studies were performed on rat liver mitochondria which were the classical mitochondria for studying oxidative phosphorylation. First using the centrifugal silicon layer filtration technique [25,26,39], the ADP/ATP transport across the inner mitochondrial membrane was scrutinised by analysing the changes of endogenous pool composition on addition of ^{14}C-ADP and ^{14}C-ATP. The uptake and the release of ATP, ADP, and AMP were measured for monitoring the specificity of the influx and of the efflux.

Basically the transport was established as a 1:1 exchange of added ADP or ATP [26,42] with endogenous ADP and ATP in the mitochondrial matrix. AMP remained excluded from both uptake and efflux. With further techniques the time course of the 1:1 exchange was directly demonstrated by labelling the endogenous nucleotides with ^{3}H and the exogenous with ^{14}C [2]. The example demonstrates a simultaneous, approximately equal release and uptake (Fig. 2). In experiments using 8-^{14}C- and γ-^{32}P-ATP, ATP is shown [42] to be taken up as an intact molecule, thus refuting a proposed *trans*-phosphorylation of γ-P across the membrane to internal ADP [23]. The exchange does not require Mg^{++} [42].To the contrary, added Mg^{++} was found to partially inhibit exchange indicating that the free ADP and ATP are transported. The influence on the exchange of uncoupling, of manipulating the phophorylation level by arsenate, of ionophores and oligomycin, was scrutinised for segregating the effects of the nucleotide composition changes from the membrane potential, as reviewed below.

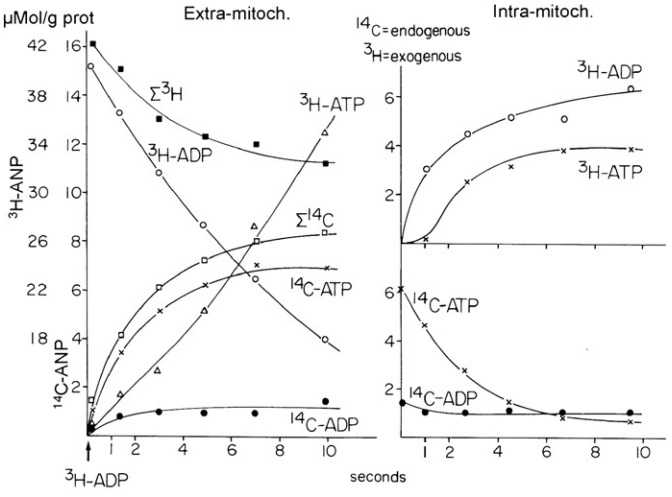

Fig. 2. The ADP/ATP exchange between external and endogenous adenine nucleotides of mitochondria. The results demonstrate (a) the 1:1 exchange, (b) the endogenous pool as an obligatory intermediate (no micro-compartmentation) and (c) the preference of ADP_{ex}–ATP_{int} exchange mode in the energised state of mitochondria. The exogenous nucleotides are labelled with ^{3}H, the endogenous nucleotides with ^{14}C. The exchange is started by addition of ^{3}H-ADP to respiring and phosphorylating liver mitochondria. The decrease of external ^{3}H-ADP is paralleled by the immediate appearance in mitochondria of ^{3}H-ADP and after a delay it is phosphorylated to ^{3}H-ATP. In exchange for ^{3}H-ADP endogenous ^{14}C-ATP is immediately released, followed by a continuous ^{3}H-ATP export. From [83].

3.1. High substrate selectivity

Dealing with ATP and ADP places the ADP/ATP transport into a special situation. The transported substrates are large, hydrophilic, highly charged molecules, averse to low dielectric membrane environment. In cells the ubiquitous ADP and ATP interact with more proteins than any other ligand. Thus it is not surprising, that in nature and in the laboratory more analogue compounds of ATP and ADP are created than for any other substrate, providing a large arsenal to explore the specificity of the transport. The ADP/ATP transport exhibits unusually high selectivity [2,42,44], as compared to most ATP utilising proteins, and also compared to other more recently identified nucleotide transporters which are also members of the mitochondrial carrier family [45–48]. Each part of nucleotide structure seems to be recognised by the AAC. The metabolically important exclusion of AMP shows that the phosphate moiety requires at least 2 phosphate groups. It may seem paradox, that while the less charged AMP is rejected; transport requires adenine nucleotides with their full charge as ATP^{4-} and ADP^{3-}.

The finding that the prevailing Mg^{++} nucleotide complexes are not transported [34,43] has important consequences, e.g. the concentration limitations by the much less abundant free ATP^{4-} and ADP^{3-} and the electrophoretic exchange of ATP^{4-} against ADP^3. This contrasts to the large universe of proteins involved in ATP hydrolysis or phosphate transfer requiring Mg^{++}. The underlying ADP/ATP carrier (AAC) is distinguished by the absence [49] of the binding site motifs common in ATP utilising phosphate transferring proteins, into which first the ADP/ATP carrier was mistakenly included [50]. Also other proteins such as uncoupling proteins, in which the ATP or GTP (ADP or GDP etc.) function as regulators rather than in phosphate transfer, have no requirement for Mg^{++} or Ca^{++}[51].

It was early concluded that the high specificity of oxidative phosphorylation in mitochondria is conditioned by the transport [26] rather than by ATP synthesis. At variance, assuming that in digitonin particles the access to the ATP synthase does not require a transport, it was suggested [44] that other factors controlled the specificity. However, these results were critically reinvestigated and not confirmed [52]. Adenine nucleotide analogues with variations in the phosphate moiety such as replacing –O– in the P–O–P bridge by –NH–, CH_2– [2,42,44], or omission of –O– in phosphonate [53] are still somewhat tolerated by the exchange. The phosphate groups must be in the 5′ position [42] as judged from the inactivity of AMP-3′P and ADP-3′P. d-ADP and d-ATP are transported at 10–15% the rates of their ribose homologues. Concerning the base moiety, a stringent specificity for adenine is maintained, excluding most other bases [42]. Useful for its fluorescence are the close adenine analogues formycin -DP, -TP which have 10% transport activity [2].

3.2. Transport of small solutes by the AAC

The AAC can also transport some small anionic solutes at much lower rates, as shown with isolated mitochondria and reconstituted AAC. Phospho-enolpyruvate (PEP) is transported in mitochondria preferentially by the citrate carrier but there is also an ATR sensitive PEP transport [54] with an activity of only a few % of ADP–ATP transport. Pyrophosphate (PP_i) transport by the AAC has been noted [55] first as a PP_i driven AdN export from mitochondria which is inhibited by ATR. In Ca^{++} loaded mitochondria PP_i drives Ca^{++} out of the mitochondria, which is again sensitive to CAT [56]. The effect is much stronger in mitochondria from heart than liver. The PP_i transport was clearly characterised [57] as a 1:1 exchange of PP_i against ADP with AAC reconstituted vesicles. Depending on the in- or outside location of PP_i, the rates can reach about half of ADP/ATP exchange. However the K_M ~1 mM is about 100 times higher than ADP or ATP. PP_i is generated during fatty acid (FA) activation and should be imported in mitochondria only when ATP and ADP levels are low. It

may be noted that PEP and PP_i share 3 or 4 negative charges with ADP and ATP, which should be essential for the interaction with AAC. Creatine phosphate (CrP) was shown [58] to be taken up in heart mitochondria by a slow CAT sensitive process. With AAC proteoliposomes the CrP transport was characterised as an exchange against ADP or ATP, however, at a 500 times lower rate than the ADP–ATP exchange. The suggested close linkage of AAC to creatine kinase (see Chapter 6) may facilitate CrP uptake in vivo.

3.3. Kinetics of ADP/ATP exchange

Maximum rates of ADP/ATP transport are of interest not only for assessing the capacity of transport of the underlying carrier, but also because of the possible rate limitation for the ATP supply in the eukaryotic cells. Measurements of ADP/ATP exchange kinetics in mitochondria can be experimentally difficult since they depend on the exchange with a limited endogenous adenine nucleotide pool. Liver mitochondria are favourable because they contain less transport sites in relation to the nucleotide pool size. Even at low temperature the time interval from the addition of the labelled ADP or ATP until equilibration is only up to 50 s, requiring high time resolution techniques for measuring the initial rates. In fact, initial rates, although important, are rarely measured in mitochondria. Ideally the exchange follows a first order reaction to equilibrium between internal or external labelled nucleotides, and the absolute rate has to be calculated on the basis of the exchangeable endogenous pool size, i.e. the ADP and ATP content since AMP is not exchanged [43]. The ADP and ATP content can vary widely in different metabolic states of mitochondria. The first order rate will be composed of the different exchange rates of endogenous ADP and ATP. A major factor controlling these differences is the membrane potential. Because of these problems it is not surprising that in the literature data on the rates of the ADP/ATP transport in mitochondria varied considerably.

In the first reports time courses were poorly [26] or not resolved [23,33,59]. Some artefacts lead to the conclusion of a two stage exchange [59]. Rigorous measurements of the exchange kinetics were performed with a time resolution of 1 s, using rapid pressure filtration or the inhibitor stop method [43]. This resolution was sufficient for rat liver mitochondria to measure initial kinetics at 2° where 90% to equilibrium is reached at about 50 s. The kinetics were approximately first order related to the ADP+ATP pool. The time course was homogenous and the reported oscillations [44] of the rate during the time course were not observed. In heart mitochondria the size of the endogenous pool related to the carrier (AAC) content is about 3 times smaller, requiring higher resolution by subsequent developments of new automated rapid mixing, sampling and quenching apparatus with a time resolution down to 0.1 s [40,41], as well a rapid filtration technique [60]. This enabled measurement of kinetics of exchange at higher temperatures, up to 27° in rat liver mitochondria and up to 20° in rat heart and yeast mitochondria [40].

A detailed study of the temperature dependence of the exchange rates in liver mitochondria shows that the rate increases steeply between 0° and 14° about 30 fold from about 3 to 90 μMol/min/g protein and from 15° to 27° only 3 fold. In the Arrhenius plot a temperature break is at 14.5° where the activation energy decreases from 34 kcal to 13.8 kcal. An extrapolation of the temperature dependence allows estimation of the rates at 37°. The same temperature break was observed with heart mitochondria. Measurements down to −10° (using 10% glycol) showed no further temperature break, indicating that a phase transition of phospholipids is not involved. Therefore an intrinsic conformation change of the carrier protein may occur around 14°. The significance of this activation energy in the transport mechanism will be elucidated below (Chapter 16.1). Initial rates measured at 22° of transport into heart mitochondria [60] by a rapid filtration technique were lower than those previously reported [40] possibly caused by nucleotide leakage from broken mitochondria deposited on the filter.

The strong temperature dependence of transport below 14° was first noted [61] by comparing the phosphorylation rates of endogenous and exogenous ADP using a fast sampling technique. Below 14° endogenous ATP is formed much faster than external ATP. As a result low temperature has a similar effect as ATR on oxidative phosphorylation. Also the hydrolysis of exogenous ATP stimulated by uncoupler at low temperature was much slower than the endogenous rate. A difference in phosphorylation rates of endogenous and exogenous nucleotides at 0° was also noted in other studies [62]. These results show that at lower temperatures oxidative phosphorylation is rate limited by the transport. Similar temperature breaks reflecting ADP-ATP transport are found [61] in Arrhenius plots of the ADP stimulated respiration and in the oxidation rate of endogenous NADH+NAPDH on addition of ADP.

Transport rates at 37° are extrapolated assuming "linear" continuation to 37° in the Arrhenius plots. These "maximum" rates are obtained under conditions, where the exchangeable portion of internal AdN (low AMP content) and the membrane potential is high in order to optimally drive ADP uptake against ATP release (see below) [40,43,63]. For calculating the turnover number, the content of AAC sites in mitochondria was determined by binding of CAT (see Chapter 7.3). Comparatively low turnover values are measured characterising the AAC as a slow carrier. However in reconstituted vesicles with optimal phospholipid composition, the AAC works at a 4 fold higher turnover [64,65]. These higher turnovers have been observed not only with bovine heart AAC but also with reconstituted AAC from *Saccharomyces cerevisiae* [66].

A special effort was made to measure the initial phase of the ADP/ATP exchange in the ms range [67]. Using a quench-flow apparatus with starting and stopping mixing chambers and an intervening reaction tube, a time range between 20 and 400 ms was covered, using an inhibitor stop with ATR. A biphasic time course with an initial rapid uptake between 25 and 100 ms was observed and identified as a quench delay. Its size depended among other factors on the ADP concentration and on the time between ADP and ATR addition, indicating a delay due to the competitive removal of ADP by ATR. No time delay was observed in the de-energised state (valinomycin, FCCP), where the AAC tends to assume the c-state, showing that the reorientation from the m- to the c-state is the major rate limiting step for the ATR access and thus responsible for the quench delay. As evident from mitochondrial contraction recordings [68] and from NEM interaction [2,69,70] (see Chapter 8.6), in the energised state on addition of ADP, the AAC is converted mostly to the m-state. In accordance with this model involving the carrier reorientation steps, the quench delay was computer simulated with m- to c-state transition turnover time of 50 s^{-1}.

Another kinetic parameter burdened with variability is the K_M. Widely different values are reported, partially explained by experimental problems. For example leakage of endogenous nucleotide gives a higher true concentration than the added concentration, an error most serious at low concentrations. As a result a too low K_M is determined unless the concentration is corrected for the leakage [71,72]. The error can be minimized with the back exchange method, which allows using more dilute mitochondria producing a low leakage concentration [43]. The K_M depends on the energy state of mitochondria influencing the phosphorylation state of the endogenous adenine nucleotide (AdN) pool and the membrane potential. A biphasic relationship of V on the ADP or ATP concentration, corresponding to two K_M is obtained in V versus V/S, or $1/V$ versus $1/S$ plots [43]. In another work a non-linear relation is also observed [71], but two reports found only a linear relation [72,73]. The rate measurements were often based only on a single time point [71,73].

Evaluating rates obtained from the full time course, for ADP two K_M varying by a factor of about 10 are obtained: in the "energised" state K_M=3/27 μM, in the "de-energised" K_M=0.8/11 μM, [2]. The two

K_M are attributed to two variable portions of the participating AAC, varying with ADP or ATP and the energy states. These K_M values are at variance with those reported by Duyckaerts et al. [72] in energised/de-energised states: K_M (ADP) 1–2 μM, K_M (ATP)=140 and 1 μM, or by Barbour and Chan [71]: K_M (ADP)=2–4 and 20–30 μM, K_M (ATP)= 100–120 and 6–DEL id="del79" orig="- ">10 μM.

In reconstituted vesicles the variables affecting the kinetics and thus the K_M can be better controlled [64,65]. A wealth of information is obtained by segregating the exchange modes (ADP_e against ADP_i, ADP_e against ATP_i, etc). The concentration dependence is linear with a single K_M and the K_M values do not show the large increase on "energisation" as observed in mitochondria. With $\Delta\psi$=180 mV the K_M for ATP increases only 1.5–2 fold. Most interesting are studies of the competition between ADP and ATP, which can best be performed with reconstituted vesicles where inside and outside the concentration of ADP and ATP can be freely adjusted in a "competitive" ADP_e+ATP_e against ADP_i+ATP_i exchange mode, indicating the strong preference for ADP under the influence of $\Delta\psi$ (see below).

3.4. Kinetic models of transport mechanism

In two studies of the kinetics criteria were applied for differentiating between single sites (ping-pong) or dual sites (sequential or simultaneous) mechanisms. For this purpose, in addition to the external also the internal concentrations of ADP and ATP were changed by inhibiting oxidative phosphorylation to varying degrees [71,72]. In 1/V versus 1/S plots the lines intersected with the ordinate in accordance with a sequential mechanism. A non-linear biphasic relation was found which varied with the endogenous nucleotide composition. This was interpreted as a cooperation between two binding sites, for example the two binding sites in an AAC homodimer [71]. A ternary complex of AAC with both exchanging substrates had to be postulated in the transport cycle and both groups proposed that the release of the substrate from the AAC at the matrix side requires binding of endogenous ADP or ATP destined for export. Originally deduced from kinetic work on the aspartate–glutamate exchange in mitochondria [74], this variant of the sequential mechanism avoids the simultaneous existence of an outer and inner facing binding site, and is more compatible with other data of inhibitor binding to be elucidated below. However the evaluation of the experimental data may be flawed in several respects. Duyckaerts et al. [72] accounted only for the endogenous ADP and ignored that ATP is also released. Moreover, they assumed that the ADP–Mg complex is the endogenous exchanging substrate contrary to evidence that only the free ADP and ATP are transported. Further, the leakage of endogenous nucleotides will skew the concentration dependence so that the K_M are underestimated. Barbour and Chan [71] were aware of this error but did not correct for the leakage of ADP.

It should be mentioned, that kinetic studies in reconstituted vesicles with other mitochondrial carriers such as the glutamate–aspartate carrier also seemed to support the sequential (simultaneous) mechanism [75,76]. However, there is a suspicion that low "concentrations" in the μM range inside the vesicles and mitochondria are kinetically not meaningful since in the small matrix space only a few, down to one or less molecules are present. Notably in all cases the apparent K_M for internal substrate are measured in the mM range contrasting to the μM range of the external K_M, which at face value would indicate that the inner side of the carrier exhibits a much lower affinity than the external side. However, in vesicles part of the carrier molecules are in a reversed configuration, so that one would have to conclude that the membrane configuration rather than the different faces of the carrier determine the affinity. A more rational explanation seems to be that in the "μM" range the low number of molecules present in the vesicles does not permit to define a kinetically relevant concentration.

3.5. The ADP–ATP exchange in submitochondrial particles

To investigate transport characteristics from the m-side of the inner membrane, sonic particles from BHM (SMP) [73,77,78] were loaded with ^{3}H-ADP or ^{3}H-ATP for measuring simultaneously uptake and release [78]. The kinetics were followed by a rapid filtration or the BKA stop method. A 1 to 1 exchange between the endogenous ^{3}H and external ^{14}C was demonstrated. The rates were 10 times lower than in mitochondria [73,78], indicating that only part of the sonic particles are tight and inside-out. ATR and CAT did not or only partially inhibit the exchange unless added prior to sonication. BKA completely inhibited exchange, without showing a time delay and a pH dependence as in mitochondria. The temperature dependence measured between 0° and 35° showed a steep increase up to 25° with an activation energy of 24 kcal [78]. The rates were only weakly influenced by energisation [78], but a strong influence on the K_M of ATP uptake was reported [73]. The selectivity for substrates was still higher in SMP than in mitochondria, with exchange rates of dADP, dATP, AMPPNP, AMPPCP amounting to only 2 to 3% of mitochondria. The enhanced specificity reflects the different configuration of the binding site at the m-side in line with the single site reorienting mechanism (see below). SMP were also used to demonstrate the electrogenicity of the ATP–ADP exchange [79] as reviewed below. In an early report on ATP transport in SMP by Shertzer and Racker [80] the rates were actually 35 times lower than in mitochondria, although equal rates are claimed.

4. Energy control

4.1. Energy dependent shift of ADP versus ATP transport in mitochondria

Some aspects of the influence of the mitochondrial energy state on the ADP/ATP exchange were reviewed above, such the phosphorylation state of the endogenous pool on the K_M. Here we will concentrate on the differentiation between ADP and ATP transport, the important consequence for the control of the ADP–ATP exchange, and the force and mechanism involved. Basically the ADP/ATP exchange is not energy dependent. It proceeds with high activity when the mitochondria are completely depolarised by uncouplers [42,43] and ADP and ATP are transported in both directions at nearly equal rates. Since in this state the carrier purely acts as a facilitator, the exchange should be symmetric in both directions. In mitochondria energisation shifts ADP and ATP transport rates in opposite manner such that the ratios $V_{ADP\rightarrow}/V_{ATP\rightarrow}$ of the influx increases and $V_{ADP\leftarrow}/V_{ATP\leftarrow}$ of the efflux decreases [42,81]. This effect is most dramatic, when, similarly to in vivo conditions, ADP and ATP are offered simultaneously at varying proportions. ADP uptake is preferred to such an extent, that equal uptake rates are obtained only when the mixture is skewed to 8% ADP and 92% ATP [81,82].

In the energised state internal ATP is the preferred exchange partner as deduced [42,81] from varying the endogenous ATP content by alternating incubations with P_i and oligomycin, signalling that energy drives efflux of ATP in preference to ADP. With advanced sampling techniques [40] individual uptake and release rates were simultaneously measured, directly showing that in the energised state ATP is released preferably to ADP but in the uncoupled state ADP leaves the mitochondria more rapidly than ATP [81,83], although equal rates should be expected, just as observed for the uptake. This can be explained by considering that in the mitochondria the nucleotides are present mostly as the Mg^{++} complexes which are not transported and thus the concentration of free ATP is decreased about 10 times more than of ADP because of the stronger Mg^{++} binding constant. After correction of this effect the ratio of the efflux rates in the uncoupled state is $V_{ATP\leftarrow}/V_{ADP\leftarrow}\approx 1$ and in the energised state ≈20 [81].

By promoting uptake of ADP and release of ATP, the transport is modulated such that it would cooperate with oxidative phosphorylation.

The total AAC activity can be divided up into four exchange modes (D=ADP, T=ATP) where D_e-T_i are the productive, T_e-D_i counter-productive, and D_e-D_i, T_e-T_i neutral modes. The combinations must be regarded as statistically independent whether the AAC operates in a ping-pong or sequential mechanism. As shown in Fig. 3A in the energised state the productive mode reaches nearly 60% and surpasses the counter-productive mode 15 fold. The partitioning of the capacity among these modes represents the most important regulation of the AAC. It was earlier recognised that the principal force for this regulation is the membrane potential $\Delta\psi$. Since the exchange uses the "free" nucleotides without Mg^{++}, ATP^{4-} and ADP^{3-} appeared to be the transported species and as a result the D_e-T_i and T_i-D_e modes are electrically imbalanced. The difference of one negative charge is the anchor for $\Delta\psi$ driving the exchange D_e-T_i. The final proof of correlating $\Delta\psi$ with the electrically imbalanced transport came from the reconstituted system [64,65,84] as reviewed below. Here the single exchange modes could be segregated or combined and various $\Delta\psi$ imposed at will. Fig. 3B shows the dramatic shift of the modes by $\Delta\psi$. At "physiological" +180 mV the D_e-T_i mode share amounts to more than 60% whereas at −120 mV the opposite mode pattern is seen.

The conclusion of the electrical nature of the ADP/ATP exchange was not immediately accepted, partially, as it would upset the textbook P/O ratios. Mitchell and Moyle believed to have shown [85] that the exchange is neutral, corresponding to $ADP^{3-} \leftrightarrow ATPH^{3-}$, with a parallel H^+ release, or that it is neutralised by Ca^{++} co-transport [86]. Kemp and Out suggested an energy dependant conformation change which differentiates between ADP and ATP [87]. Vignais et al. assumed [88] that the energy transduction to the ATP synthesis also drives the ATP export, implying a localised release of newly synthesised ATP (see Chapter 6). Other arguments against an electrogenic exchange were based on claiming that energisation changes K_M rather than V_{max} [73] and a conformational change of the AAC by $\Delta\mu H^+$ was invoked.

Direct measurements of the electrical charge transfer became important to further ascertain the electrical nature of the exchange. In mitochondria permeabilised for K^+ by valinomycin, the uptake or efflux of K^+ or H^+ was measured parallel with ATP or ADP uptake [89]. Experiments were performed to determine the electro-neutral share of transport by measuring the H^+ movement which would accompany an $ADP^{3-}/ATPH^{3-}$ exchange, and to determine the share of electrical ADP^{3-}/ATP^{4-} exchange by measuring the parallel H^+ or K^+ transport in mitochondria which are permeabilised for H^+ (uncoupler) or for K^+ (valinomycin) [89]. The electro-neutral share amounted to about 0.2 H^+/ATP and the electrical share to 0.6 to 0.8 K^+/ATP. In other experiments, by inducing ATP uptake by K^+ or H^+ additions, the ratios H^+/ATP or K^+/ATP reached 0.8 to 0.9 [90]. Since due to back leakages some of those values may be underestimated, with Occam's razor one negative charge per ATP has been extrapolated and thus a fully electrical ADP^{3-}/ATP^{4-} exchange postulated [90]. However, a fractional charge, corresponding to a partial $ATPH^{3-}$-transport cannot be ruled out and may serve to adjust the energy share used in the exchange in dependence on $\Delta\psi$ and ΔpH. At any rate, the ADP–ATP exchange is a primarily "electrophoretic" ATP export, and can be classified as a "secondary active" transport. In another work [91] using a fluorescent indicator, the change of $\Delta\psi$ was recorded during the import of ATP into ADP loaded SMP from BHM, showing that the reversed exchange is electrogenic. Applying valinomycin and nigericin the changes of ΔpH were attributed to other side reactions rather than to $ATPH^{3-}$ transport, substantiating a fully electric ATP^{4-}–ADP^{3-} exchange.

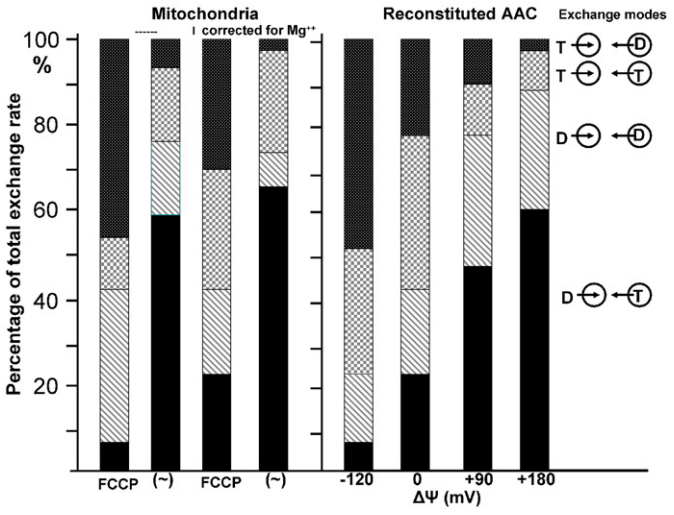

Fig. 3. The influence on the partitioning of the total exchange transport capacity into the four exchange modes: productive D_e–T_i (ADP_{ex}–ATP_{int}), unproductive D_e–D_i, T_e–T_i, counter-productive T_e–D_i modes. Energised (~) and de-energised state (FCCP) of mitochondria, and of the equivalent $\Delta\psi$ variation in reconstituted AAC proteoliposomes. Strong preference of the productive mode in energised state and at +180 mV and near equal mode distribution on uncoupling and $\Delta\psi$=0. The data in mitochondria are given without and with correction for endogenous Mg^{++}. The corrected values are to be compared to those of the reconstituted Mg^{++}-free system. From [81,64].

4.2. Electrophoretic control of the reconstituted AAC

In the reconstituted system, without interference by other mitochondrial components, $\Delta\psi$ and separately ΔpH can be set arbitrarily over a wide range even in the "opposite" direction for determining their influence on transport [64]. Internal nucleotides are not complexed with Mg^{++} as in mitochondria and the nucleotide composition can be chosen such that the 4 single modes can be separately measured. The $\Delta\psi$ is set by the K^+-gradient and valinomycin over a theoretical but actually a somewhat smaller range of −120 to +180 mV. By varying the $\Delta\psi$ a linear relation between $RT\ \log(V_{ADP\rightarrow}/V_{ATP\leftarrow})$ and $nF\Delta\psi$ is obtained with a slope of n=0.6. Only the hetero-modes ATP_e–ADP_i and ADP_e–ATP_i were markedly influenced by $\Delta\psi$, according to the polarity and ATP versus ADP location. At equal in- and outside concentrations of ATP and ADP the ratio of uptake rates V_{ATP}/V_{ADP} is radically changed from 5 to 0.2, when $\Delta\psi$ was varied from −120 to +180 mV. By double labelling the efflux was also measured and thus the partition of the total exchange into the 4 modes was determined, showing at $\Delta\psi$=0 a near equality of the 4 modes (each 25% of total exchange) and at −120 mV or +180 mV a preference for the hetero-modes D/T or T/D (60% and 65% of total) (cf. Fig. 3B). Varying the ΔpH (at $\Delta\psi$=0) from −1 to +1 decreased the rate ratio V_{ATP}/V_{ADP} only 1.5 fold, providing evidence that the electro-neutral mode ADP^3-$ATPH^{3-}$ plays only a minor role as compared to the electrical mode ADP^3-ATP^{4-}.

In a further rigorous study of the reconstituted system the question was settled [65], in view the different results reported on mitochondria, of whether the K_M (affinity) or the maximal rate V_{max} are changed by the $\Delta\psi$. The kinetic parameters were analysed by two different kinetic models according to whether $\Delta\psi$ controls the affinity (K_M) or velocity. The concentrations of external and internal ADP or ATP were varied for determining the K_M and the four uptake and efflux rate constants $k^D_{\rightarrow}$, $k^T_{\rightarrow}$, $k^D_{\leftarrow}$, and $k^T_{\leftarrow}$. The changes of the rates by $\Delta\psi$ evaluated according to the K_M model were not compatible with the measured data, but agreed well when calculated according to the V_{max} model. In this model any changes of K_M with $\Delta\psi$ result from an influence of the velocity, whereas the affinity ($1/K_D$) remains unchanged. A strong asymmetry of c- versus m-state distribution of the AAC under the influence of $\Delta\psi$ emerges from the evaluation of the kinetic data, caused by the strong asymmetry of the rates. These results show that $\Delta\psi$ lowers the activation barrier of the c–m transitions rather than the conformation of the carrier. The data are supported by results with mitochondria, showing that the influence of $\Delta\psi$ on K_M is small [2]. Reports by other groups of a

large influence of $\Delta\psi$ on K_M in mitochondria [71,73,91,92] could not be confirmed and may be partially explained by experimental problems.

4.3. Direct measurement of electrical currents of ADP/ATP transport

Electrical currents associated with the electrogenic ATP/ADP transport could be directly measured [93,94], using a technique [95] in which the capacity currents across a planar membrane between two bath electrodes are measured. Reconstituted AAC containing vesicles were adsorbed at the planar membrane, caged ATP or caged ADP were added to the medium. These are not transported but are bound and are competitive inhibitors of ADP/ATP transport. The exchange was initiated by photolysis liberating ADP or ATP from the caged compounds. On flash photolysis transients current were measured which were abolished by combined CAT and BKA. The currents reflect several cycles of ATP–ADP exchange until the external level of liberated ATP or ADP subside and excess caged ATP or ADP reclaims the AAC. Opposite currents were measured with caged-ADP_{out}–ATP_{in} and caged-ATPout-ADP_{in} vesicles. Unexpectedly, currents were also generated with the homo-exchanges in the mode caged-ATP_{out}–ATP_{in}. The currents were shorter and weaker and were visualised to reflect the redistribution of carrier sites, initially all trapped outside by the caged substrates, after the photolytic release of ATP or ADP. The measurements of the influence of pH, of K^+ gradient, $\Delta\psi$ and of temperature on these currents [94] all concurred with the former transport measurements. With excess Mg^{++} the translocation currents were suppressed, again underlining that Mg^{++}–nucleotide complexes are not translocated.

Still smaller but distinct currents were observed with unloaded vesicles after photolysis of caged ATP and ADP, interpreted as the one way translocation of carrier sites loaded with ATP or ADP from out-to inside, to be caused by half cycle translocation of the ATP- or ADP–AAC complex. The fact that not only with caged ATP but also with caged ADP currents are measured, although in an opposite direction, called for a new view of the charge balance at the binding site. Formerly it was proposed [84] that at the binding center the charge balance is neutral in the putative $ADP^{3-}AAC^{3+}$ complex and negative in the $ATP^{4-}AAC^{3+}$ complex. The current measurements [96] indicated that ADP–AAC also carries a fractional although positive charge. While the total charge movement of −1 for ATP^{4-}/ADP^{3-} exchange is well established, the results call for a fractional charge distribution at the binding center according to $(ATP–AAC)^{-0.7}$ and $(ADP–AAC)^{+0.3}$. Fractional charge shifts are also observed on binding of ATR and BKA initiated by photolysis of caged ATP (N. Brustovetsky, unpublished).

High time resolution measurements revealed the current stages of the initial in situ conversion of caged ATP^{3-} and caged ADP^{2-} into ATP^{4-} and ADP^{3-} ($1100\ s^{-1}$) followed by the slower translocation of the in situ generated ADP or ATP to the inside (ADP, $400\ s^{-1}$, ATP $160\ s^{-1}$) [96]. Since caged ATP and caged ADP bind tightly to the AAC, ADP or ATP are released at the binding site and immediately mobilise the AAC. The translocation kinetics were independent of the flash strength, i.e. of how much free ATP and ADP were generated in the surrounding solution, showing the preferred status of the in situ released ATP and ADP. This differs from similar experiments with a typical ATPase [97], such as the Na^+/K^+-pump where caged ATP and in situ released ATP are in rapidly equilibrium with the surrounding photolysed ATP. Probably the binding charge difference, ATP^{4-} in AAC versus $(ATP\ Mg)^{2-}$ in the ATPase are responsible for this difference. In conclusion, the high sensitivity of this method revealed the detailed working of the AAC such as the forward and back flow of sites loaded with ADP or ATP as reflected in currents with opposite polarity, the trapping of the carrier outside by caged ATP and ADP and the competition between external ATP and ADP and the caged derivatives.

4.4. The phosphorylation potential gradient across the mitochondrial membrane

The electrochemically driven ADP-ATP exchange produces a gradient of the T/D ratio between the mitochondrial matrix and the cytosol by elevating external T/D over the internal T/D. This prediction, which at first seemed to be a paradox because ATP is generated in the mitochondria, was substantiated by a variety of experimental results, showing that the external ratio $(T/D)_e$ can be up to 20 times higher than $(T/D)_I$ [98,99]. In contrast, opposite gradients with a higher $(T/D)_i$ had been reported by Duee and Vignais [62] and were interpreted as a build up of internal ATP. However this $(T/D)_i$ may be in error since it was calculated as a difference of supernatant and pellet without a subtraction of the surrounding "sucrose space", necessary to determine the matrix space and the T/D ratios therein [98]. Whereas with phosphorylating mitochondria the $(T/D)_e/(T/D)_i$ ratio is 5 [1,98], the $(T/D)_e/(T/D)_i$ can reach even 20 under conditions where substrate level and oxidative phosphorylation were halted with oligomycin and rotenone, allowing equilibration of ADP and ATP across the membrane. Since under these conditions the $(T/D)_e$ is "clamped" due to its large excess, the energisation of the membrane causes primarily a decrease of $(T/D)_i$ by largely depleting the matrix of ATP. It may seem to be a surprise, that on uncoupling $(T/D)_i$ became nearly 3 fold higher than $(T/D)_e$. However, this can rationalised by noting that the high internal Mg^{++} concentration lowers the free $(T/D)_i$ as compared to total $(T/D)_i$ approximately 5 fold, because Mg^{++} binds about 5 times tighter to ATP than to ADP. Consequently, by adding 2 mM Mg^{++} the external and internal T/D become equal [98].

The T/D gradient could be varied over a wide range, under non-phosphorylating conditions, by imposing a K^+ diffusion potential [100] in the presence of valinomycin, generating $\Delta\psi$ from 50 to 160 mV. The internal K^+ concentration was measured by radioactive Rb^+. At 160 mV $(T/D)_i$ was decreased to 0.01, resulting in $(T/D)_e/(T/D)_i \approx 100$ with $(T/D)_e = 1.2$. The linear relation of $\log (T/D)_e/(T/D)_i$ to the K^+ diffusion potential was determined to follow the relation: $\log (T/D)_e/(T/D)_i) = (0.65 \text{ to } 1) \times \log (K^+_i/K^+_e) - (0.2 \text{ to } 0.5)$. Since this relation was shown to hold for all energy states of the mitochondria, e.g. uncoupled, energised or electron transport inhibited states, the $\Delta\psi$ dependent T/D gradient was concluded to be a function only of the electrophoretic ADP/ATP exchange. This finding again contrasts to the seemingly evident picture that the T/D gradient across the membrane with a high internal T/D drives the ATP export [62].

4.5. Oxidative phosphorylation and the free energy of cytosolic ATP

The T/D gradient produces a cytosol/matrix difference of the phosphorylation potential of ATP $\Delta^{i-e}G_{ATP}$, which adds to the matrix $\Delta G^{mit.}_{ATP}$ to yield the cytosol potential ΔG^{cyt}_{ATP}. The additional "transport energy" can be calculated by 4 different methods [1,82]. The calculation has to also include the P_i distribution which depends on the transmembrane ΔpH since it is transported as "P_iH^+". With $\Delta pH \leq 0.5$ under most conditions $(P_i)_i,/(P_i)_e$ is close to 3, thus influencing only little $\Delta^{i-e}G_{ATP}$. With $(T/D)_e/(T/D)_i = 20$ the difference of free energies between extra and intramitochondrial space amounts to $\Delta^{i-e}G_{ATP} = 2.5$ kcal [82]. This corresponds to a typical setting with isolated mitochondria in "state 4". Other values are estimated from the ATP to ADP ratio of the import and export rates to $\Delta G^{i-e}{}_{ATP} = 3.2$ kcal, from P/O stoichiometry 3.4 kcal, from $\Delta\psi$ and ΔpH 2.9 kcal. $\Delta G^{transp.}_{ATP}$ can reach up to −4.2 kcal with $\Delta\psi = 180$ mV. Given an internal $(T/D)_i = 0.1$ and Pi = 0.1 mM a matrix $\Delta G^{mit.}_{ATP} = -10.4$ kcal and $\Delta G^{cyt}_{ATP} = -14.6$ kcal are calculated. In this extreme "static head" case up to 30% of the free energy of cytosolic ATP comes from the uphill transport from the mitochondria. Under most steady state conditions the transport share will be at 20 to 25%.

The energy distribution can be numerated in terms of the H^+ electrochemical energy "units" represented by the H^+ pumped by the

respiratory chain (Fig. 4). If n H^+/ATP are used by the ATP synthesis, one charge transferred by the ATP/ADP exchange, and 1 H^+ co-transported with P_i, $(n+1)$ H^+/ATP are required for the supply of cytosolic ATP. With $n=3$, the energy needed for the transport amounts to 1/4 of the total energy. A total of 10 H^+/2 e^- are assumed [101] to be pumped by the respiratory chain through the 3 complexes in mammalian mitochondria. With this value, $n=3$, and one H^+ for transport a maximum of 2.5 ATP/2 e^- could be accommodated in oxidative phosphorylation for the ideal case of no leakage and uncoupling. In the reversed reaction, in ATP hydrolysis of ATP added to mitochondria a ratio of H^+/ATP=2 was measured [102]. Compared to 3 H^+/electron transport site, one H^+/ATP was concluded to be consumed by the ATP translocation, corresponding to 1/3 of the total energy.

As predicted by the in vitro results with mitochondria and reconstituted proteoliposomes, in vivo large phosphorylation potential differences between mitochondria and cytosol were found. The most relevant data were obtained by a freeze lyophilisation and non-aqueous fractionation procedure [103,104]. In perfused rat liver the maximal difference of $\Delta^{i-e}\Delta G_{ATP}$=2.5 kcal was determined. This gradient was abolished by dinitrophenol to 0.1 kcal, and by CAT to 0.15 [104]. In perfused heart the ΔG_{ATP} are deduced from the creatine-P/creatine ratio as $\Delta^{i-e}\Delta G_{ATP}$=1.8 and 3.0 kcal [105]. A summary of these and further data on the in vivo phosphorylation potential gradient is given in [106]. These findings can be summed up in the important conclusion that in eukaryotes oxidative phosphorylation has evolved to generate less ATP for the sake of ATP with a higher energy potential.

4.6. Regulation and rate limitation

For controlling the supply of ATP to the cytosol where a higher phosphorylation potential reigns, the AAC has developed a simple "auto-regulation" with a passive response to the factors reflecting the ATP demand, such as the surrounding T/D ratios and the $\Delta\psi$. Without membrane potential the AAC can catalyse the 4 exchange combinations of ADP and ATP, two homo-exchange modes T–T, D–D, and two hetero-exchange modes T–D, D–T at nearly equal rates. Only the D–T mode (ADP uptake−ATP release) is productive for the ATP delivery whereas the T–D mode is counter-productive and the homo-modes D–D and T–T are diverting exchange capacity. The distribution between the modes should be trivially controlled by the D/T ratios on both sides of membranes. The membrane potential skews this distribution in favour of the D–T mode and suppresses the T–D mode. In the reconstituted system it was elegantly demonstrated [107] how the membrane potential redistributes the modes such that at +180 mV the D–T exchange can use 60% of the total capacity under "normalised" conditions, in which the T/D ratios are preset to be equal on both sides (see Fig. 3). At "static head" the net ATP export will be down regulated to approach zero when the T/D ratios across the membrane diverge so much that the D–T mode becomes equal to the T–D mode. The AAC is constantly exchanging nucleotides, but within its capacity the rate of various exchange modes may strongly vary under the influences of the T/D ratios and of $\Delta\psi$.

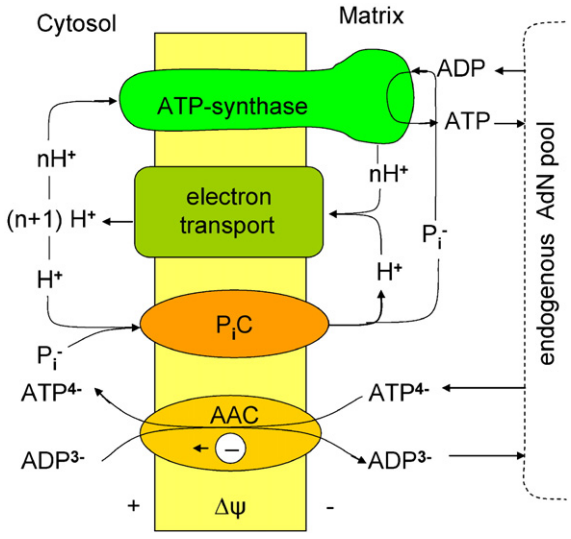

Fig. 4. The H^+ transport balance for the synthesis of cytosolic ATP. Out of $(n+1)$ H^+ pumped by electron transport, n H+ are used for synthesis of endogenous ATP and 1 H^+ for the $\Delta\psi$ driven export of ATP. This H^+ is returned by neutralising the P_i^- import and thus compensates the export of one negative charge in the ADP^{3-}–ATP^{4-} exchange. From [81].

The question of whether or to what extent the ATP transport is rate limiting in the supply of external ATP has been a contentious issue [8,108–112]. One problem broadly discussed is how rate limitation by the AAC is defined in a sequence of reactive steps. Any influence on one step will shift the sequence of reactions into a new steady state and thus spread to other steps by changing the substrate concentration and electrochemical potential. However, the major issue of how AAC responds to the varying energy demand is insufficiently accounted for. As pointed out above, the redistribution among the exchange modes provides an autonomous regulatory response to the changing demands. The obvious "external" regulatory factor is the $\Delta\psi$. But in vivo changes of $\Delta\psi$ are normally confined to a range of 130 to 180 mV, and thus the preference of the D_e–T_i exchange mode is always supported unless under extreme deranged conditions. Stronger changes of the ATP export rate are caused by variations of T/D ratios on both sides of the inner membrane. In particular the cytosolic ADP level can vary several fold between rest and activity in muscles [105,113] thus strongly influencing the exchange mode pattern. As a result the AAC uses its gateway role for adjusting both the ATP supply and the phosphorylation potential of ATP in accordance with the requirements of the cytosol.

4.7. AAC and endosymbiosis

The increase of the free energy of cytosolic ATP by the AAC exchange can be regarded as a vestige of the prokaryotic origin of mitochondria. It may be assumed that in the precursor eukaryote cell ATP operated at a high phosphorylation potential whereas in the mitochondrial precursor prokaryotes a lower ΔG_{ATP} prevailed. The AAC by virtue of its electrophoretic transport of ATP bridges the energy gap and thus is a critical factor for the endosymbiosis. The thermodynamic differences extend also to the redox state represented by the universal NADH–NAD system. In mitochondria as in the ancestral prokaryotes the NADH/NAD ratio is linearly related to the ATP/ADP ratio in the oxidative phosphorylation system, whereas in the cytosol this ratio is inversely correlated by the substrate level phosphorylation of glycolysis [114–117]: $(NADH/NAD)_{cyt}\times(ATP/ADP.P)_{cyt}$=const. The redox potential in the mitochondria is about 100 mV more negative than in the cytosol, possibly reflecting the origin of the mitochondrial precursor from a more reducing environment. The coupling of the phosphorylation potentials to the divergent redox potentials of the NADH/NAD system may be the principal reason for the divergence of the phosphorylation potentials. The AAC and the aspartate–glutamate exchanger permit the symbiosis of the different thermodynamic environments via their electrophoretic mode of transport.

5. Micro-compartmentation?

The ADP/ATP transport was characterised as an exchange of exogenous with a pool of endogenous nucleotides. On the basis of comparing the rates of ATP synthesis or of hydrolysis of endogenous and exogenous nucleotides [20,42,61,62,118], this pool of ATP, ADP and AMP was shown to be intermediary between transport and intramitochondrial phosphate transfer reactions, such as ATP

synthesis, substrate level phosphorylation etc. Subsequently, alternate types of the organisation of the ADP/ATP exchange and ATP synthesis were envisaged [92,87]. As reported by Vignais et al. [92], addition of ^{14}C-ADP to phosphorylating mitochondria immediately released endogenous (unlabelled) ADP and ATP and, in agreement with the pool function, newly synthesised ^{14}C-ATP after a delay. However, in the intramitochondrial pool, calculated as a difference between total and exogenous AdN, no ^{14}C-ADP, but only ^{14}C-ATP appeared without delay. In contrast in non-phosphorylating mitochondria added ^{14}C-ADP appeared immediately inside but endogenous ATP was released only as ADP. Both results were interpreted [92] to show a direct cooperation of ATP synthase and AAC, such that entering ADP is handed over directly to the ATP synthase and released as ATP into the endogenous pool. Correspondingly, in non-phosphorylating conditions endogenous ATP hydrolysed by ATP synthase is directly exported. As a result a reversible association of ATP synthase and AAC was postulated with the AAC moving across the membrane. Kemp and Out reported [87] that on ^{14}C-ADP addition ^{14}C-ATP is released without time delay bypassing the endogenous pool. However, in the uncoupled state the added ^{14}C-ADP seemed to enter the pool. They concluded that entering ADP passed through the ATP synthase and that, the newly formed ATP is immediately released, and that the ATP synthase–AAC association is dependent on the energisation of the membrane. The absence of a time delay, at variance to Vignais et al. [92] and Klingenberg [83], can be explained by the 5 times slower rate of ^{14}C-ATP formation than exchange rate in these experiments which will obscure a time delay. Probably a not instantaneous quenching of the supernatant lowered the ATP level. Delayed quenching of the mitochondria may also explain the failure of Vignais et al. [92] to observe the crucial uptake of exogenous ADP.

Kinetic experiments using a sophisticated automated rapid mixing, sampling, separating and quenching apparatus, in which after rapid pressure filtration both the supernatant and the mitochondria are immediately quenched, did not confirm [83] those results arguing for a functional AAC–ATP synthase association. By double labelling in mitochondria a rapid entry of exogenous ^{3}H-ADP and exit of endogenous ^{14}C-ATP is measured (Fig. 2). In the external medium endogenous ^{14}C-ATP appears immediately and exogenous ^{3}H-ATP after a delay. The steady state rate of ^{3}H-ATP formation is only 1.6 time slower than the total exchange rate. Similarly, in uncoupled mitochondria on addition of ^{3}H-ATP, endogenous ^{14}C-ADP exits immediately and after a delay ^{3}H-ADP is released, again demonstrating that the endogenous pool is on the main pathway between AAC and ATPase. These results confirm the previous experiment based conclusions of a central role of the endogenous pool. They also point out the importance of fast quenching after separation of mitochondria [42] to avoid the reported artefacts.

The association of a 29–30 kDa protein with ATP synthase preparations has been reported [119], but later found to be absent in better defined detergent solubilised oligomycin sensitive ATPase preparations [120], confirming in hindsight the suspicion that the large abundance of AAC amounting to about 2.5 AAC/cytochrome oxidase based on CAT binding [2], may easily have caused this contamination. Recently a complex comprising ATP synthase, AAC and P_i carrier, was described [121,122] as an electron microscopic particle identified by antibody staining. Citing the above mentioned, but not confirmed, kinetic evidence of an association between AAC and ATP-synthase, a "synthasome" was envisaged to foster the direct collaboration of the three entities in transport and synthesis of ATP. Not only the operational evidence but also stoichiometry problems, such as the molar ratio of AAC/Pi carrier ~4 estimated in mitochondria, argue against the existence of this entity. At this point one may cite an evolutionary argument for the independence of AAC from ATP synthase: in the predecessor bacteria the ATP synthase operated only with the endogenous AdN pool. Only after the endosymbiotic transition to mitochondria, AAC arose, most probably as independent entity.

Different from the situation at the matrix side, there is some evidence for a limited direct cooperation of the AAC in the intramembrane space with phosphate transferring enzymes such as with the mitochondrial creatine kinase (CrK). First proposed on the basis of a mitochondrial CrK isoform [123], the "creatine phosphate shuttle" [124] was elaborated and established [125,126] by postulating a preferred transfer of exported ATP to CrK followed by the diffusion of creatine phosphate (CrP) to the cytosol. Hereby a cooperation of the intramembrane CrK with the AAC is implicated [127,128]. However due to the excess of AAC over CrK only a small portion of AAC can be physically coupled to octameric CrK. A complex between AAC, CrK and VDAC (porin) at the contact sites between inner and outer membrane has been envisaged [129]. Whereas the CrP-shuttle should be confined to muscle and neuronal cells, in other cells such as liver the intermembrane adenylate kinase may play a similar role. In the CrP shuttle the diffusion of a smaller sized CrP poses an advantage over ATP. This is further underlined by the finding that the passage of ADP and ATP through VDAC may be a rate limiting and regulated step [130]. All these considerations of functional micro-compartmentation must be viewed on the background of the physical organisation of the intermembranes space and the inner membrane into two sections, the inner cristae membrane surrounding the cristae space and the inner boundary intermembrane space and inner boundary membrane facing the outer membrane. The exit from the cristae space into the surrounding transmembrane space is provided by small tubular structures [131,132] whereas the AAC and the respiratory complexes must be located in the cristae membrane, and the location of the phosphate transferases is not clear.

6. Inhibitors of transport

Nature has provided the AAC with exceptionally specific and effective inhibitors furnishing unique tools for elucidating the transport mechanism. These natural inhibitors fall into two groups: those containing atractylogenine and its homologues and bongkrekic acid (BKA) with the isoform iso-BKA. Both these inhibitor groups are highly specific for the AAC and up to date no other transport system including various other nucleotide transporters have been found to respond to them [15]. Besides differentiating AAC linked transport, these inhibitors were also crucially important for isolating the AAC. No other transporter is known to be endowed by nature with such selective and high affinity inhibitors which also excel by an extraordinary opposite membrane side specificity.

6.1. Atractyloside-group

Atractyloside (ATR) was found to be the toxic principle of the thistle *Atractylis gummifera* from the Mediterranean region (see monography [133]). The inhibitory effect of ATR on oxidative phosphorylation was early recognised [27,28]. However, its specific role of inhibiting the ATP export from mitochondria could only be identified after the existence of the ADP/ATP exchange was established [25]. Erroneously the notion is propagated in the literature, e.g. [9], that the discovery of the ADP/ATP transport is due to the inhibition by atractyloside. Bruni [32] found that ATR inhibits (^{32}P)-ATP "binding" to mitochondria and made the logical conclusion that ATR binds to the ADP binding site of the ATP synthase. However, as first reported at the IUB congress in 1964, ATR was shown to inhibit the ADP/ATP transport already previously established [25]. Similar conclusion were inferred subsequently by Chappell and Crofts [22] and Kemp and Slater [30] Thus the inhibition target of ATR was shown to be the transport rather than the ATP synthesis per se.

The homologue carboxy-ATR (CAT), found in the same plants [134], also called gummiferin [135,136], proved to be a very useful tool for characterising the underlying transporter because of its very high binding affinity. CAT^{4-} with two glycosidic sulphate groups and two carboxyls in the aglycone provides one more charge than ATR^{3-} for binding which may explain the higher binding affinity. However, the stereo-isomer epi-ATR^{3-} generated by decarboxylation of CAT and thus carrying only 3 anionic groups [137], has nearly as high binding affinity as CAT ($K_D = 1 \times 10^{-9}$ M for CAT, 1.8×10^{-9} M for epi-ATR) [138]. In epi-ATR the carboxyl group is in equatorial [139] and in ATR in axial position in the kaurine moiety, indicating that the stereochemistry of the charge groups is equally important as their number. Elimination of $-SO_4$ group from ATR abolishes binding [140]. The minimum of 3 negative charges is reminiscent of ADP^{3-} and the exclusion of AMP^{2-} from transport, supporting the notion that ATR binds to the same site as ADP and ATP. However other atractylogenine containing glucosides such as wedeloside [141,142] and "coffee-ATR" [143,144] which carry no sulphate groups, but instead hydrophobic side chains at the glucose, are also high affinity inhibitors of the ADP/ATP exchange, indicating a greater complexity of the inhibitor binding site where hydrophobic interactions can compensate for the lack of ion bonds.

The inhibition of transport by ATR is quasi competitive with ADP or ATP [2,140]. For example, a 10 fold increase of the ADP concentration increases the apparent K_I of ATR only 2 fold. A slow dissociation rate of the first bound ATR may prevent equilibrium with ADP added for the exchange. Fully uncompetitive is the inhibition by CAT, in line with a high binding affinity (see below) and with epi-ATR [137]. Present both in epi-ATR and CAT, the equatorial carboxyl seems to be more effective for binding [138], perhaps due to greater proximity to the sulfate groups.

6.2. Bongkrekic acid

A different, very effective transport inhibitor, bongkrekic acid (BKA), comes from a bacterial source, *Pseudomonas cocovenenans* [145–147]. Together with its less effective isomer, iso-BKA, the tricarboxylic acid has a complex poly-isoprenoid structure [147]. In iso-BKA one isoprenoid segment carrying a CO_2^- group is in *trans*- instead of *cis*-position. It is generated by alkaline treatment of BKA [148] or by intermediate methyl-ester formation [149,150]. The only common structural denominator of BKA with ATP and also ADP seems to be displaying three negative charges. With the hydrophobic backbone BKA contrasts to the more hydrophilic structure of the ATR group with their carbohydrate moiety.

In the first direct demonstration of BKA as a transport inhibitor [151] complete inhibition was reached at 0.2 to 0.3 nMol/mg prot. in liver mitochondria, at the same level as with ATR. The $K_I < 0.2 \times 10^{-8}$ M was lower as for ATR. Whereas the inhibition by BKA is quasi non-competitive with ADP, inhibition of transport by iso-BKA is competitive, with a 2 to 4 times higher K_I than BKA [148]. Different from ATR the inhibition by BKA required preincubation at higher temperature (20°) because of a time delay of the inhibition onset [151]. A strong pH dependency of BKA inhibition of respiration was noted [152] and quantitatively investigated [68,153] in conjunction with other BKA effects such as ADP binding and the contraction of mitochondria. These and other data reflecting the interaction of BKA with the AAC could be explained [153] by assuming that BKA, before reaching the AAC, has to diffuse through the inner membrane. Since the electro-neutral form $BKAH_3$ should by easily permeating, it is mainly the concentration of $BKAH_3$ which is rate limiting the onset of inhibition. As a result, in mitochondria, the interaction rate of BKA with the AAC increases nearly 100 fold from pH 7 to 6.3 [68]. In accordance in SMP, which are mostly inside-out, nearly no time delay is observed [78]. Based on this and other types of evidence, it was concluded that the anion BKA^{3-} is the inhibitory form.

6.3. Acyl-CoA

Acyl-CoA, in particular the long chain palmityl- and oleyl-CoA, were found [154,155] to inhibit the ADP/ATP transport in mitochondria from rat liver and from rat heart with remarkable efficiency. The inhibition could be reversed by ADP [156] or by carnitine addition [154]. This finding also explained [155] the early observation [157] that oleate partially inhibits the ADP–ATP transport in mitochondria since oleate is converted to oleyl-CoA in the matrix. When CoA is recruited into succinyl- or acetyl-CoA by addition of ketoglutarate or pyruvate, oleate is unable to inhibit ADP translocation. As amphipathic molecules the long chain acyl-CoA absorb at the membrane so that concentrations are not meaningful and the reported K_I of 0.2 μM [158] and 1 μM [159] are conditional values. Acyl-CoA effects are competitive with ADP and ATR as shown by binding studies on mitochondria [160,161] indicating that acyl-CoA bind close to the carrier binding center. In condensed phase transitions of mitochondria (see below) palmityl-CoA has the same effect as ATR, emphasising the similarity to ATR interaction with AAC. However, different from ATR, acyl-CoA inhibit ADP uptake into SMP [73,78,160] showing that they also interact from the m-side.They do not compete with BKA, probably because of lower binding affinity. The lack of side specificity may also suggest that binding of acyl-CoA to AAC must be weaker than of ATR and BKA.

Long chain acyl-CoA also inhibits other mitochondrial carriers and several mitochondrial enzymes, suggesting at least partially an unspecific amphipathic effect. An inhibitory role of acyl-CoA on ADP–ATP transport in starved liver was suggested [162]. However loss of endogenous nucleotides may have been the main cause of the observed effects. More conclusive were studies on cardiac ischemia showing that endogenous nucleotide loss rather than acyl-CoA were responsible for the impeded ADP/ATP transport [163,164]. For this reason and the dilemma that the high acyl-CoA levels should inhibit the AAC during active fatty acid oxidation and ATP generation [8,165], a physiological regulation by acyl-CoA of the ADP/ATP transport has to be questioned.

7. The ADP/ATP carrier as a molecular target

7.1. ADP and ATP binding and the influence of atractyloside

Studies of the underlying carrier AAC of the ADP–ATP exchange can be divided into two parts: (a) the carrier in the native membrane of mitochondria, and (b) the isolated carrier in detergent solution, in reconstituted vesicles and in crystals. Before the AAC protein was identified, it was an obvious goal to determine the number and properties of carrier sites in mitochondria. Bovine heart mitochondria (BHM) were the favourite target, since they were available in large amounts and could be expected to have a higher content of carrier sites than liver mitochondria in analogy to the higher content of respiratory chain components.

Carrier binding sites were first probed with their natural substrates ADP and ATP [166]. For this purpose BHM were depleted of endogenous ADP and ATP by freeze-thawing and washing with 0.1 M phosphate. In some experiments arsenate was employed to convert endogenous ADP and ATP into AMP. Specific carrier related nucleotide binding was defined as the difference of binding in the presence of ATR added either before (ATR sensitive portion) or after the (ATR removable portion) labelled nucleotide. This extensive work proved to be a gold mine, not only for the first determination of the AAC sites in mitochondria but also unexpectedly providing a first insight into the flexibility of a carrier site concomitant with the translocation mechanism. The conditions and controls had to be carefully elaborated to avoid pitfalls as in some parallel work [167] when using ATR for differentiating and detecting the AAC sites on the background of other binding sites and the matrix pool. EDTA was

routinely added since it enhanced AAC linked binding and suppressed Mg^{++} dependent binding sites, whereas Mg^{++} addition had the opposite effect. This showed, as to be expected from the transport studies, that ADP and ATP bind to the carrier sites without Mg^{++}, in contrast to the universal Mg^{++} requirement of ATP utilising enzymes.

The concentration dependence of the ATR removable ADP and ATP binding yielded non-linear relations both with ADP and ATP, which could be well resolved in terms of two types of binding sites, high affinity sites with K_D=0.1 to 0.5 μM, amounting to 20 to 30%, and low affinity sites with K_D=5 to 12 μM for the rest [166]. Two or more affinities are not unusual for ligand binding to membrane bound receptors, and were commonly interpreted as high and low affinity sites, but the results with membrane bound "mobile" binding sites called for a more incisive explanation. The high affinity sites were interpreted to be not in an open equilibration with the added labelled ^{14}C-ADP or ^{14}C-ATP but represent carrier sites which on loading with external ^{14}C-ADP and ^{14}C-ATP flip into the inner side where the radio-labelled ^{14}C-ADP or ^{14}C-ATP are mixed with residual endogenous nucleotides. This "attraction" and "trapping" of ^{14}C-ADP or ^{14}C-ATP occur at low external concentrations thus mimicking high affinity. The larger portion of low affinity sites comprise those directed outside which are in direct binding equilibrium with the external ^{14}C-ADP or ^{14}C-ATP thus exhibiting the "true" affinity. According to this view, the non-linear binding reflects a mobilisation of carrier sites by substrates to be reoriented to the inner side. With this interpretation [166] the results visualise for the first time the reorientation of carrier binding sites between the inner and outer site.

Membranes prepared by sonication of mitochondria had low ATR sensitive binding. This loss of ATR effect was explained by assuming that ATR binds only from the "c-side" and these vesicles consist largely of inside-out membrane. In other reports of measuring nucleotide binding, rat liver mitochondria treated with the detergent Lubrol were used [59]. The high values of ADP binding (>1 μM/g prot.) suppressed by prior ATR addition were reinterpreted [166] to reflect in part ADP uptake rather than binding to carrier sites since the "bound" ^{14}C-ADP could not be removed by subsequent ATR addition or by "cold" ADP.

7.2. Influence of BKA on ADP/ATP binding

With the advent of BKA a new dimension in determining ADP binding to mitochondria emerged. Under the influence of BKA the binding patterns were unusually complex but finally revealed an underlying simple mechanism of carrier site flexibility, thereby providing a more penetrating insight into the "single site reorienting carrier mechanism" [2]. However, results with BKA were persistently used as arguments against this mechanism, claiming to show a separate binding site for BKA [168,169]. As an inhibitor of transport BKA was expected to remove ADP from the AAC, similar as ATR [166] but, surprisingly, was found to increase the binding of ATP [170]. These effects were studied with the same type of depleted bovine heart mitochondria preparation used for the ADP binding under the influence of ATR [153,171].

The most pertinent results are as follows: (a) Apart from increasing the binding of ADP, BKA enhances the apparent affinity for ADP [170]. As a result, under the influence of BKA, the biphasic mass action plots of ADP binding with an average of K_D=2.7 μM were converted into a mono-phasic plot with single high affinity K_D=0.2 μM [171]. (b) More ADP was bound when BKA was added after than before ADP. In double labelling experiments, internal nucleotides labelled with ^{3}H, more ^{3}H-nucleotides remained bound when BKA was added before ^{14}C-ADP than when added after ^{14}C-ADP. (c) The BKA induced binding increase of ADP is strikingly slow, indicating an intervening step. Pre-incubation with BKA only partially eliminates the slow time course of ADP binding, indicating that transition to the high affinity state requires synergism of BKA with ADP. (d) The rate and the level of BKA induced ADP binding was strongly pH and temperature dependent. The pH dependency indicated that the penetration of BKA through the inner membrane becomes rate limiting due to the low concentration of the membrane penetrating $BKAH_3$ that is formed in the dissociation equilibrium $BKAH_3 = BKA^{3-} + 3H^+$ at an average pK=4.5. Thus the presence of BKA on the matrix side appears to be a pre-requisite for the ADP binding increase. (e) The BKA induced binding enhancement of ADP and ATP has strong temperature dependence, reminiscent of ADP transport, indicating that the translocation is involved in the binding increase. (f) CAT prevents, ATR only retards the BKA effect. On the other hand, once BKA has induced binding of ADP, it becomes resistant, even to the subsequent addition of the high affinity ligand CAT rendering the binding quasi irreversible.

These and other scrutinising experiments designed to elucidate the nature of the BKA effect were initially interpreted according to two different models of BKA interactions (Fig. 5) (a) BKA binds to the same site as ADP and thus displaces ADP forming a binary complex with AAC [153,171], and (b) BKA binds to an allosteric site separate from ADP, forming a ternary complex with AAC, thereby increasing ADP binding affinity [170]. In the two models the state of the tightly bound ADP is radically different: it is either fixed to the AAC by BKA or free

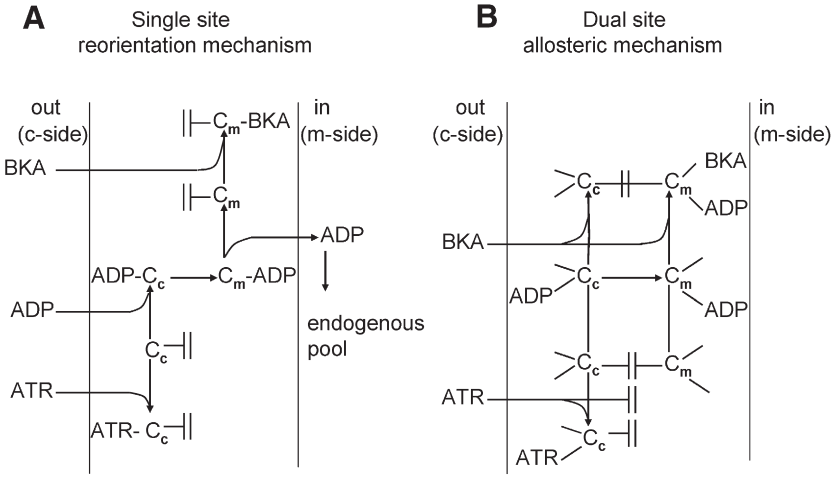

Fig. 5. The single site reorientation mechanism and the dual site allosteric mechanism of inhibitor binding to the AAC. A. Single binding site for ADP, ATP and the inhibitors atractyloside (ATR) and bongkrekic acid (BKA). AAC (C) in two states (c- and m-state) with different affinities for inhibitors. Transition between the two states is possible only when loaded with ADP or ATP. BKA can diffuse inside and binds only from the m-side. ATR binds only from c-side. B. Dual site mechanism. ADP and the inhibitors bind to different sites of the AAC. By "allosteric" interactions with the ADP binding site BKA increases binding affinity and ATR decreases affinity. BKA may bind preferentially from the m-side. From [2].

but entrapped in the matrix since BKA blocks the AAC. Whereas in the allosteric version, to comply with the binding results, in principle BKA can bind both from out or inside, in the competitive version BKA must bind exclusively from the inside. As shown above, the results strongly suggested that BKA acts exclusively from the inside. In the allosteric model a ternary complex BKA–ADP–AAC_i is formed with the inside oriented binding nucleotide site, whereas in the competitive model BKA occupies the inside oriented ADP binding site, leading to the following reaction sequence: binding sites are mostly in the external "c-state". On receiving added ^{14}C-ADP the sites are "mobilised" and reorient to the internal "m-state" where BKA, due to its higher affinity, displaces ^{14}C-ADP into the matrix. Here ^{14}C-ADP is inaccessible to external ATR, CAT and "cold" ADP because the AAC is trapped by BKA. The apparent BKA induced binding increase corresponds to the number of AAC sites transported from the c- to the m-side. The term "reorienting site mechanism" has been used synonymously with "single binding center gated pore mechanism" (SBCGP).

As mentioned above, the "single site reorienting mechanism" as an explanation of the BKA effect was repeatedly criticised [8] based on several results, for example that much less ADP is bound under influence of BKA than to be expected from the reorientation of the binding sites. This is true only for high concentrations of ADP (50 μM) which drives most sites inside so that none or few sites are left to be pulled subsequently by BKA inside. At low ADP (2 μM) nearly all sites can reorient only with BKA [153]. Other objections will be discussed below.

Since the state of ADP fixed by BKA is critical for differentiating between the two models, experiments were designed to elucidate whether the ADP "bound" by BKA is inert or reacts with intramitochondrial enzymes [172]. ^{14}C-ADP added to mitochondria converted into a similarly composed mixture of ATP, ADP and AMP as the residual endogenous nucleotide prior to ^{14}C-ADP addition and this composition was nearly the same with and without "binding" by BKA. Further in the presence of BKA the "bound" ^{14}C-nucleotides reacted freely as monitored by adding ^{32}P-phosphate under different phosphorylation conditions, induced by oligomycin or uncouplers. All these results prove that the added ADP or ATP fixed by BKA in the mitochondria is in a state similar as the endogenous nucleotide pool, contrary to what is to be expected for ADP fixed in a ternary BKA–ADP–AAC complex.

7.3. Binding of ATR and CAT

Radio-labelled ATR and CAT were used to identify and characterise AAC binding sites. By growing seedlings of *A. gummifera* on ^{35}S-sulfate, ^{35}S-ATR, ^{35}S-CAT, and a third compound identified as epi-^{35}S-ATR were extracted from these plants [173,174,137,175]. Higher specific activities were later obtained by chemically introducing ^{3}H into ATR [176] and into CAT (Klingenberg unpublished) [177] or by substituting ^{14}C-acetyl into CAT [178].

In initial binding studies with ^{35}S-ATR on rat liver mitochondria maximum binding was measured to 0.10 [179] and 0.16 nMol/mg prot [174]. Higher ^{35}S-ATR binding (1.1 nMol/mg prot.) is observed in depleted BHM [174]. The K_D is increased by ADP from 1.65 to 4.0×10^{-7} M^{-1}. In sonic particles [180] ATR binding is strongly increased when added before sonication. Relating the ATR binding to the content of cytochrome oxidase gives a molar ratio of 2.4, illustrating the high content of the underlying AAC.

A non-linear concentration dependence of ^{35}S-ATR binding was observed [179,180] which was converted to linearity by ADP addition. In depleted mitochondria an inversed non-linearity is seen which again is rectified by ADP resulting in decreased affinity for ATR. In inner membrane preparations [179] ADP lowered affinity more strongly but increased maximum binding of ATR, whereas in mitochondria no increase of maximum binding and a smaller affinity decrease was observed. Vignais et al. [179] interpreted the ADP effects on ATR binding to show separate, allosterically connected sites for ATR and ADP, Klingenberg et al. [180] maintained that the same site is competitively shared by ATR and ADP, provided it is in the c-state. Sites in the m-state can bind ATR only after they have been reoriented to c-side by first binding ADP. In double labelling experiments the displacement of ^{3}H-ADP by ^{35}S-ATR binding was titrated in depleted BHM [174] at different given ADP concentrations. The titration with ATR reaches a ratio of ΔADP/ΔATR of nearly 1, when binding sites are saturated with 20 μM ADP.

Subsequently, the high affinity ^{35}S-CAT was introduced. In a titration of rat liver mitochondria a linear correlation between inhibition of transport and binding was demonstrated [175,181]. At the saturation point of ^{35}S-CAT binding the inhibition is complete, showing that all CAT binding sites in mitochondria are identical with the transporter sites. Identical maximum binding for ATR and CAT was determined in mitochondria from bovine heart (1.2 to 2.0 μMol/g protein) and from rat liver (0.3 μM). The concentration dependence of CAT binding showed complicated convex mass action relations simulating a cooperativity [175,178,182]. Vignais et al. interpreted the non-linear dissociation plots as a cooperation of CAT binding sites. However, our observations indicated that the non-linear relation is due to an incomplete equilibration artefact caused by very slow residual binding under conditions of extreme bound/free ratios caused by a very low K_D. Therefore, only a rough evaluation of K_D was possible for CAT (<2 nM [175] or 5 to 10 nM [182]). The dissociation rates of ^{35}S-ATR and ^{35}S-CAT, induced by excess unlabelled CAT, furnished another indication of the widely different K_D [175]. A more than 100 times lower rate for CAT dissociation was measured that can be translated into an up to 100 times higher binding affinity of CAT. Related to the electron transport system, there are 2.2 to 3 CAT sites/Cyt aa_3 in heart and 0.6 to 1.5 in rat liver. Thus the ratio of AAC binding sites to the respiratory chain varies among different cells, in line with a higher proportion of ATP used in liver mitochondria than in heart mitochondria which are geared for a nearly exclusive ATP supply to the myofibrils.

7.4. Binding of bongkrekate

For binding studies BKA was ^{3}H labelled at an allylic position between two carboxylic groups by $^{3}H^{+}$ exchange in alkaline ^{3}H-H_2O yielding ^{3}H-BKA at high specific activity [149,168]. As a by-product ^{3}H-iso-BKA was obtained in nearly equal amounts to ^{3}H-BKA [148,149]. Identification of BKA binding to the AAC is complicated by the uptake of BKA into the membranes and matrix of mitochondria. In work on rat heart mitochondria saturable high affinity sites (~1/μMol/g prot) could be segregated from non-saturating BKA binding [168]. The latter part could be largely eliminated by a pre-treatment of the mitochondria with Mg^{++} and phosphate. In another work with intact BHM, a linear non-saturation increase affinity binding of ^{3}H-BKA binding without a high affinity portion site was measured. Only after sonication or after freeze-thawing of the BHM or by permeabilisation with Lubrol WX a saturable high affinity binding was unmasked. These results show that non-saturating binding corresponds to internalised BKA which is released on breakage of mitochondria. The variant results with rat heart mitochondria can be explained by assuming that the heart mitochondria were partially broken. This is further supported by using CAT as a tool for discriminating the AAC specific ^{3}H-BKA binding. In mitochondria from rat heart CAT suppressed high affinity binding of ^{3}H-BKA whereas it had no effect on BHM [150]. Only after damaging the BHM by freeze-thawing or sonication, ^{3}H-BKA binding became CAT sensitive. In all these cases ^{3}H-BKA binding is strongly increased by addition of ADP, in line with the synergism between BKA and ADP binding. Double labelling experiments using ^{3}H-BKA and ^{14}C-ADP showed directly that ^{3}H-BKA and ^{14}CADP binding are inter-dependent [150]. The synergism between ^{14}C-ADP and ^{3}H-BKA uptake is more pronounced, when by prior addition of ATR, ^{14}C-ADP is removed from binding sites. The CAT sensitive portion of ^{3}H-BKA binding to fragmented BHM was

evaluated in mass action plots to give $K_D = 6.0 \times 10^{-8}$ M. Thus the BKA has a binding affinity between those of CAT and ATR. IsoBKA exhibits similar binding characteristics as BKA, however, the affinity of iso-BKA is about 10 times lower as judged from competitive binding measurements [150].

These binding results of the inhibitors of the AAC can be accommodated with the SBCGP mechanism by assuming that in intact BHM nearly all AAC are in the c-state driven by the endogenous ADP and ATP and thus in a non-binding state for BKA. By added nucleotides these carrier sites can switch to the m-state where they are trapped by BKA binding. If CAT is added, the AAC remains in the c-state also after breakage of mitochondria. This explanation is at variance to the model proposed by Lauquin and Vignais [168] who postulated that BKA acts on ADP or ATR from an allosteric site, different from the nucleotide and from the CAT binding site.

7.5. Interactions between BKA, iso-BKA and CAT, ATR

BKA was early shown to remove ^{35}S-ATR bound to depleted BHM [180]. This removal was found to require catalytic amounts of ADP with a surprisingly low K_M of only 1.2 μM ADP. This can be explained according to the single reorienting binding site, where BKA removes ATR from its binding site only by intervention of ADP which first displaces ATR allowing the site to reorient into the m-state where it is trapped by BKA. Titrations showed that the removal of ATR by BKA is uncompetitive. In contrast ^{35}S-CAT binding to depleted BHM was resistant to BKA [175]. When BKA was added first without ADP, ^{35}S-CAT binding was partially and with ADP nearly total prevented. Applying ^{14}C-acetyl-CAT and ^{3}H-BKA [178] to rat heat mitochondria, ^{14}C-acetyl-CAT was not removed by BKA although ^{3}H-BKA was bound. When first added, twice as much ^{3}H-BKA was bound and subsequently a third ^{14}C-acetyl-CAT was bound without lowering ^{3}H-BKA binding. These results were interpreted [178] to show that BKA and CAT bind to different sites. However the interpretation ignores the unspecific uptake of BKA into mitochondria [150, 168]. In the depleted BHM preparation a clear prevention of ^{3}H-BKA binding by ^{35}S-CAT binding was shown [150]. BKA could remove ^{35}S-ATR but not ^{35}S-CAT [150]. Importantly, both the removal and prevention are strongly dependent on the presence of ADP. BKA alone prevents only about half and with ADP more than 90% of ^{35}S-ATR or ^{35}S-CAT binding. In titrations the activation constant for ADP was about 1 μM. In titrations with ^{3}H-BKA [150] the replacement of ^{35}S-ATR, amounted to approximately ΔATR/ΔBKA = −1, providing evidence for an equal number of BKA and ATR binding sites. Using ^{3}H-iso-BKA the reversed reaction i.e. displacement by ^{35}S-CAT could be demonstrated [150] with a ratio of Δiso-BKA/ΔCAT = 0.7. Probably, some of the displaced iso-BKA was redistributed into the membrane. In both cases the complete replacements are dependent on ADP. Based on these results the hierarchy of binding affinity is CAT > BKA > ATR > iso-BKA.

The two crucial results, the mutual displacements and replacements of the inhibitors and the dependence on ADP agree with postulates of the SBCGP, thus supporting this mechanism. Deviations from 1:1 stoichiometry can be explained by unspecific binding of BKA and of ADP to leaky membranes through which ADP can be recycled. The results do not support an allosteric fixation of ADP by BKA [8,168,178]. Further the data are not consistent with the contention [8,168,178] that BKA and ATR (CAT) bind to different sites. In this respect the finding that BKA binds to the AAC only from the m-side [153] is in agreement with the reported interactions between BKA, ATR and CAT.

7.6. Binding to the isolated and reconstituted AAC

The isolated and reconstituted AAC provided a platform for studying ligand binding independently from interferences in mitochondria. In the initially isolated CAT–AAC complex the bound ^{35}S-CAT could be slowly replaced by unlabelled CAT [183], indicating that binding is less tight than in mitochondria. ATR replaced only 30% and excess BKA required ADP for replacing CAT to 70%. In the isolated ^{3}H-BKA–AAC complex CAT was able to displace ^{3}H-BKA only when ADP was added [184]. The specificity for this catalytic nucleotide effect agreed with the high specificity observed for the transport. The isologous replacement of ^{3}H-BKA by BKA did not require ADP. In the unliganded carrier isolated with LAPAO the amount and affinity of CAT binding ($K_D = 0.31$ μM) and of BKA binding (1.5 μM) was much lower [185,186]. Binding affinity of ADP and ATP is so low to the soluble AAC (estimated $K_D > 0.1$ mM) that it cannot be measured even at high protein concentration. More binding results to the isolated AAC using fluorescent derivatives are reviewed in Chapter 9.2.

On incorporation of AAC into vesicles the amount and the affinity of ^{3}H-CAT and ^{3}H-BKA binding are dramatically enhanced ($K_D = 13$ nM for CAT, 100 nM for BKA) nearly to the level in mitochondria [186]. In this system the dependence of the inhibitors binding on ADP or ATP and their mutual displacements could be most convincingly demonstrated. Nearly no BKA and little CAT bind unless ADP is added. Isologous replacements of the inhibitors were complete but heterologous replacements required ADP and reached about 70%. Intricate combinations of ligands showed that in the reconstituted AAC binding of BKA and CAT are exclusive and provide no evidence for a ternary BKA–ADP–AAC complex [186]. CAT and BKA binding indicate the directionality of incorporated AAC, by showing a variable outside location of the c- or m-state as to whether ADP is included in the vesicle formation. Since CAT is not permeating, it can only reach the AAC portion with the c-side outside, whereas BKA can bind to both m-side populations. More inner sites resist CAT binding on prior BKA addition, indicating less c-side outside. In both cases maximum binding requires ADP for the conversion to the c- or m-state. The interconversion rates are biphasic, the slow portion possibly reflecting imperfect incorporation into unilamellar vesicles, as shown by the reconstitution. All these results thus support the SBCGP model also in the reconstituted system [186].

7.7. Non transportable nucleotide derivatives and analogues

Nucleotide analogues may be bound to the carrier sites, without being transported. But a clear differentiation of binding from a slow uptake has rarely been made. These compounds may inhibit transport of ADP or ATP depending on their binding strength. Binding versus transport activity data can be rationalised within the “ITF” mechanism [187] reviewed below. The apparent binding strength is the difference of the intrinsic binding energy and the conformational change energy in the AAC. If the ligand structure does not permit transport, i.e. is not tolerated in the transition state, the intrinsic binding energy will prevail. At variance, with transported substrates such as ADP and ATP the high intrinsic binding energy is largely consumed by the induction of the transition state, resulting in low apparent binding strength. Thus the moderate binding strength of most analogues indicate a moderate fit to the ground state and a poor or no fit to the transition state due to disturbance by the substituted groups or configuration restraint.

In the first binding study of analogues based on the displacement of ^{14}C-ADP [166], binding and exchange were not distinguished. Derivatives with varied phosphate position or ribose substitution (ADP-3′P, ADP-ribose) were found to be inactive. ATP-5′-P (“tetra-ATP”), AMPPCP were also active as a transported substrate. Among nucleotides with other bases only GDP and ITP were weak binding competitors. Among the series of derivatives synthesised by the group of Schlimme and Schafer [188], 1-*N*-oxides of ADP and ATP were weak competitors. 8′Br-ATP was the first example of an analogue with a higher binding affinity but no transport activity [189]. Oxidative cleavage of the ribose ring of ADP and ATP [190] produced weak inhibitors with $K_I = 10^{-4}$ M, but the reported data do not allow to

exclude a slow transport. Also 2-amino-ADP seems to bind but not inhibit [191]. Dinitro- and trinitrophenol substituted ATP were not transported [192] but are effective inhibitors [193] of transport with K_I of 2 and 1 μM. 3′*O*-dimethylamino-naphthoyl-(DAN-) esters of AMP and ADP bind to AAC in mitochondria but much stronger to SMP [194,195] with K_I of 12 and 8 μM, exhibiting a large fluorescence signal on binding only to SMP (see Chapter 9.2). The DAN-derivatives are minimally transported in mitochondria. 3-*O*′-naphthoyl-ADP and -ATP (N-ADP, N-ATP) are also good inhibitors binding both from the c- and m-side and appear not to be transported [196]. Caged (nitrophenyl-ethyl) ADP or ATP are good ligands to the AAC [93,96] as shown by the in situ hydrolysis, different from their binding to ATPases [197]. ^{1}H-NMR measurements show that caged ATP binds stronger than ATP [198]. In conclusion, the large substituent size seems to be the reason that most of these derivatives are non-transportable.

Another factor influencing binding is the intramolecular mobility of the nucleotide [8,199]. *Anti*- and *syn*-configuration at the glycosidic bond appear to be relevant for transport and binding. Substitution with Br or azido at the 8-position of ADP or ATP [189,200] favour the *syn*-conformers which are poorly bound and not transported [199]. However, ADP and ATP being preferentially *anti*-conformers may bind in this conformation. The *anti*-configuration can be stabilised in 2-azido-ADP or -ATP that are good inhibitors of transport (K_I=8 μM) [201] and claimed to be not transported although the evidence is not entirely convincing. At any rate, the results indicated that flexibility between *anti*- and *syn*-conformers is necessary for transport. Covalent fixation in the *anti*-conformation by bridging 2-C with 4-C′ still allowed binding but was stated to block transport [199].

Interestingly, ATP analogous were generated in vivo from drugs of the biphosphonate group by converting the phosphate moiety of ATP to β,γ dichloro-methylene tri-phosphate [202] or by substituting isopentenyl to the γ-phosphate [203]. These reactions were catalysed by aminoacyl tRNA synthetase. Both compounds are inhibitors of ADP/ATP exchange and thus were suggested to enact apoptotic cell death.

Employing ^{1}H-NMR, nucleotide binding to BHM incubated in D_2O was studied [198]. The binding constants were derived from relaxation times which give the off rates. With the non-hydrolysable AMPPCP a rapid binding equilibration was demonstrated with an off rate more than 1000 times higher than the transport rate. According to transient nuclear Overhauser effect measurements (TRNOR), the nucleotide could be shown to adopt the *anti*-conformation on binding, in agreement with conclusions from 2-azido-ADP binding [201]. ^{1}H-NMR line broadening showed that AMP also binds to the AAC from the c-side although it is not transported. By comparison with other nucleotide monophosphates the relative binding strengths were evaluated to show that GMP, dAMP, FOMP and IMP also exhibit some binding strength, pyrimidine nucleotides are virtually non-binding.

8. Physical and chemical probes of AAC structure

8.1. Fluorescent nucleotides and derivatives

The high selectivity of the AAC for the adenine nucleotides is reflected also in the limited choice of fluorescent nucleotide derivatives to be used as probes for monitoring binding and possibly reorientation of binding sites. Well propagated highly fluorescent derivatives of nucleotides such as etheno-adenine nucleotides do not bind to the AAC [204]. Formycin nucleotides (FoDP, FoTP), with a base very similar to adenine, are weakly fluorescent. They are transported by AAC with 15% of the ADP rate [2]. Apart from the low fluorescence yield, a disadvantage is the overlap with the fluorescence of endogenous tryptophane, which allows only approximate measurements formycin nucleotide fluorescence in mitochondria [204], whereas with the isolated AAC, FoDP could be directly used [205]. To overcome the obstacles in mitochondria, secondary high yield fluorescence probes were introduced which received and enhanced the signal from FoDP by resonance transfer [204]. For this purpose, anthracene derivatives (vinyl-anthracene (VA) and anthracene maleimide (AM)) were absorbed into the mitochondrial membrane or covalently attached to SH groups. Thus a convenient fluorescent recording of FoDP binding was possible. The fluorescence is quenched by ADP at concentrations which differ widely according to whether ADP is added before or after FoDP (K_I=0.2 μM versus 40 μM). This can be explained by the reorienting carrier mechanism assuming that the fluorescence indicates m-side location of FoDP: external ADP added before FoDP, even in small amounts, are sufficient to switch AAC sites to the matrix side rendering them unavailable to the 5 times slower FoDP, whereas when first FoDP is allowed to go inside, higher amounts of external ADP are required to remove FoDP by exchange. The m-side localisation of the fluorescent FoDP is compatible with the probable attachment of anthracene maleimide to a matrix oriented-SH (C56) group. The complete quenching of FoDP fluorescence by BKA shows that BKA displaces FoDP from the AAC in the matrix, supporting the view that BKA binds to the same site as ADP [153]. Particular attention was paid to measure the fluorescence decay time which on binding was prolonged from 0.95 to 1.95 ns indicating an unpolar environment of the formycin binding pocket. A depolarisation decay time >15 ns shows that the bound FoDP is immobile at this time period.

Introducing fluorescent groups to the ribose of adenine nucleotides provided some very useful probes for the AAC as first noted by Schäfer and Onur [194]. From the comparison of various substituants carrying naphthoyl- and benzyl-groups, 1,5-dimethylamino-naphthoyl-3′*O*-AMP, -ADP and -ATP (DAN-AMP, -ADP and -ATP) emerged as the most useful fluorescent probes to monitor binding to the AAC [192, 206]. Being largely quenched in solution, on binding they emitted a large and conveniently located fluorescence with a maximum at 520 nm [192,206]. Naphtoyl-3′*O*-ADP and -ATP were also extensively used [196], however opposite to the DAN-AdN, the relatively weak fluorescence of the free probe was partially quenched on binding and thus could only be monitored as a fluorescence difference.

DAN-AdN fluorescence depended on binding to the inner (m-) side of the membrane: on addition to mitochondria from heart only a small or from liver no fluorescence signal was observed [192], whereas with sonicated patricles (SMP) the DAN-derivatives rapidly displayed a strong fluorescence signal which was totally reversed by BKA [195], indicating that the fluorescence reflects specific binding to the AAC. The apparent affinity was high with K_D=2 μM for DAN-AMP and 1.5 μM for DAN-ADP. CAT could reverse fluorescence slowly and not more than by half and only when ADP or ATP was present. ADP or ATP alone could only partially inhibit or reverse the fluorescence requiring surprisingly high concentration of >2 mM. In conclusion, the fluorescence of the DAN-nucleotides corresponds to m-side specific binding and permits recording the reorientation of binding sites under the influence of ADP and the inhibitors BKA and CAT between the c- and m-states.

DAN-nucleotides are competitive inhibitors of the ADP transport both in mitochondria with a K_I=12 μM and in SMP with K_I=2 μM, indicating that DAN-nucleotides also bind from the c-side to the AAC [192]. By introducing radio-labelled DAN-nucleotides, binding was measured parallel to fluorescence [195]. In SMP a total of 2.8 μMol/g protein ^{3}H-DAN-ADP were bound and 1.6 μMol/g prot. were removed by BKA which should represent binding to the AAC sites. The difference of 1.2 μMol/g of non-fluorescent binding is probably to the ATP synthase. The same base level was obtained in SMP prepared from CAT loaded mitochondria. The parallel determination of CAT sensitive binding of ^{3}H-BKA gave the same value (1.6 μMol/g protein), showing a 1:1 binding capacity for BKA and DAN-nucleotides.

Also in mitochondria distinct although weaker binding of ^{3}H-DAN-ADP could be measured, however, without a fluorescence response. The $K_D \approx 15$ μM was close to the K_I. From the total binding of 0.8 μM/g prot. CAT could remove only about 25%, but when added before ^{3}H-DAN-ADP, CAT decreased binding by 70% and BKA by 75%. Obviously a small portion (20%) of ^{3}H-DAN-AMP is bound to the c-side and the larger portion is translocated inside where it is mostly replaced at the AAC by endogenous ADP and ATP leaving DAN-AMP in the free non-fluorescent state. This is supported by the same experiments with liver mitochondria. ADP addition reduced binding not only to AAC but also by driving out ^{3}H-DAN-ADP from mitochondria by exchange.

The different interactions of DAN-ADP with the c- and m-state of the AAC can be explained with a single but flexible binding site. On binding to the m-state the DAN group contributes to the affinity by binding to a partially hydrophobic niche signalled by the strong fluorescence, whereas in the c-state by the same criteria the DAN moiety seems not to participate in binding. In the case of DAN-AMP the additional binding energy even overrides the usually poor binding of AMP as compared to ADP or ATP. Thus DAN-nucleotides provided the most persuasive illustration for the difference of the binding center interfaces between the in and out state, as postulated in the original concept of the single site reorienting carrier [2,3]. Further DAN-nucleotides allowed to visualise the distribution and the transition of the AAC between the c- and m-state in accordance with the reorienting (alternating) carrier mechanism [206,207].

The more easily synthesised fluorescent 3-*O*′-naphthoyl (N-) derivatives show a different interaction pattern with the AAC [192,196,208]. Opposite to DAN-nucleotides, free N-ADP and N-ATP have exhibit fluorescence in solution which is decreased on binding. Probably due to the stronger hydrophobicity of the unsubstituted N- as compared to the DAN-group, N-ADP and N-ATP fluoresce without binding. Therefore not the binding but only the displacement of bound N-AdN by CAT or BKA producing a fluorescence increase can be recorded [196,208]. Further the low fluorescence yield results in a lower signal to noise ratio as compared to DAN-nucleotides. N-ADP was found to bind to AAC both in mitochondria and in SMP as monitored by the fluorescence increase by CAT and BKA [208]. In mitochondria CAT alone seemed to release most of N-ADP whereas the BKA induced release depended largely on ADP. In SMP the fluorescence increase, reflecting N-ADP displacement, reached maximum at 2.0 μMol BKA/g prot., but was small with CAT. Thus the responses of N-ADP binding to inhibitors were similar as with DAN-nucleotides but, at variance, N-ADP appeared to have the same affinity to mitochondria and to SMP, i.e. to c- and m-state. Using radio-labelled ^{14}C–N-ADP maximum displacement of N-ADP binding by CAT was determined at 1.3 μMol CAT/g protein in mitochondria which agrees with the parallel measured value of ^{14}C-acetyl-CAT binding [196]. In SMP the AAC attributable ^{14}C–N-ADP binding was 1.2 μMol/g prot.

These results were interpreted [208] in terms of the "CATR"- and "BA"-state of the binding site, including an ADP requirement for the transition between these states. Thus the authors [208] essentially implement and support the c- and m-state binding site change and their catalysis by ADP [153,209], however with the specification that the binding sites for CAT and BKA are not shared with the nucleotide site. As an inconsistency with the single site model the removal of internal N-ADP in the presence of CAT by external ADP and vice versa in SMP in the presence of BKA was quoted [208]. Instead two anti-cooperative sites in AAC were envisaged which are out- and inside located in line with the simultaneous kinetic mechanism, reviewed in Chapter 4.4. However, it may be argued that the partially leaky membranes permit ADP to diffuse inside and thus to switch the carrier sites to the outside. Another explanation would be that, similarly to DAN-ADP, N-ADP may catalyse a slow c- to m-transition.

A fluorescent derivative of ATR, 6-*O*-dansyl-aminobutyryl-ATR supplements these results [210]. High fluorescence appears on binding and can be largely suppressed by CAT or by BKA only in conjunction with ADP. Opposite to the DAN-nucleotides, this compound provides a sensitive fluorescent probe specific for the c-state of the AAC.

8.2. Fluorescent ligands for the isolated AAC

Binding of DAN-AMP, -ADP, -ATP to the solubilised AAC produced fluorescence, similar as in SMP, only in the m-state [207]. The absorption of DAN-nucleotides to the detergents induced a fluorescence which was subtracted from the signal due to AAC binding, after a careful calibration of the fluorescence yield. The fluorescence changes of DAN-AMP, -ADP, -ATP in response to ADP, CAT and BKA additions were recorded and combined with binding measurements of ^{3}H-DAN-AdN, ^{3}H-BKA and ^{3}H-CAT. The decrease of ^{3}H-DAN-AMP binding corresponded approximately 1:1 to the replacement by ^{3}H-BKA or ^{3}H-CAT in conjunction with ADP. The equal binding level is in agreement with the assumption that these diverse ligands bind to the same adaptive site. An additional application of DAN-nucleotides is the convenient determination of the a priori distribution of the solubilised AAC between the c- and m-state, based on the fluorescence response. Thus, for example, salts (Na-sulfate, NaCl) were found to shift the AAC more to the m-state.

The fluorescence response of N-ATP binding was also probed with the solubilised AAC [211]. A total of two, one high and one low affinity sites for N-ATP per one CAT binding site was extrapolated from the titrations whereas the BKA sensitive N-ATP binding has only low affinity sites. However, these fluorescence titrations seem less reliable than the binding measurement with ^{14}C–N-ATP in mitochondria, where a displacement ratio N-ATP/CAT = 1.2 was directly measured [196]. The different affinities were interpreted to reflect anti-cooperativity between two binding sites. No correction for the fluorescence of N-ATP absorbed to the detergent is reported although it may strongly influence the calibrations. Another factor for the discrepancies of binding numbers might be that analogues to DAN-AdN N-AdN also have a different fluorescence yield on binding to the c-state or m-state and the two affinities may reflect binding to the c- and m-states.

The binding of ^{3}H-DAN-AdN to the isolated AAC has some peculiarities seemingly at odds with the AAC binding site [207]. DAN-AMP binding is highly sensitive to BKA but its displacement does not conform to the hierarchy of transportable substrate. High concentrations (>5 mM) of ADP and ATP are required for the removal of DAN-AMP, in contrast to the μM concentration needed to activate the fluorescence by shifting the AAC from the c- to m-state. ATP is more effective in the removal whereas ADP is more effective in the activation. Poorly transported GDP and GTP and salts, such as Na_2SO_4, pyrophosphate are effective competitors with DAN nucleotide binding. These findings and that DAN-AMP has even a higher affinity than DAN-ATP and DAN-ADP argue that the binding site assumes a state close to the abortive m′-state induced by BKA with little affinity for ADP and ATP [212] (see Chapter 16.1).

It can be concluded that the various models based on the work of N-ATP binding to the AAC [196,205,211,8], such as high and low affinity sites, separate sites for the inhibitors and nucleotides, tetrameric AAC, are not sufficiently founded to revise the reorienting single site (SBCGP) model. Similar reservations can be applied to the conclusions drawn from FTP binding to the isolated AAC [205], where 4 classes of binding sites for FTP are claimed. Here binding is further complicated by the ability of FTP as a transportable substrate to catalyse switching between the c- to m-states although less efficiently than ATP. This may explain the biphasic binding competition between FTP and ATP and would argue against the proposed model [205] that employs different sites for inhibitors and nucleotides interacting in

an anti-cooperatively manner. The contrasting BKA effects on FTP and N-ADP binding are compatible with the SBCGP mechanism.

8.3. Intrinsic fluorescence of AAC

Intrinsic fluorescence based on constituent tryptophanes was extensively used to probe the interaction of ligands with the AAC [213–215]. Fluorescence decreased on addition of ATP and CAT and increased by BKA. Based on titrations only about 4 nMol/CAT/mg protein bind (as compared to 14 for pure AAC), similar as with N- and DAN-AdN, reflecting the low yield on isolation of "free" AAC. In the eye of the reviewer, the response of intrinsic fluorescence to CAT, BKA and ATP basically supports the SBCGP model. A biphasic kinetic response to ATP at low temperature is interpreted to show segregated rapid binding and slower translocation. The fact that sub-stoichiometric amounts of ATP can convert all AAC into the high fluorescence form shows that the conformations can exist independent of ATP binding, in agreement with c–m-state model. Also in studies on yeast AAC2 mutants [216] with single eliminations of Trp for elucidating the individual contributions of the three Trp, the fluorescence response to the various ligands could be rationalised within the framework of the c–m-state mechanism (called here C–C*). Based on the intrinsic fluorescence change, an influence of the detergent type on the initial state of the isolated free AAC was noted: in LAPAO the c-state, in CHAPS the m-state is preferred [213]. In dodecyl-maltoside yAAC2 appears to exist mostly in the c-state [217].

8.4. Probing the AAC in mitochondria with spin labels

Spin labels (SL) attached to ligands of the AAC were primarily used to explore mobilities. In the first applications SL were attached to various positions at long chain acyl groups linked to ATR and CoA. With SL-acyl-ATR essentially the mobility of the acyl chain in the lipid environment of the AAC in mitochondria were probed [218]. Short chain SL-acyl-ATR was less mobile on binding to the AAC in mitochondria than long chain acyl-ATR. The long chain acyl groups were less mobile than free SL fatty acids indicating interactions of the acyl chains with the AAC similar as previously seen with SL-acyl-CoA [219]. However the immobilisation of long chain acyl appears to be too small for protein interaction and thus a model was invoked where the AAC in the center of the membrane has a lipid niche or is divided into two subunits in tandem across the membrane in the ATR-state.

Positioning the spin label at the glucose moiety of CAT should give information on the mobility of CAT on binding to the AAC, or on the mobility of the CAT–AAC complex in the membrane [220]. Different from SL-acyl-ATR, with SL-CAT exclusive binding to the AAC was observed without a background as monitored by the hyperfine splitting (HFS). Two HFS components were found, one major with extreme HFS and another smaller with lower HFS value. The major component reflects extreme rigidity, with a reorientation time $\tau < 10^7\ s^{-1}$, and the other $\tau \approx 10^8\ s^{-1}$. This surpassed the hyperfine splitting measured in solubilised-frozen SL-CAT–AAC complex, or frozen free SL-CAT at −100°. A highly polar environment of the NO-group was reflected in the very narrow line width. Anisotropic motion of the SL-CAT–AAC complex or two binding states of SL-CAT were proposed to explain the lower HFS component. In continuation of these results, saturation transfer ESR studies with SL-CAT allowed to extend the measurable time range [221] and to determine in mitochondria the rotational time τ of the AAC molecule to 20 μs (0°) and 2 μs (30°). In reconstituted vesicle τ was 1–5 μs which can be accommodated with a size of the AAC > 60 kDa in line with an AAC dimer.

8.5. Covalent modifications and probes

SH reagents turned out to be most interesting and revealing probes for the dynamic and structure of the AAC. Initially ADP/ATP exchange appeared to be insensitive to SH reagents, such as mercurials or maleimide (NEM), in contrast to the high sensibility of the P_i carrier towards these reagents [222–225]. This changed with the significant observation that NEM requires the presence of ADP or ATP for becoming an inhibitor of ADP/ATP transport in mitochondria [69]. ADP enhanced ^{14}C-NEM incorporation into mitochondria and ATR inhibited this effect [226]. Vice versa once NEM had reacted, ^{35}S-ATR binding was irreversibly inhibited [227]. Opposite to ADP, uncoupling counteracted the inhibition of transport and ATR incorporation by NEM.

The important finding that ADP induces specific configuration changes of the mitochondrial structure which are reversed by ATR [228] and which are reflected in turbidity changes in a suspension of mitochondria [229], enabled to record the cumulative influence of NEM and ADP on the turbidity [69]. These results suggested that the ADP induced configuration change of the mitochondrial membrane was involved in unmasking SH groups. The turbidity change was further explored under the view of AAC involvement [230] and finally shown to reflect the reorientation of the AAC binding center [68] (see Chapter 8,7). The kinetics of the NEM induced turbidity increase was correlated to the increase of ^{3}H-ADP binding and the simultaneous decrease of ^{35}S-CAT binding capacity. On this basis the NEM effect could be consistently integrated in the SBCGP by concluding that NEM reacts with the AAC only in the m-state and thus required ADP for catalysing the transition from the c- into m-state [2,231], rather than explained by an unspecific ADP induced "allosteric" change. The unmasking of the SH group in the m-state provided the first evidence for the postulated conformation changes between both states. This superseded the seemingly obvious conclusion that the different reactivity toward some amino acid reagents is evidence for different binding sites for ATR (CAT) and BKA [232]. Interestingly, the NEM effect, as monitored by the inhibition of ^{35}S-ATR binding, is inhibited by uncoupling of mitochondria [233]. This agrees with the evidence from the turbidity changes that transition to the m-state requires coupled mitochondria [68,230].

Whereas NEM is a penetrating SH reagent, the derivative eosin maleimide (EMA) is poorly membrane permeable. It was introduced to measure rotational diffusion of the AAC in mitochondria [234] but then realized to react with the AAC only after access to the matrix side in broken mitochondria or sonic particles [235]. Similarly to NEM, EMA incorporation is inhibited by ATR or CAT. Phenylglyoxal (PheG) and butanedione were used to probe the involvement of arginines in binding to the AAC [231,232]. These reagents are membrane permeable but require higher concentration than NEM to inhibit the transport and to influence CAT and BKA binding. Basically similar to NEM, PheG was found to inhibit binding of ^{3}H-CAT but not of ^{3}H-BKA [231], again supporting the concept that the conformation changes associated with the reorientation unmask residues involved with the binding center. At variance Block et al. [232] reported that butanedione and also PheG inhibit both CAT and BKA binding. At prolonged incubation time and high concentration, the effect of these reagents become less selective and may affect Arg or other groups also in the c-state of the AAC. In fact, determination of ^{14}C-PheG incorporation into the AAC showed that for the inactivation of ATR binding only 1 PheG per AAC dimer but for the inhibition of BKA binding, 2 PheG were incorporated [237]. Also by UV irradiation a differential inactivation of ATR versus BKA binding was observed [238] which was enhanced by ADP and interpreted as evidence for segregated sites for ATR and BKA binding. However, applying the same reasoning as for the amino acid reagents, the differential inactivation by UV does not contradict the single site c–m-state conformational activation concept [231].

8.6. Probing SH group in the isolated AAC

A definite assignment of the ADP stimulated NEM incorporation to the AAC was achieved by isolation of the ^{3}H-NEM labelled protein [239].

It also enabled to quantify the amount of NEM incorporation. In SDS gels of BHM the ADP stimulated ^{3}H-NEM incorporation was concentrated against a broad background in a 30 kDa fraction and was fully suppressed by CAT but not by BKA. With CAT or by uncoupling the incorporation into 30 kDa band was largely prevented. Double labelling permitted [239] to differentiate the ADP or ADP+BKA stimulated ^{3}H-NEM incorporation from the ^{14}C-NEM labelled background fractions. By chromatography Triton solubilised AAC was isolated with a low background and high ^{3}H/^{14}C-NEM ratio and shown to contain about 1 NEM/30 kDa protein.

The reactivity of SH groups of the isolated AAC was studied in the isolated CAT-, ATR- and BKA–AAC complexes [226]. Although AAC contains 4 Cys, the incorporation of only one NEM or DTNB/30 kDa was measured by various methods. The isolated ATR–AAC complex turned out to be most useful for studying ligand driven c–m-state conversions, since it can be isolated in a comparatively stable state protected by ATR. Different from isolated "free" AAC, the ATR–AAC preparation has a high purity and homogenous c-state population. Because of the lower affinity, ATR can be displaced and the AAC converted into other states. Under the premise that DTNB reacting with the SH group traps the AAC in the m-state, recording of the absorbance increase of DTNB was used as a convenient and precise method to elucidate the role of nucleotides in the c- to m-state transition [226]. Very small concentration of ADP or ATP, with a K_M for ADP of 0.5 μM, for ATP of 3 μM, were able to catalyse the SH-unmasking, BKA alone was inactive. Similar as in transport, the catalytic effect was highly specific; GDP, GTP, ITP, etc did not initiate the reaction but dADP and dATP activated with a K_M 10 times higher than ADP and ATP. The results also excluded a ternary complex of AAC with BKA and ADP.

Pyrenyl-NEM (PYM) was used as a fluorescent SH reagent to monitor the influence of phospholipids (PL) on the access to the SH–group in the isolated AAC [240]. By competition the hydrophobic PYM was shown to react with the same site (C56) as NEM. Starting with the ATR–AAC complex, addition of ADP activated the reaction of PYM as monitored by fluorescence increase. After phospholipase treatment the reaction became largely ADP independent. In contrast cardiolipin addition at >70 mol/mol AAC inhibited completely the ADP stimulated reaction, even when the AAC was fixed in the m-state by addition of BKA, where otherwise the access to C56 is most favoured. The effect is quite specific, other PL inhibiting only up to 70% as compared to 99% inhibition by CL. Obviously CL does not act by shifting the AAC into the c-state but occludes C56 by virtue of its specific structure. Since the isolated ATR–AAC contains bound CL, the additional CL may bind to additional positive charges thus encasing the m-side of the AAC.

8.7. The relation of AAC to the macroscopic inner membrane structure

Unique conformation changes of the mitochondrial cristae reticulum between "orthodox" and "condensed" conformation were first attributed to de-energised versus energised states of the inner membrane [241,242]. Weber and Blair [228,243] made the observation that ADP alone could induce a transition to the condensed conformation, independently of energy transfer, as judged from the insensitivity to oligomycin. Features of the condensation such as, requirement of only μM ADP or inhibition by ATR were evidence for the involvement of the AAC [229]. These transitions could be conveniently studied by recording the absorbance increase in a mitochondrial suspension, reflecting the change from the relaxed to the condensed state of the matrix. Notably, these reconfigurations appear not to be accompanied by matrix volume changes [244,245]. The nucleotide specificity coincided with that for transport [230]. In contrast to ATR, BKA was found to increase the absorbance still further as ADP thus for the first time demonstrating opposite effects of ATR and BKA on the AAC [246,247]. The absorbance changes were quantitatively correlated [68] with the binding of ^{14}C-ADP, showing that the absorbance increase is nearly proportioned to the ADP binding, both alone and when enhanced by BKA. By titrating the absorbance change, K_D=4 μM for ADP, 3 μM for ATP and 0.03 μM for ATR were determined. Interestingly, BKA added after ADP causes a rapid transient decrease of absorbance with a half time strongly increasing with pH. No transient decrease is observed when BKA is added before ADP. ^{14}C-ADP binding is also transiently decreased indicating that externally added BKA may transiently and loosely bind to the c-state binding site. The rate V of absorbance increase by BKA has an extremely steep pH dependence and was evaluated to follow the relation $\Delta \log V/\Delta pH = -2.4$. This relation provided quantitative evidence that the concentration of the hydrophobic form $BKAH_3$, required for the permeation through the membrane, is rate limiting [68], in agreement with the evidence for BKA binding at the m-side [153]. In accordance, the transient weak binding of BKA to the c-side, recorded by the rapid initial absorbance decrease, is largely pH independent.

The role of the AAC in these macroscopic configuration changes is intriguing. Two groups assumed a general conformation change on binding ADP and ATR without specifying a mechanism [228,230]. Stoner and Sirak interpreted [247] the pH effect to show accumulation of BKA in the membrane and changes of the phospholipid organisation and thus indirectly affecting the AAC. An entirely different interpretation was based on the reorientation mechanism with the following postulates [248]: (a) The structural changes reflect the distribution of the AAC between c- and m-state, independently of whether or which ligand is bound to the AAC. (b) The absorbance increase reflects binding sites accumulation in the m-state and vice versa in the c-state. (c) Mobilising ligands such as ADP and ATP enable redistribution of the carrier between the c- and m-state. Immobilising ligands (ATR, CAT, BKA) produce one sided distribution, in either the c- or m-state.

Without added ligands of AAC, mitochondria are in a low absorbance "relaxed" state, where most binding sites are trapped in the free form on the c-side after being translocated by the endogenous ADP or ATP and release of nucleotides. ADP addition partially reorients the sites to the inside reflected in a partial contraction. ATR and CAT fix the AAC in the extreme c-state with low absorbance and BKA fixes the AAC in the extreme m-state with highest absorbance in the contracted state. All sites are trapped by BKA only after being mobilised by external ADP. BKA^{3-} is considered the binding form, as confirmed by absence of pH dependence of BKA effects on sonic particles [73,78,150]. Thus the absorbance change of the mitochondrial suspension provides a unique visualisation of the extent and rate of the redistribution of the AAC between the c- and m-state.

The molecular basis of these morphologic changes is still a matter of conjectures. They can be visualised to result from the expansion of one membrane surface relative to the opposite surface; in the c-state the outer face is larger than the inner face and in the m-state vice versa, thus enforcing a more vesicular (relaxed state) or vacuolar (condensed state) configuration of the matrix [68]. As a possible driving source, excess surface charges were proposed for the expansion. On this line the curious initial rapid decrease of the absorbance on BKA addition [68,249] may be due to the absorption of BKA^{3-} at the outer membranes surface rather than a weak transient binding of BKA^{3-} to the c-side of the AAC. It was also discussed [68] that due to the drastic conformation change of the AAC structure between c- and m-state, the AAC molecule may occupy more space either on the c- or on the m-surface of the membrane and, combined with the high density of AAC corresponding up to 12% the inner membrane protein, this molecular change translates into macroscopic changes. This version is supported by the finding that the AAC is the major protein exposed at the c-side membranes as shown by to lactoperoxidase-iodination [250]. The crystal structure of the CAT–AAC complex with the wide c-side funnel seems further to vindicate this speculation. Revisiting this discussion on the basis of 3D tomography of the mitochondrial structure, Mannella [251] suggested that the surface charge changes are caused by a rearrangement of

cardiolipin associated with the c–m transition of AAC since the AAC recruits a high amount of cardiolipin molecules [252,253].

9. Isolated AAC

9.1. The solubilisation and isolation of the intact AAC

The AAC from bovine heart mitochondria (bAAC1) was the first membrane transporter to be isolated in a non-denatured form [77,183,254]. The success was based on the high abundance of the AAC in these mitochondria and the use of the tightly binding inhibitor ^{35}S-CAT for tagging the AAC. The working hypothesis that CAT binding stabilises the AAC proved to be a crucial advantage for the isolation of an intact carrier. Dealing with a deeply embedded transmembrane protein, the solubilisation of the AAC required full disintegration of the membrane phospholipid. Whereas with mitochondria cholate had been the preferred detergent for solubilising membrane proteins, this and some other popular detergents failed to preserve the CAT binding. Non-ionic detergents were little used because their removal was difficult. Among the polyethylglycol (PEG) detergents, only those with an aromatic moiety such as Triton X100 were found to easily solubilise mitochondria in conjunction with high ionic strength. Under these conditions Triton X100 solubilised and preserved the CAT–AAC complex. In other work the branched alkyl detergent emulphogen was applied [176]. The more aggressive aminoxide detergents lauryl-aminoxide (LDAO) and laurylpropyl-aminoxide (LAPAO) were introduced [185] requiring less salt for solubilising the AAC. Attempts to purify the AAC by affinity chromatography with ADP or with ATR failed to produce intact AAC [183] or gave only a partially pure protein [176]. Other reports claiming purification without CAT binding, actually failed to isolate the AAC. In the procedure described by Egan and Lehninger [255] using the alkyl PEG Brij 56, in fact no solubilisation of AAC could be achieved [256]. SDS and affinity chromatography, applied for the isolation [257], abolish CAT binding and thus the criterion for intactness. Shertzer and Racker [80] reported purification and reconstitution using cholate and ammonium sulfate fractionation. To the contrary, the gel electrophoresis of this fraction showed a selective decrease of the 30 kDa content.

A great advance was the introduction of hydroxyapatite (HTS) as a first step for separating the AAC from the bulk of mitochondrial protein [183]. Bound ^{35}S-CAT was the convenient marker for following the purification steps. An important pre-requisite was CAT or ATR binding for obtaining a high yield of AAC in the initial "break-through" fraction. Otherwise and particularly with ADP much less or only a small yield of AAC appeared in the break-through. During the subsequent further purification by sepharose chromatography most of the detergent and accompanying phospholipid were removed. In SDS gel a single band with a MW = 30 kDa was identified. Starting from BHM the CAT–bAAC1 complex was purified about 14 fold as based on the CAT/protein ratio. The ^{35}S-CAT content of 16 μMol/g prot. in the purified protein gives a MW ≈ 60 kDa [137, 256] corresponding to a dimer. With CAT binding of 2 μMol CAT/g prot. in BHM [3] and with a MW 60 kDa the AAC share amounts to about 12% of membrane protein and thus has the highest content of any BHM membrane protein. The abundance of the AAC also became evident by the finding that lactoperoxidase catalysed radio-iodination of the c-surface of inner membrane preparation of BHM primarily targeted a 29 kDa protein [258] estimated at 10% of total membrane protein and later identified as the AAC [250].

The AAC was also isolated and purified as the ^{3}H-BKA–bAAC1 complex [184] until the HTS stage, since the BKA–bAAC1 complex was too unstable to survive the subsequent sepharose chromatography. The amino composition of this product was nearly identical to the isolated CAT–bAAC1 providing direct evidence that CAT and BKA bind to the same protein. The greater sensitivity of BKA–bAAC1 to endogenous or exogenous proteases was demonstrated by the near complete (>98%) degradation of the 30 kDa protein by trypsin after 24 h, while CAT–bAAC1 is degraded only by 5%. On solubilisation without ligands the AAC was degraded in the extracts by endogenous proteases, to 50% within 3 min at 0° as measured by CAT binding [256]. The affinity of CAT binding was weaker in the solubilised protein than in mitochondria as shown by the ability of ADP or ATP to slowly displace the bound ^{35}S-CAT. As a consequence the isolated CAT–bAAC1 was destabilised by ADP and the protein was degraded with a half time of 6 hours.

The stability of various ligand–bAAC1 complexes in Triton X100 follows the sequence: CAT–AAC > ATR–AAC > BKA–AAC > AAC > ADP–AAC [184,185,256,259]. Without inhibitors the AAC stands out in its instability as compared to other major mitochondrial membrane proteins, as monitored by the 30 kDa band in SDS gels of Triton X100 solubilised mitochondrial membranes. Whereas the solubilised major components of cytochrome complexes and of ATP synthase are stable for hours, the 30 kDa band is rapidly decreased unless stabilised by prior addition of CAT. The AAC from bovine liver and kidney (bAAC2) turned out to be still more labile and barely stabilised by CAT [260].

In the cholate based extraction of mitochondria a major fraction called "structural protein" containing a 29 kDa band [261] is probable identical to denatured AAC. N-terminal acetyl-serine was also found in the sequence work of authentical AAC [49]. The same band was regarded as a constituent of ATP-synthase preparations [119] but was later eliminated in purer preparations.

Because of its lability, the AAC afforded an early test to grade detergents according to their efficacy in handling the intact AAC [259]. Weaker solubilisers, requiring high ion strengths, are C_{12} $E_{8,}$ $C_{13}E_{6-12}$ (Emulphogen), and dodecyl-maltoside. Good solubilisers requiring moderate ion strength were in the order of AAC stability: Triton X100 > lauroyl propyl aminoxide (LAPAO) > lauryl-aminoxide (LDAO) > (unstable) lauryl-betaine > octylglucoside > cholate. Thus non-ionic detergents were the least aggressive, and the stronger "dipole" aminoxide detergents preserved the CAT–AAC complex but were less tolerable towards BKA–AAC. The need for high ionic strength (>0.5 M NaCl or >0.1 M Na_2SO_4) for the solubilisation of the mitochondrial membrane was presumably caused by the high content of charged phospholipids, in particular of >10% cardiolipin in BHM.

9.2. AAC as a dimer

On isolation of the bovine CAT–bAAC1 complex [183] a general investigation of the composition, size and shape of the protein detergent micelle was undertaken. Further the question was addressed of whether AAC forms a dimer [262]. Initially a dimer was inferred from CAT binding amounting up to 18 μMol/g protein corresponding to a MW 56 000, suggesting that about one molecule of CAT binds to two AAC monomers. ^{35}S-CAT–bAAC1 complex detergent micelles labelled with ^{3}H-Triton X100 were separated from bulk Triton by sucrose gradient or gel filtration. The CAT–bAAC1 complex was monitored by the bound ^{35}S-CAT. 1.5 g Triton X100/g protein were measured to be associated with the AAC corresponding to 150 molecules Triton X100 per AAC dimer. The Stokes radius was determined by gel filtration to 53 Å for the "free" Triton micelles and 65 Å for the protein Triton micelle, equivalent to a diffusion coefficient $D = 3.43 \times 10^{-7}$ cm^2 s^{-1}. The same value was obtained from boundary broadening in analytical ultracentrifugation. Sedimentation coefficients were determined by two ultracentrifugation methods to S = 3.85 or 3.93×10^{-13} s. From these data and the specific weight of 0.85 g/ml a MW = 182 kDa for the AAC micelle was calculated. Independent sedimentation equilibrium measurements gave a MW = 172 kDa. The specific volume was determined by centrifugation in H_2O/D_2O gradients and agreed with that calculated from the composition of the mixed micelles. Subtracting the Triton and phospholipids, MW = 67 kDa (sed.-velocity) and 64 kDa (sed.-equilibrium)

respectively were calculated with the low figure having a smaller methods error.

Including measurements of the phospholipid content, the AAC was concluded to form a dimer in the isolated CAT–bAAC1 complex surrounded by 150 molecules Triton X100 and 16 "phosphate units" of phopholipids [262]. The combination of sedimentation, diffusion coefficient and MW allowed to determine the frictional ratio $f/f_o = 1.56$ indicating a strong deviation from a sphere. These data were modelled into an oblate elongated ellipsoid structure with the short axis at the two fold axis of the dimer surrounded by a detergent shell. Similar measurements with CAT–bAAC1 isolated using the aminoxide detergents LAPAO and LDAO gave Stokes radii of 44 Å and 46 Å and MW of 141 kDa and 130 kDa respectively [263], and for the protein share MW of 59.5 kDa and 60.4 kDa. The low density of these detergents leads to a broader error range for the specific volume and thus for the MW determination in particular with LDAO. When Triton X100 was replaced by SDS in the isolated AAC, a MW of 32 kDa was determined by gel filtration, and 29 kDa and 28.5 kDa by the two hydrodynamic methods in agreement with the expected monomer formation in SDS. It might be mentioned that applying the same hydrodynamic studies to the homologue uncoupling protein (UCP1) a dimer structure was also assigned to the isolated UCP1 [264]. Small angle neutron scattering measurements of the CAT–bAAC1 and BKA–bAAC1 complex isolated in LAPAO were performed [265], again providing evidence for the dimer structure of the solubilised bAAC. The detergent micelle contribution was eliminated by adding 10% D_2O to the solution. Depending on the assumed degree of D to H exchange in the protein MW 53 to 61 kDa were determined.

The topography of carriers in the membrane had been barely addressed in the literature, but required attention with the arrival of the dimer structure of the AAC. In a general essay [266] the principles governing the orientation of two subunits to each other and to the membrane were elucidated. Taking into account the protein–lipid interaction and an asymmetry of the lipid composition between the inner and outer membrane leaflet, simple calculations show that dimerisation of proteins in the membrane has an energy advantage over the monomer state and that the twofold axis of a dimer membrane protein must be perpendicular to the membrane. The formation of one translocation path at the twofold axis or of two separate paths in each subunit and the implications for the transport mechanism were discussed (see chapter 16).

Crosslinking of the isolated AAC with bifunctional lysine reagents produced protein with MW of 65 kDa in SDS gel [263]. The crosslink yield of 30% was independent of protein concentrations, showing intra-dimer crosslinking and providing independent evidence for at least a dimer state of the isolated CAT–AAC complex. Crosslinking of bAAC1 in the native membrane by Cu^{++}-phenanthroline forming S–S bridges [267] or by using bifunctional maleimides [268] leading to a covalent dimer involving the C56–C56 bridge are reviewed in Chapter 14.5. These results provide evidence for the existence of AAC as a dimer in mitochondria. Other members of the mitochondrial carrier family were also crosslinked showing their dimer state. Isolated UCP1 can be easily crosslinked by forming an S–S link between C304 near the C-terminal. The yield is 90% and independent of protein concentration. The isolated oxoglutarate carrier (OGC) could also be oxidatively crosslinked between C184 located in TMH4, indicating their proximity at the dimer interface [269].

Using native gel electrophoresis of mitochondria a dimer state of yeast yAAC2 was demonstrated [270]. Interestingly the MW was 80 kDa whereas a slightly lower MW was found for yAAC2 from a cardiolipin (CL) deficient mutant [271]. The difference probably reflected the retention of tightly bound CL in isolated yAAC2 from wt yeast, supporting the NMR data [272]. With this method the dimerisation of the oxoglutarate carrier (OGC) after import into mitochondria membrane was also shown [273]. A monomer–dimer dissociation equilibrium of OGC in the membrane is proposed with about 2% monomer for accepting newly imported OGC molecules to form dimers. This equilibrium would indicate that the association of the dimer is not strong. The dicarboxylate carrier in yeast mitochondria was also reported to form a dimer [274]. For the isolated citrate carrier from yeast a dimeric state by native and by charge shift electrophoresis was determined [275].

Recently it has been reported that yAAC2 and yAAC3 isolated in alkyl-maltosides of various length and in LAPAO exist as monomers [276]. The Stokes radius determined by gel filtration and extrapolated to zero alkyl lengths were compatible with a monomer state. Also equilibrium centrifugation measurements indicate a MW = 30 kDa. Conceivably, the oligomer state depends on the detergent used. No results are available on the MW of yAAC2 in Triton X100 but bAAC1 isolated in the alkyl-ethylene-oxide detergent emulphogen (tridecyl E_{6-14}) has a Stokes radius compatible with a dimer [263]. The reason why the isolated yeast yAAC2 might differ from bAAC1, may reside in the weaker protein–lipid interaction of the yeast AAC and an easier dissociation into monomers. A lack of lipids may be suggested in view of the more stringent requirement for cardiolipin addition in reconstituting yAAC2 [272] than bAAC1. No data on the lipid content in the yAAC2 micelles were given in this work [276] although they may alter the MW calculations. In the bAAC1 micelle PL contribute about 17% to the weight [262]. Another discussion point is that CAT binds less tightly to yeast AAC and it would be important to assure that CAT binding persists in the exclusion chromatography. The two first crystal structures of the bAAC1 did not contain dimers. Also the lattice of the two-dimensional crystals of yeast yAAC3 did not show preferred contacts between the single molecules [277]. However, in a second crystal structure of bAAC1 [278] dimers were discerned in which CL are located at the interface being probably essential for mediating the monomer contact.

10. Reconstitution of isolated AAC into vesicles

10.1. Methods of reconstitution

One of the most important applications of carrier isolation is the reincorporation into PL vesicles. This reductionist approach allows determination of the various factors influencing and determining the transport, free from the complications in the original membrane. A reconstitution was deemed useful when the carrier was reasonably pure and the amount of carrier reconstituted was defined [279,280], since only under these conditions the measured results could be convincingly assigned. This was not the case in the first claims of reconstitution using cholate extracted " particles" [80] or cholate solubilised preparation [281,282]. Without data on the purity, although called "highly purified", the ATR sensitive transport activity was in the "particle" reconstitution less than 0.2% and in the soluble fraction 2% of that reconstituted with purified AAC preparation [283]. Cholate had been found to largely degrade the AAC, explaining the failure of this approach [254].

In initial reports on a defined reconstitution, LAPAO was introduced besides Triton X100 as a solubiliser [185]. First the reconstitution efforts focussed on ways to cope with an AAC preparation not stabilised by CAT. For shortening the steps after solubilisation, the hydroxylapatite (HTS) column passage was replaced with HTS mixing, and the 80% pure AAC supernatant was immediately mixed with sonicated PL. Thus only a few minutes passed before the product was stabilised by the reincorporation. The identity with the CAT–AAC complex was assured by immuno cross-precipitation with antibodies against the CAT complex or unliganded protein [185]. In further work ATR–bAAC1 and BKA–bAAC1 complexes were used for reconstitution [279]. Different from CAT, these ligands were displaced by the high ADP or ATP concentration (≈10–20 mM) present for loading the vesicles. In particles the ATR–AAC complex is stable for several days and permits purification by HTS and sepharose columns before

reconstitution. The methods for the incorporation into phospholipids were improved (see review [11]), varying the conditions of sonication, freeze-thawing the phospholipids protein mixture for the incorporation of protein and the solutes [107]. Later proteoliposomes were formed by first solubilising the phospholipids with excess detergents, addition of the AAC extract and subsequent stepwise removal of the detergent by polystyrene beads. Krämer and Heberger [284] elaborated a technique of using a short column of polystyrene beads through which the phospholipid–detergent–protein mixture is passed repeatedly. In this application $C_{12}F_8$ was the preferred detergent for solubilising the AAC, Triton X100 but not LAPAO could also be handled by this method if the amounts of detergent, lipid, and protein were carefully chosen. In another method applied first for the reconstitution of the uncoupling protein, but then also for the AAC, detergent was removed by additions of polystyrene beads stepwise in 5 to 6 portions [285,286] in 30 min intervals. As with the column method an excess of beads (Amberlite XAD-2 or Biobeads) were required for the removal of detergents from the PL and AAC containing mixture. Vesicles had larger size than in the sonication method but if more protein was incorporated vesicle size decreased. Finally for removing the solutes added for loading the vesicles, filtration through ion exchange column (Dowex) or, with improved yields, short sepharose column was applied.

An important objective is the elucidation of the phospholipid requirements. Egg yolk PL proved to be the most suitable basis [280], whereas asolectin as used by Shertzer and Racker [80,282] failed to sustain a marked transport activity. Admixture of anionic PL particularly of cardiolipin (CL), or mitochondrial PL were found to enhance activity [283]. The activation by CL was confirmed [287] and the beneficial effect of PE was noted in this [287] and parallel work [283]. However the maximum rates [287] were 10 times lower than determined under the optimised composition of PC/PE/CL=58/31/11 (7000 μMol/min/g protein) [283]. CL could be replaced partially by PS indicating the requirement primarily for anionic PL. All this reconstitution work was performed with the standard bAAC1. In later work on mutants of yAAC2, an absolute requirement for CL was noted [272].

The reconstitution of an isolated AAC provided the opportunity to study the mechanism of protein insertion into PL vesicles by ^{31}P-NMR [288]. The method demanded a higher protein/PL ratio than for transport. The results were interpreted by a mechanism where the AAC Triton micelle is incorporated into the outer layer of the multilayer liposome and part of the detergent is released as a mixed PL–detergent micelle. At a molar ratio of about 200 PL/AAC further incorporation is stopped. On contact with the detergent absorbing polystyrene beads, a unilamellar proteoliposome is peeled off the liposome and a new protein detergent micelle can insert into next PL layer. Thus a uniform proteoliposome population is generated. By freeze fracture electron microscopy protein particles were visible on both aspects of the vesicles providing evidence for their transmembrane insertion. These results must be considered on the background of ^{1}H- and ^{31}P-NMR studies of how detergent micelles are inserted and dissolved into PL bilayers [289]. Non-ionic detergent micelles of LAPAO were found to be readily incorporated into PL vesicles, at first producing a heterogeneous population. This is followed by a slow uniform distribution of the detergent molecules in all vesicles. Up to a weight ratio of 0.3 detergent/PL, vesicles remain intact. These results are also important for understanding the protein solubilisation as a replacement of PL with detergent molecules.

10.2. Ionic effects

The reconstituted AAC provided a well defined platform for elucidating ionic effects on the AAC. The inhibition of transport by divalent cations such as Mg^{++} is clearly shown [290] to be caused by the diversion to non-transportable Mg^{++}–nucleotide complexes, as originally concluded for mitochondria [42]. Besides complexing nucleotides, the Ca^{++} influence has more facets some of which are reviewed in the Chapter 12 on pore formation. In addition to inhibition, an activation of transport by some divalent cations was observed [291] in the reconstituted system and also in mitoplasts, but not in mitochondria. Depending on the conditions, activity could be stimulated by Ca^{++} and in particular by divalent organic cations, such as spermine, as well as by polylysine. The direct interaction of these cations with the AAC could be excluded, suggesting instead an interaction with lipid components. CL being tightly bound to the isolated AAC [252] seems to be a logical target for divalent cations. By binding to CL they could change the AAC conformation, “loosen” the carrier “halfway” to the complete opening of the channel, as hypothesised for the Ca^{++} effect [292] (see Chapter 12).

Anions were found to both inhibit and activate the reconstituted AAC [293]. A competition of anionic molecules with ADP and ATP for the cationic residues of the binding site and thus inhibition of transport seems feasible. This effect should increase the K_M for ADP and has to be segregated from rate activation by anions. The inhibition depends strongly on the number of anionic charges, with K_I ranging from 100 mM for Cl^- to 0.9 mM for pyrophosphate and 0.015 mM for ATP. More intriguing and less clearly understood is a strong activation of reconstituted transport by anions. This effect can be well segregated from the activation by divalent cations and from the inhibition by anions. The activation has a different specificity, following in part the lyotropic series; SO_4^-, ClO_4^- are strong activators. Judged from Hill plots, 2 anions have to bind to their target for activation. A competition for the AAC with negatively charged PL was excluded. Since anions increase the translocation rate constant, it has been suggested that the anions enhance carrier “mobility” possibly by binding to some of the excess cationic groups of the AAC.

Surface potential is another factor influencing the AAC activity [294]. It can be changed by addition of positive or negatively charged acyl derivatives to be absorbed by the bilayer or by varying the ion double layer changing the NaCl concentration. Negative surface potential on the c-side, measured by indicators, increased only little the K_M of ADP and ATP and the K_D of CAT, but had a stronger activation effect on the m-side of the AAC. This is interpreted to indicate that in the c-state the intruding binding site is more distant from the membrane surface whereas the m-side is closer to the membrane surface. In conclusion, in mitochondria negatively charged PL, with CL as the most prominent one, might influence the AAC activity via the surface potential.

10.3. Lipid requirements of the AAC

The reconstitution system afforded a first insight into the lipid requirements of the AAC. Egg yolk PC produced tight vesicles but AAC transport activity required inclusion of acidic PL [295]. Cardiolipin (CL) was the best activator, as supported by another work [287]. Additional enhancers are PE and cholesterol [283,287,296]. Both these neutral lipids were regarded to influence the AAC activity by modulating the bilayer structure rather than by interacting with the AAC, at variance to the interpretation by Brandolin et al. [287] who postulated a specific PE interaction with the AAC. In the inner mitochondrial membrane the PE content is low and cholesterol largely absent, but the activation by negatively charged PL, most notably by CL, is shown to be caused by interaction with the AAC (see below). Notably, the activation of the reconstituted AAC was side specific [294], the acidic PL had to be at the m-side of the membrane.

The AAC–Triton micelle contains 16 molecules PL per AAC dimer [262] in a composition similar to the total mitochondrial PL. However, in the analysis of these PL by ^{32}P-NMR a signal for CL was suspiciously low [252]. Only on denaturation with SDS a marked signal for CL appeared next to unchanged bands of PE and PC indicating a strong immobility of CL in the native protein. The size of the CL signal corresponded to 6 CL molecules per dimer, tightly bound to the isolated AAC. The amount of CL binding exceeds that reported for other mitochondrial components.

It is the only case known to the reviewer of such extreme ^{32}P-line broadening of a PL. The unusual excess of 18 positively charged residues in AAC monomer [49] may be responsible for the CL binding, in particular at the interface between protein and PL head groups. The specific binding of CL to the AAC was vindicated [297] by the crystal structure of the CAT–bAAC1 complex isolated in LAPAO where 2 CL molecules/AAC monomer and in a second crystal 3 CL/monomer were identified [278].

Using spin labelled PL [253] strong binding of CL and of phosphatidic acid to soluble AAC was measured but no interaction of PE and PC. In addition to the endogenous CL, one added molecule CL was bound per monomer AAC. From these and other data it was concluded that besides electrostatic also hydrophobic forces contribute to CL binding. Further it was shown [298] that added ^{14}C-CL did not exchange with the endogenous CL on an hour scale, marking the unusual binding strength. Also hydrogenation of bound AAC CL did not loosen the binding as shown by ^{31}P-NMR, indicating that a specific acyl chain is not required. ESR studies of the interaction of various spin labelled PL with AAC incorporated into lipid vesicles at a high protein/PL ratio showed [299] that the AAC is surrounded by a shell of 50 PL at the membrane interface, thus indicating out an unusually large PL binding surface for the protein size. Acidic PL, PA and CL were preferred ligands measured at a dissociation time range that does not include the tightly bound CL, and thus represents additional binding. The surface of a rotational ellipsoid as deduced for the AAC dimer [262] could accommodate only 34 PL molecules suggesting surface invaginations of the AAC. As reviewed above (Chapter 8.6), the SH group activated by ADP can be masked specifically by addition of CL in the isolated ATR–AAC complex [240].

Studying mutants of the yAAC2 (e.g.C73S-yAAC2) from *S. cerevisiae* an absolute dependence on CL addition was noted [272]. The specificity for CL was strict, the acidic PA and PS did not activate transport, different from the less specific activation of bovine AAC. The binding of ^{3}H-CAT to C73S-yAAC2 was unchanged as compared to wt AAC and was not altered by CL addition. Both mutant and wt AAC contained nearly equal amounts of CL. However according to ^{32}P NMR analysis in mutant AAC the tightly bound CL was decreased to 2.9 as compared to 6.4 CL molecules/AAC dimer in wt AAC. These results show that the complete set of tightly bound CL is necessary for transport. On reconstitution of recombinant AAC from *Neurospora crassa* (ncAAC) expressed in *E. coli* the dependence of transport on CL had a threshold dependency on CL [300] starting at 4% with a maximum of 12% CL whereas in the reconstitution of the yeast mutant AAC the dependence was linear with a maximum at 8% CL. Obviously, because of the total absence of endogenous CL in the ncAAC expressed in *E. coli* inclusion bodies, a minimum level of CL is required for any transport activity. In a *S. cerevisiae* mutant devoid of CL by disruption of CL synthesis, this defect could be partially compensated by increased levels of other acidic PL in particular PG, enforced by growth on non-fermentable substrate [301]. Despite a near normal level of AAC and cytochromes, oxidative phosphorylation was reduced in isolated mitochondria indicating that PG can only partially compensate the lack of CL. In isolated and reconstituted AAC from this CL deficient yeast transport is virtually inactive but can be stimulated to half the activity of wt by 8% CL.

11. Pore functions of AAC

11.1. The role of AAC in mitochondrial pore transition

Apart from the 1:1 exchange, in isolated mitochondria the AAC can mediate an efflux of nucleotides. Leakage of nucleotides induced by Ca^{++} and P_i additions had been early reported [302–306]. In rat liver mitochondria AdN efflux induced by Mg^{++} and P_i [307] was specifically associated with the AAC. ATP was the preferred leakage substrate and the efflux was inhibited by external ADP but not by ATR, which even protected against ADP. Similar observations of Mg^{++} stimulated, ATR insensitive leakage of ATP were subsequently reported [62]. Although the P_i+Mg^{++} driven ATP efflux strongly resembles the function of the Mg^{++}–ATP/P_i exchanger [47,308,309] the role of ADP and ATR argued for an involvement of the AAC. The role of AAC in leakage was further strengthened by the advent of BKA. In contrast to ATR, BKA inhibited the Mg^{++}+Pi induced nucleotide efflux [246] and also the efflux induced by low Ca^{++} concentration.

Ca^{++} induced a less specific efflux from mitochondria comprising all nucleotides and NAD. At increasing Ca^{++} concentration the efflux became insensitive to ADP [307]. Large Ca^{++} induced morphological changes of the cristae were recorded as a decrease of light scattering [310, 311]. This membrane transition was associated with the formation of a leakage pore in the inner membrane. The inhibition of the transition by ADP and BKA and the promotion by ATR suggested the involvement of AAC [311,312,329]. The role of the AAC was more clearly discerned by showing that ATR counteracted the inhibition by ADP plus NEM as well as by BKA [313]. This effect of ATR was interpreted to show that the c-state of the AAC is required for the pore formation.

Research on the conditions and the factors involved in the "mitochondrial pore transition" (MPT) also called "permeability transition pore" was discussed in several reviews [314–319]. A more precise definition of the MPT came from the discovery that it can be inhibited by cyclosporine (CsA) [320,321]. This observation was then rationalised by results indicating that binding of mitochondrial cyclophilin-D (CyP-D) to a component of the pore, possibly the AAC, promotes pore opening [322–324] and that on CsA binding CyP is released from the pore which thus becomes closed. The requirement for MPT of CyP is not absolute, as high matrix Ca^{++} concentrations and Ca^{++} in conjunction with prooxidants can overrule the inhibition of MPT by CsA, thus qualifying CyP as a conditional modulator of MPT [325–327]. At low sub-μM Ca^{++} the transition, as monitored by light scattering, can also become CsA insensitive when PP_i is accumulated in the matrix via the AAC during fatty acid oxidation [322,328]. PP_i seems to stimulate K^+ influx resulting in CsA insensitive matrix swelling. In this context an early observation is to be cited that the leakage of H^+ and K^+ into mitochondria accompanying swelling in NH_4NO_3 medium was controlled by AAC as judged from inhibition by ADP and its prevention by CAT [330]. By affecting the SH groups of the AAC with various agents such as NEM, EMA and by stabilising the c-state with crosslinking agents diamide and phenyl arsine oxide, it is further substantiated [331] that the c-state of the AAC is essential for the MPT. These results overlap with the diverse oxidation effects on the MPT (see reviews [315,332]) where "prooxidants" including the SH crosslinkers enhance the sensitivity to Ca^{++} and may abolish the sensitivity to CsA. Prooxidants such as oxygen radicals (ROS) can also affect the AAC by oxidising cardiolipin associated with AAC as discussed in Chapter 10.3.

A "mega-channel" observed by patch clamp of the inner mitochondrial membrane in the presence of Ca^{++} has been associated with the MPT [333,334]. However the identity of the components involved remained unclear. Single channel patch clamp studies with isolated reconstituted AAC showed Ca^{++} dependent large channels of up to 600 p-Siemens [292]. The single channels were voltage gated at >150 mV and closed at pH<5.5, similar as the MPT [335,336]. ADP+BKA closed the channel, CAT had no effect. Having the same earmarks as MPT, the patch clamp AAC channel strongly supported the pore forming role of AAC in MPT. The inability of CsA to inhibit the channel could be explained by the absence of CyP in the reconstituted system in agreement with the observation that in MPT high Ca^{++} can overcome the inhibition by CsA.

Using recombinant *N. crassa* AAC (ncAAC) expressed in *E. coli*, in patch clamp studies of reconstituted AAC, Ca^{++} induced channels were also observed [337]. This preparation was absolutely free of CyP, arguing against the possibility that traces of CyP may have activated the reconstituted bAAC1 channels. Interestingly, on addition of CyP the voltage gating at >150 mV was abolished. When the channel was

closed by ADP, CyP opened the channel in the whole voltage range into a flickering state. CsA reversed the effect of CyP and ADP closed the channel. The results show that recombinant AAC devoid of any other component can form a large pore and that CyP can directly interact with the AAC. It was concluded that CyP has a modulating rather than obligatory role in the pore formation of AAC, consistent with evidence for the MTP. These results suggested that the role of CyP is to regulate the voltage gating of the MPT.

The prooxidant tert-butylperoxide, known to activate the MPT, was applied to the patched channel. Interestingly, it also abolished the voltage gating, and this could be recovered by DTE, indicating the involvement of SH groups in the gating mechanism. This response of the AAC channel to tert-butylperoxide is consistent with the enhancer role of prooxidants for the MPT and the involvement of dithiol groups [332,338,339]. At this juncture it is important to note that mersalyl was shown to be an efflux inducer [340] in reconstituted AAC vesicles loaded with ADP or ATP. Mersalyl also increased fatty acid dependent uncoupling of reconstituted AAC [341]. Similar as EMA [342,343] mersalyl was thought to target C159 surrounded by basic residues possibly near the binding center and the m-side gate.

The MPT has been suggested to be a complex of several proteins, including VDAC (porin), CyP, AAC, hexokinase, creatine kinase, peripheral diazepine receptor [344–346]. A role of the octameric creatine kinase in MPT was considered [129,347]. These complexes were thought to be at the contact points between the outer and inner mitochondrial membrane [348].

Although the central role of the AAC in the MPT has been supported by various types of evidence [317,319], this role was lately refuted by showing that mouse liver mitochondria, whose mAAC1 and 2 have been deleted, still exhibit a Ca^{++} activated MPT, sensitive to CsA [349,350]. Based on this and other evidence a regulatory rather than pore forming role of AAC in MPT was inferred [351]. In defence of a central role of AAC in "wt" mitochondria one may call attention to the more than threefold higher threshold of Ca^{++} concentration required for the MPT in AAC deleted mitochondria [349], suggesting that this MPT may involve another mitochondrial carrier [350]. It might be speculated that the MPT is comparatively unselective in also employing other mitochondrial carriers but has a a normal preponderance of the AAC. The pore formation under the influence of mercurials of the reconstituted carriers for aspartate/glutamate and for P_i as well as of AAC [340,352,353] may support this view. The role of MPT and thus of AAC in cell death such as apoptosis and necrosis has been abundantly reviewed [317,319,344]. Important pathologic roles of MPT are well documented in the ischemia reperfusion damage in heart cells [317,319] and the excito-toxicity in neuronal cells [354–356].

11.2. Basal uncoupling and fatty acids

Another class of "leakage" inducers, by which the AAC can be taken hostage to be diverted from its proper function, are long chain fatty acids. Endogenous FA were early known to be a major cause of uncoupling in isolated mitochondria, as reflected in the recoupling by serum albumin (see review [357]). Added FA, e.g. oleate, stimulated a H^+ leakage, as reflected in uncoupling, that was much stronger than could be explained [358] by a slow FA^-/FAH cycle across lipid bilayers [359–361]. It was concluded [358] that a membrane component catalyses the cycle, by accelerating the rate limiting FA^- transfer. The finding, that CAT partially inhibited the FA induced uncoupling [362,63], pointed out an involvement of the AAC. The effect is weak in liver and stronger in muscle mitochondria [363,364] which have a higher AAC content [174]. Similarly, enhanced levels of AAC in hyperthyroid livers increase the FA mediated uncoupling [365].

The AAC was envisaged to translocate the FA^- anion by virtue of its role as a transporter of anionic substrates [366]. A similar mechanism was proposed for the FA dependent H^+ translocation by uncoupling protein (UCP) [366] which reaches, however, two orders of magnitude higher rates than the AAC dependent H^+ translocation. Besides AAC, other carriers such as for aspartate/glutamate [367] and for dicarboxylates [368] also seem to contribute to the FA dependent uncoupling. Thus the FA induced uncoupling in mitochondria is the sum of contributions from various carriers and possibly other membrane components, with the AAC having a major share. To complicate the picture further, under certain conditions, such as low Ca^{++} loading, the uncoupling by FA assumes properties of MPT as defined by the sensitivity towards CsA [369,370]. AMP is reported to activate the basal metabolic rate by 15% in skeletal muscle mitochondria [371]. Similarily as with FA, the effect is largely abolished by CAT. A possible involvement of FA is not tested. AMP, althogh not transported, can bind to AAC [372] but in vivo would be in competition with ATP and ADP.

It should be stressed that the FA induced H^+ conductance is weak as compared to uncouplers or as compared to UCP and might play, if any, only a minor role in cell metabolism. A role in thermogenesis of muscles has been proposed. However, only few muscles, e.g. heart, have a sufficiently high content of mitochondria and the constantly working heart does not need generation of heat in addition to that produced by myofibril contraction. An alternative or additional role of the FA linked uncoupling is to lower ROS production [373] as discussed in a broader context also for the uncoupling by UCP.

In vesicles, co-reconstituted with isolated AAC and cytochrome oxidase for generating a H^+ electrochemical gradient, a clear assignment of FA mediated uncoupling to AAC could be made, free of any possible cofactors [341]. H^+ efflux was only about 30% inhibited by CAT as compared to inhibition by albumin (BSA) and further inhibited by BKA, indicating that a large portion of AAC is incorporated with c-side inside. The $\Delta\psi$ recordings with vesicle containing COX only and COX + AAC, showed the critical role of AAC in partial uncoupling by FA, even if the weak uncoupling amounts to only a minor fraction (5%) of total $\Delta\psi$. Interestingly, mersalyl known for generating a small pore in AAC, markedly potentiated the FA effect. It sensitised the uncoupling against nucleotides, even allowing GDP to become inhibitory and restitutes the sensitivity towards ATR. Also in mitochondria the partial inhibition by GDP of uncoupling by FA was recently reported [374]. The skewed inhibitor specificities of the AAC mediated uncoupling attest to the different translocation mechanism in the FA effect.

A possible role of AAC mediated FA uncoupling in kidney tubular cell reperfusion damage was reported [375]. By ablating AAC1 in murine muscle mitochondria and in insect cells, basal uncoupling was decreased by half and elevated by overexpression of AAC1 [376]. These changes were observed both for the CAT sensitive and insensitive portions, indicating that more than half of basal uncoupling, although dependent on AAC presence, was not associated with CAT sensitive FA induced uncoupling. Instead a H^+ conductance by polyunsaturated PL acyl chains at the AAC interface was suggested [376]. Tightly and more loosely bound CL with their high degree of unsaturation [298] surrounding the AAC [252,253] may be responsible for the H^+ conductance.

11.3. Side effects of AAC in mitochondria result from its principal function

In summary, the AAC is obviously involved in a wide variety of leakage phenomena of the mitochondrial inner membrane. These may be categorised (a) by the size of the underlying channel and the selectivity of the leaked solutes and (b) by the sensitivity and specificity towards specific AAC ligands. Under the provision that in most cases the AAC constitutes the pore (channel) forming component, it seems that the AAC is highly flexible in response to the pore inducing factors, ranging from a partially "native" state with ATP specific leakage to a state with a wide pore of ~20 Å under the influence of Ca^{++} that allows unselective solute passage and becomes irreversible with additional

prooxidants. Gating, i.e. coordinated closure at either side of the binding center, is still rudimentary at the first leakage stage and abolished at the stage of MPT. Typically in this category, ADP and BKA are pore inhibitors, but CAT and ATR are pore promoters by preventing ADP and BKA binding, indicating that the pore state resembles the c-state of AAC but with an opened m-gate. With CAT bound, the pore opening requires the existence of a channel around the binding site which is difficult to visualise. In a functional AAC dimer model acting in "half site reactivity" with only one subunit occupied by CAT, the "free" subunit might form a pore. The FA induced weak, partially CAT sensitive, H^+ leakage may be due to a H^+ diffusion facilitated at the AAC surface layer by virtue of its high CL content. Some of the effects reflect the abundance of AAC, others, like the MPT, a strategic localisations of selected AAC at the peripheral inner membrane.

Why would the AAC have these side roles, besides its primary function necessary for the existence of the eukaryotic cell, featuring some roles endangering and executing the demise of these cells? It seems reasonable to argue that the secondary roles are a consequence of the primary function and that, to make the best of this situation, negative side effects of the AAC function have been evolved to be exploited to some extent for useful purposes. In other words, on the basis of its role in ADP/ATP transport the AAC is captive of forming pores in mitochondria. The reasoning is as follows. The AAC handles large substrates and thus must have a wide translocation path with wide gates requiring large conformation changes on opening and closing. These large rearrangements involve high energy barriers which require unusual measures to be surmounted. The strong interactions of AAC with cardiolipin [252,272] have been proposed to involve "collar" lysines at the m-side of AAC at the membrane surface [292] which may be one reason for the high content of lysines in AAC. The binding energy of CL is thought to provide catalytic energy in addition to the intrinsic ATP or ADP binding energy needed for facilitating the large gating rearrangements. With a high affinity for CL [377] Ca^{++} can derange the CL–AAC interaction by sequestering CL (Fig. 6). The released excess positive charges should enforce the opening of the m-side gate. Inherited from the primary function, AAC has the potential for a large pore that manifests itself, when the coordinating gating is disturbed. Thus with the evolution of the precursor prokaryotes into mitochondria, eukaryotic cells acquired with AAC an "Achilles heel". Another weak spot caused by the high CL requirement is the unusually high degree of acyl chain unsaturation, rendering CL most vulnerable to oxygen radicals. ROS affect CL bound to the AAC forming aggressive aldehydes such as malondialdehyde and hexenal [378, 379]. These react in situ with lysines of the AAC to form Schiff base adducts. This was demonstrated by exposing isolated mitochondria to tert butyl-OOH. The only major protein detected to change the MW was the AAC [379] illustrating the exceptional vulnerability of this membrane component.

12. The structure of the AAC

12.1. The sequence of the AAC and its structural highlights

The primary structure (amino acid sequence) of the AAC was first established with the standard bovine heart AAC (bAAC1) [49,380]. The ability to isolate large quantities of the AAC protein as the stable AAC–CAT complex enabled amino acid sequencing with technologies of the 1970–80 ties when c-DNA cloning of mammalian proteins was still in its infancy. The amino acid sequence was the first primary structure of any transporter, preceded by the c-DNA sequence at the bacterial lactose permease [381]. Adverse conditions made sequencing difficult: unfavourable positioning of standard CNBr cleavage sites, acetylation of the N-terminus (acetyl-ser), the necessity for citraconylation etc. of the hydrophobic peptides to facilitate peptidases work. The peptides were separated by gel chromatography with formic acid and other solvent mixtures and sequenced by the liquid phase Edman procedure.

The sequence comprising 297 amino acids surprised [49] by consisting of more than 20% (total of f56 acidic and 39 basic) charged residues, which was unexpected for an integral membrane protein. Due to the excess of 18 basic residues, bAAC1 has a high isoelectric point $pK_i > 9$. In 8 positions positive and negative residues are adjacent suggesting charge pairs. The scattering of numerous charged residues over the sequence gave mixed results in algorithms searching for hydrophobic transmembrane segments. In the first report [49] three candidate transmembrane helix segments were noted, W111-L133, G 171-Y195, and I209-F230. In particular the region to become TMH2 barely qualified for intramembrane regions showing too low hydrophobicity coefficients. Only by using internal homology plots, segmentation into 3 similar domains, each featuring 2 helices with a total of 6 transmembrane helices, were predicted [382] (Fig. 7).

The striking sequence RRRMMM regarded as an AAC signature [383] was present in all forthcoming AAC sequences, such as of *N. crassa* [384], *S. cerevisiae* [385], *Zea mays* [386], now all based on c-DNA sequencing, and in the first forthcoming mammalian cDNAs [387–390]. The conservation in the three domains of helix breaking prolines and glycines was emphasized [49]. In addition acidic and basic residues fully or partially are conserved in putative TMH terminating positions. The subsequently elucidated [391] amino acid sequence of uncoupling protein (UCP1) turned out to be similar to AAC also featuring a three repeat domain structure. The comparison of the two structures and the search for conserved residues facilitated to discern essential elements. Both sequences of AAC and UCP were aligned in a 3 repeat arrangement, by allowing for insertions or deletions, for finding maximum of conserved residues in the combined six repeat domains. Six fold conserved prolines were found and placed as helix breakers at the matrix end of the uneven

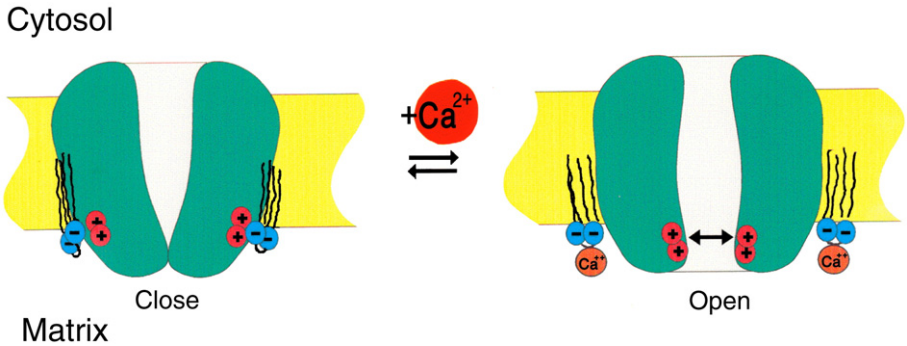

Fig. 6. Mechanism of pore formation in the AAC under the influence of Ca^{++}. At the matrix side cardiolipin (CL) is tightly bound to the AAC. Ca^{++} chelates with CL and thus displaces CL bound to positive charged residues from the AAC at the m-side. The liberated positive charges open the m-gate. From [292].

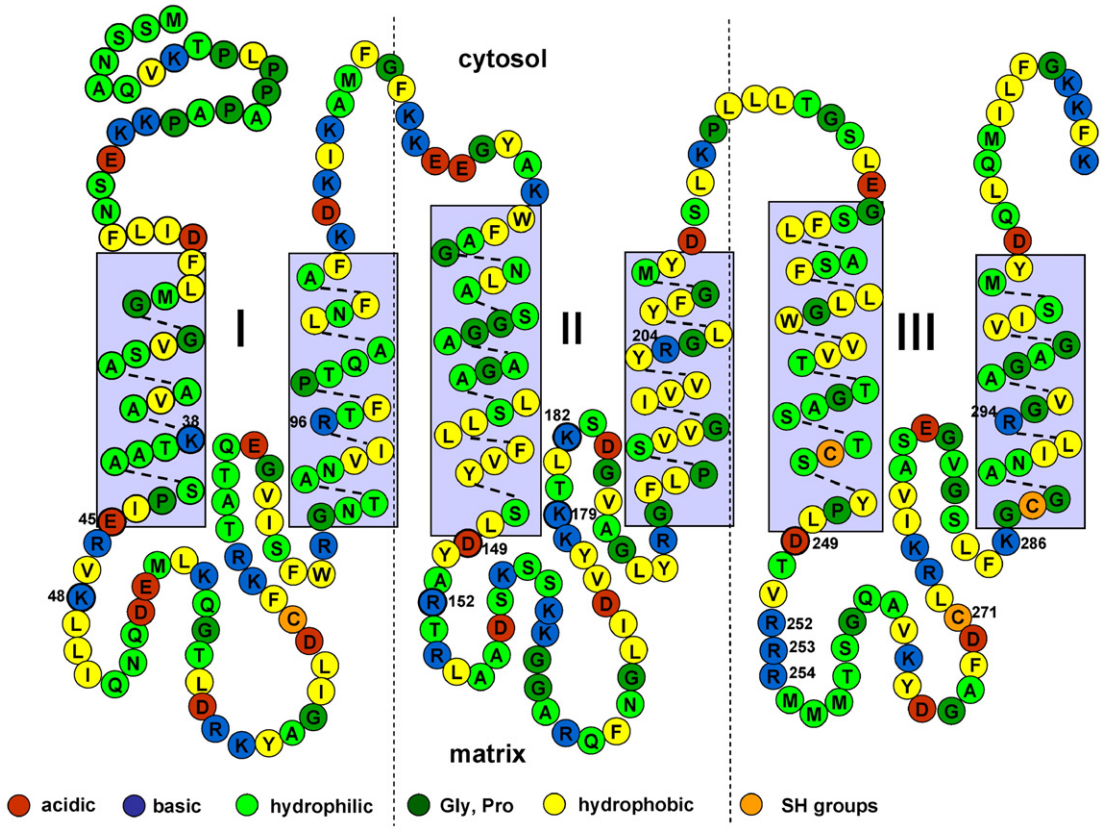

Fig. 7. The 6 transmembrane helix and 3 repeat domain model of AAC exemplified with yeast AAC (yAAC2). The structure accommodates various mapping results. Hairpin loops from the matrix side intrude the translocation path. Residues involved in site directed mutations and in spontaneous second site mutations are numbered. Modified from [477].

numbered TMH. Also structurally important 6 Gly were conserved in the even numbered TMH regions. In three positions acidic or basic residues were conserved in the repeats domains. Refined hydropathy analysis searching for amphipathic helices overcame some problems with the classical hydropathy plots and suggested 6 amphipathic helical stretches of 20 to 25 residues both in the AAC and UCP. An apparent amphipathic β-strand appeared to traverse the membrane between TM1 and TM2, turning the C- and N-terminals to the opposite side. On the basis of circular dichroism measurements 40 to 42% α-helix content was calculated for AAC and UCP [391]. This amounts to 120 residues in α-helical positions which can accommodate 6 average 20 residue long TMH. Applying FTIR spectroscopy to yAAC2 60 to 70% of the residues were evaluated to be in α-helical positions [392] which better approximates the helix content of the crystal structure [297].

In addition to bAAC1 and UCP1, the phosphate carrier (PiC) was amino acid sequenced and again found to be homologous to the AAC and UCP [393,394]. The finding that the primary structures of three mitochondrial carriers are homologous and all feature three repeat domains with two transmembrane helices (TMH) allowed for the first time to conclude that the mitochondrial carriers with AAC as a foremost member form a family [393]. Two sequence motifs were shown [393] to be conserved in isologues positions in the 3 repeat domains in all three carriers: at start of the matrix (M) loops **PXD/EXXK/RXK/R** and at the end of the M-loops **Φ+G** (Φ aromatic residue). The concept of a mitochondrial carrier family (MCF) was vindicated by the identification of numerous more sequences based on c-DNA screening, the heterologous expression and functional determination of many carriers of the MCF [12,15,395,13]. This motif, then more specifically designated **PhD/EXhK/RXK/R** (h hydrophobic residue) [396], was expounded as a marker for a rapidly expanding mitochondria carrier family.

12.2. Isoforms of AAC in mammalians

The occurrence of isoforms of AAC in various organs of mammalians was first suggested based on immunological evidence anticipating the finding of bovine AAC isoforms [390] and their tissue distribution. The antiserum against CAT–bAAC1 reacted poorly in the Ouchterlony assay with isolated CAT–AAC from rat and bovine liver, bovine kidney [397], later identified as bAAC2 [390], but reacted strongly with rat heart AAC. This difference was reciprocally found with antiserum against bovine kidney CAT–AAC by employing quantitative immuno-adsorption titrations [260,398]. Bovine liver AAC reacted at only 20% and kidney AAC at about 50% of the heart AAC. These results indicate that the immunological difference between organs is greater than between species.

First in yeast (see below) and then in other organisms, particularly in mammals, genes of AAC isoform were identified by mutational search and later the full inventory of isoforms was determined by complete genome search. In the following, AAC isoforms of some "standard" species will be listed such as of mouse, rat, cow and man. To avoid any confusion – extant in the literature – the isoforms are given according to the annotations of the Protein Bank. In mouse mAAC1(P48962) and mAAC2 (P51881) [399,400], in rat rAAC1 (Q05962) and rAAC2 (Q09073) [401], in cow bAAC1 (P02722) [390], bAAC2 (Q8SQH5) [402], bAAC3 (P32007) [390], bAAC4 (Q2YDD9), in man hAAC1 (P12235) [388], hAAC2 (P05141) [387], hAAC3 (P12236) [403], and hAAC4 (Q9H0C2) [404]. Note, there is some ambivalence in the literature in the assignment of AAC2 and AAC3 (ANT2 and ANT3). The sequence differences in these isoforms are small, as compared to those in the yeast, retaining about 85% identity [402], with the exception of h- and bAAC4 with only 66–68% identity to the other isoforms [404]. In the two or three isoforms of the cited mammalian species, the differences were noticed [402] to be concentrated in the

M regions where in positions 45, 147 and 164 (numbers based on bAAC1) specific replacements occur that are conserved in the isoforms of all these species, e.g. in position 45: Gly in AAC1, Ala in AAC2, Ser in AAC3. This emphasizes a role of these residues in the still unknown adaptations of the functional parameters to the particular tissue needs.

Only a cursory review of AAC isoform expression can be given here, omitting the literature on the regulatory elements involved in AAC expression. h- and, bAAC1 occurs exclusively in muscles [390,402,405], h- and, bAAC2 in proliferating cells [405,406], hAAC3 in all organs [389,404,405]. hAAC4 is expressed at low levels and is confined to liver and testis, marginally to brain [404]. hAAC1 expression is induced during myoblast differentiation [405]. hAAC2 expression is strongly stimulated by growth factor in fibroblasts [407] and also increased in neoplastic cells [408,409] as well in early myoblasts [405]. These results indicate that the hAAC2 level is related to enhanced glycolysis in these proliferating cells, and has the role of supplying glycolytic ATP into the mitochondria, similarly as suggested for yAAC3 in yeast [410]. In fact, substitution of hAAC2 in yeast cells devoid of yAAC3 gene sustained fermentative yeast cell growth [406]. Thyroid hormone (T3) was shown [411] to increase selectively the rAAC3-mRNA level 7 to 9 fold in rat liver and rat heart, while not stimulating rAAC1-mRNA.

13. Yeast AAC as a basis for structure/function studies

Yeast (*S. cerevisiae*) became the platform of convenient genetic manipulations of the AAC for elucidating structure–function relationships on a broad scale. In *S. cerevisiae* and *Candida utilis* the existence of ATR sensitive ADP/ATP exchange similar to that in rat liver mitochondria was early recognised [412]. A lower temperature dependence and lower affinity for ATR was noted. AAC mutants resistant to BKA were claimed by three groups screening the growth of yeast in the presence of BKA [413–415]. In isolated mitochondria ADP/ATP transport was reported to be BKA insensitive [414,415]. However these results remained unconfirmed and seemed to be caused by measurement problems. The first "native" AAC mutation was found in the classical nuclear mutant op1 (p9) [416] showing a decreased translocation rate and affinity for ADP and ATP. With ^{35}S -CAT binding a lower AAC content and a 4 times lower affinity for CAT in op1 than in wt was determined [418]. Also a major affinity decrease for ^{3}H-BKA binding was reported [419]. In this context attention is drawn to the AAC in mitochondrial DNA defective ρ^- cells. The low ADP/ATP exchange in ρ^- mitochondria was inhibited equally by CAT and BKA [418,419]. Binding of ^{35}S-CAT had the same high affinity as in wt [418]. These and other [420] results confirmed the nuclear coded biogenesis of AAC and contradicted the reported absence [421] of an ATR sensitive transport in ρ^- cells. Also in promitochondria isolated from anaerobic yeast a CAT sensitive ADP/ATP exchange was observed but CAT binding had a 4 times reduced affinity [418]. The reported inhibition of AAC biogenesis by erythromycin [421] could not be confirmed [419]. Another direct proof for nuclear coded AAC came from studying the expression of AAC in *N. crassa* [422]. In cells grown on ^{14}C-leucine and chased with ^{3}H-leucine after addition of chloramphenicol or chlorheximide, the ^{3}H/^{14}C ratio in the 33 kDa band of AAC clearly established the nuclear controlled biogenesis of AAC.

13.1. AAC isoforms in yeast

S. cerevisiae contains three genes for isoforms of AAC. yAAC1 was first discovered by sequence homology to bAAC1 [423], but it only poorly complemented the op1 defect of aerobic growth. Disruption of the yAAC1 gene revealed the yAAC2 gene which turned out to represent the op1 gene by fully complementing op1[424]. yAAC2 is the principal AAC in aerobic yeast, yAAC1 is barely expressed [425]. Disruption of AAC2 alone prevents aerobic non-fermentative growth. A third transport pathway was postulated [425] based on the finding that disruption of both yAAC1 and yAAC2 genes still allowed fermentative growth. It could be attributed to a third (yAAC3) isoform [426] that is expressed only under anaeorobic conditions. Exclusively yAAC3 is obligatory for anaerobic growth as shown by disrupting the 3 isoforms separately [427]. The role of yAAC3 is suggested [410] to act in reversed order, supplying ATP from glycolysis to the mitochondria. Although the expression in anaerobic yeast of yAAC3 can be monitored by mRNA, the level of yAAC1 protein is too low to be detected in immunoblots [427]. The apparent lack of a leader sequence lead to the unconfirmed speculation that yAAC3 might be expressed in other intracellular membranes [426]. In particular, the finding, that *yAAC1* deficient strain produced no phenotype except for a red colour of adenine precursors accumulated in vacuoles, lead to suggest a role of yAAC1 in vacuoles [428]. Notably, in the obligate aerob *N. crassa* only one ncAAC gene has been found [384].

The yAAC2 became a major vehicle for structure–function studies by introducing mutations or by searching for spontaneous mutations. For the functional characterisation mitochondria were used, but for a more incisive characterisation yAAC2 was isolated and reconstituted. In an early report the "ATR binding protein" from yeast was isolated displaying two bands with 37 kDa and 34 kDa [429]. ATR affinity chromatography produced low yield and according to other experience [256] a denatured product. For the isolation of intact yAAC2 from yeast, the HTS procedure was modified [430] in order to separate the AAC from porin. In SDS gel yAAC2 was identified to migrate as 31 kDa band and yeast porin as 29 kDa. This isolation method was also the basis for reconstitution. The higher apparent MW of 37 kDa [429] in the previous work may be caused by calibration problems and the 34 kDa band may be due to porin.

yAAC1 and yAAC2 were isolated from yeast strains constructed to overexpress these isoforms separately [431]. The reconstituted yAAC1 was still 1/3 as active in transport as yAAC2 and had the same K_M for ADP. Thus the reason, that in yAAC2 defect cells AAC1could not support aerobic growth, lies in the low expression level. The conditions and factors involved for the expression of the three isoforms were shown to be influenced by the oxygen and heme level [432–434]. The involved promoters and transcription factors were characterised and it was shown that the N-terminal extension in yAAC2 is instrumental for the high expression level [433]. Mammalian AACs such as bovine bAAC1 could be expressed in yeast only at very low levels [300,435] even with multicopy vectors. Levels of bAAC1 equivalent to the yAAC2 were expressed when the unique 5′ extension of yAAC2 gene was spliced into the expression vector construct [435]. This method was also used for the expression of the three human AAC isoforms in yeast [436].

13.2. Mutations in yeast AAC

The mutation responsible for pet9 (op1) was identified as R96H in AAC2 [385,425]. The overexpressed and reconstituted protein had a distinct but reduced transport activity [431]. In an extensive broad study 15 mostly charged residues conserved within the three repeat structure of the yAAC2 or in the AAC motif were selected for mutations [437]. In the second helix of each repeat the homologous R96, R204, R294, and in the AAC motif RRRMMM the three Arg were mutated to neutral residues (Fig. 7). In all these mutated cells non-fermentative growth was impaired indicating the importance of these charged residues in AAC function. Direct measurements of ATP synthesis in cells [438] showed a residual 8% oxidative phosphorylation rate in R294A and zero activity in the other mutants. In contrast the decrease of cytochrome content was only small. Obviously in these plasmid strains oxidative phosphorylation and respiratory capacity are poorly correlated. Similarly, in the isolated mitochondria respiration and cytochrome content were reduced to between 25 to 60% of wt [438]. ATP synthesis rates were zero in 5 R→A mutations, but still at 5% of wt in R96H and 20% in R294A. The AAC content, as measured by

competitive ELISA titrations, was reduced to zero only in R96A and R204L but retained 70% in R294A and about 25% in R96R, R252A, R253A, and R254A. Determination of 3H-CAT and ^{3}H-BKA binding in these mitochondria revealed a similar pattern. These results show that the mutations do not affect the binding of BKA and CAT. The ratio of ATP synthesis to AAC content was reduced to between 20 and 1%.

The mutant AAC was isolated and reconstituted for transport measurements aimed at elucidating not only changes in the absolute rates but also in the four exchange modes [66]. Besides stopping exchange by a CAT+BKA mixture, an automated rapid mixing and sampling device allowed rapid and precise measurements without relying on inhibitors which may be disabled in the mutants. With CAT alone in wt only 10 to 15% of the activity was inhibited indicating that the majority of AAC is incorporated with the m-side out in the vesicles. The activity of the basic electro-neutral D/D exchange mode was zero in R96A, R204L and at a few % in other mutants, with the exception of R294A reaching that of wt. This paradox to the poor aerobic growth was resolved by measuring the 4 exchange modes. Strikingly, the modes including ATP in particular the T/T mode, which are normally higher in wt, were drastically decreased in R294A compared to the D/D mode. The ratios of the homo-mode rates v(T–T)/v(D–D) inverted from 1.5 in wt to 0.2 in R294A, and the productive versus counter-productive hetero-mode rates v(T–D)/v(D–T) from 2.6 in wt to 0.2 in R294A. Since in the vesicles most of AAC was inserted m-side out, the T–D mode would be required for ATP delivery in oxidative phosphorylation, explaining that the AAC mutants because of their suppressed T–D mode did not sustain sufficient ATP delivery. A similar inversion of modes although at a lower level was determined in the other Arg mutants. These and other (Heidkämper et al. unpublished) results indicated that only the K_M of ATP is increased in these mutants indicating the importance of these Arg, in particular R294, to compensate the extra negative charge of ATP^{4-} versus ADP^{3-}. R96 and R204, as well as the Arg triplett (R252-254) seem to be more important for transport activation in general i.e. for both ADP and ATP, whereas the homologous R294 in the third repeat controls more specifically ATP with its extra charge.

Further conspicuous charged residues addressed by mutations [439], were K38 in the first helix, which is conserved in all AAC within the MCF, and two other repeat triads largely conserved in the MCF, K48A, R152A and R252 including a member of AAC signature Arg triplett R252,R253, R254, and a triad with negatively charged residues E45A, D149S, D249. These acidic residues, originally proposed to be at the helix terminal at the matrix membrane surface [437], are according to the crystal structure [297] located near the matrix surface within the bent helices (see Fig. 9). Here they form ion bridges with the positive triad residues, E45-R152, D149-R252, and D249-K48. In all these mutants ATP synthesis measured in cells and in the isolated mitochondria was inhibited [439], with the exception of E45G which retained full or in mitochondria even enhanced activity. According to ELISA the amount of AAC was reduced to 20 or 50%. ^{3}H-CAT and ^{3}H-BKA binding and inhibition of exchange were strongly reduced in K48A, D149S, D249S, indicating a role of these residues in inhibitor binding. The transport rates of the isolated and reconstituted AAC in the 4 exchange modes were very low for K48A, D149S, D249S, but nearly normal for all modes in E45G. Further a strong exchange mode inversion was observed in K38A and R152A, with little attenuated rate of the basic D–D mode but strongly decreased rates of the T–T and T–D modes. This explained that these mutants despite their only partially reduced respiratory activity could not sustain non-fermentative growth.

Taken together, the three residue triads were visualised to form three charged rings around the translocation path. Most intriguing, in each triad one residue (E45, R152, R294) is distinctly less important for transport, spiralling along the threefold axis between the three repeat domains. This was speculated to cause a rotating substrate movement along the translocation path [439]. Whereas the triad residues were regarded to have a more general structural role typical for the MCF, K38 seems to play a more direct role in ADP/ATP transport.

By enforced chemical or irradiative mutagenesis *S. cerevisiae* was screened for growth resistance to BKA [169]. Four point mutations in AAC2 were identified G30S, Y97C, L14S, G298S which partially relieved the inhibition of growth on glycerol by BKA. The AAC content in the isolated mitochondria and exchange activity were normal. The K_I for BKA was only 1.5 to 2 times increased in the mutants. Only ATP synthesis was recorded as a proxy for transport. All these mutations generated OH− or SH− groups indicating that conformational changes rather than lack of binding residues are responsible for the decreased sensitivity to BKA.

13.3. Mutations in N. crassa AAC and relevance for AAC import

In principle mutational defects can also be caused by defects of AAC import and folding. To discern this factor, the AAC was expressed in *E. coli*. Interestingly, yAAC2 has a partially different codon usage than other MCF members that does not allow yAAC2 to be expressed in *E. coli*. Instead AAC from *N. crassa* could be well expressed [300] in *E. coli* inclusion bodies. Mutations in ncAAC were introduced in positions isologous to the mutations in yAAC2. Again the rates of the 4 exchange modes were measured. In general, the ATP involving modes in particular the T–T mode were more affected than the basal D–D mode indicating the higher sensitivity of ATP^{4-} against a loss of a positive charge in the AAC, similarly as observed with yAAC2. The comparison of the basal transport rates of the ncAAC mutants isolated from *E. coli* and from mitochondria (yAAC2) should indicate whether protein import and folding are impaired by the mutation. A strong difference was found in the mutants of the first two intrahelical Arg, ncR86A/yR96A, ncR195A/yR204A, which are inactive in yAAC2 but retained distinct activity in ncAAC, supporting their importance for AAC processing. In the third repeat domain the intrahelical Arg (ncR285/yR294) were not impaired in mitochondria, indicating a different role. These results agree with the different roles of the three repeat target sequences found in import studies [440].

13.4. Revertants of yeast AAC2

Starting with selected mutations, spontaneous second site revertants were searched [441,442] based on the spontaneous conversion of yeast cells to non-fermentative growth. About 15 revertants were collected containing second mutations of neutral or charged residues into neutral residues. In the biochemical characterisation only those strains, where the first and second sites start with charged residues, showed clear reversions of AAC expression and transport activity. Further, second site rescues occurred only when the first mutation did not inactivate transport completely. (Heidkämper et al. unpublished). These were R294A+E45G/Q, R152A+E45Q, D149S+R252T, and K38A +E45G. Since the spontaneous second mutations removed an opposite charge, these residues were proposed to form salt bridges in wt yAAC2: R152-E45 were visualised to connect TMH1 and TMH3, and D149-R252 to connect TMH3 and TMH5 [396], both bridges being located at the m-side. This prediction was confirmed in the crystal structure of the CAT–AAC complex [9] where these salt bridges form a circular closure. The replacements of these ion pairs by neutral residues abolished partially the exchange mode inversion of the primary AAC mutants, although the absolute transport rates remained low. The results indicate some redundancy in this charge pair network as elimination of one charge pair still allows sufficient transport to sustain a limited aerobic growth.

14. Mapping the structure of AAC

A number of topographic methods were applied for the elucidation of binding sites, the folding of the peptide chain, and the conformation changes between the transport states. These included covalently

interacting reagents, affinity labelling with substrate analogues, antibody interactions, limited proteolysis and mutations. Besides information on the structure, more importantly, an insight in the structural changes implicated in transport was obtained. These results have to be compared and interpreted with the crystal structure of the AAC, and the crystal structures will also have to be reconciled with the mapping results. A summary of the mapping studies on the AAC are assembled in Table 1.

14.1. Mapping of lysine residues

The high content of lysines in bAAC1 made these reactive residues a favourable target for a broad accessibility investigation [443,444]. Pyridoxal phosphate (PLP) is membrane impermeable and forms Schiff base conjugates with lysine which can be stabilised by reduction with $NaBH_4$. PLP inhibits ADP/ATP transport with K_I=0.8 mM but was unable to remove CAT or BKA and only partially ATR. ^{3}H-PLP was synthesised and applied to bovine heart mitochondria to probe c-side access and to SMP for m-side access. The c- and m-states were established by loading with CAT, BKA or ATR. Access to the c-side was probed in mitochondria and to the m-side in SMP. Further, isolated CAT–AAC and ATR–AAC were probed with PLP. After fixation of ^{3}H-PLP by $NaBH_4$, the AAC was isolated and fragmented and the peptides generated by thermolysin were sequenced and analysed for ^{3}H-PLP incorporation. Among the 18 lysines analysed, the majority could be accessed from the c-side. Several lysines change their accessibility between c- and m-state. More were accessible in the m-state (with BKA) than in the c-state (with CAT or ATR). The interpretation of the access was based on a 6 TMH folding model. An additional, not confirmed amphipathic β sheet spanning the membrane after TMH1 placed the N-terminal at the m-side [391] but did not affect the targeted lysine positions with the exception of K22. K42, K48 in the first matrix loop M1 were exposed to PLP in the presence of BKA and largely masked in the presence of CAT, whereas K62 was unmasked by CAT. K146 in M2 was exposed to the m-side in both states, but K162 could be accessed only from the c-side. In mitochondria this access required the m-state but in the isolated AAC it also reacted in the c-state.

By adhering to the 6 TMH model, the c-side access of K162 led to the assumption [444] that the segment carrying K162 folds into the membrane from the m-side into the translocation channel open from the c-side. The c-side cluster K91, K93, K95 was accessible to PLP from the c-side in the m-state only and masked with CAT. Originally assumed to be in the C1 loop, according to the crystal structure [297] these residues are at the c-side of TMH2. For K91 the masking reflects the linkage in the crystal structure to the SO_4^- group of CAT. For K93, K95 the masking agrees with the evidence obtained in yAAC2 that TMH2 [445] turns 180° from c- to m-state. The analogously positioned K198 and K 205, originally in C2 but now at the c-end of TMH4, are accessible in both states from the c-side only. Thus TMH4 can be visualised to turn between the c- and m-states similarly as TMH2. The unique AAC specific K22 in TMH1 is accessible only from the c-side and masked in the c-state.

14.2. Photoaffinity labelling by azido derivatives

Photoaffinity labelling of the AAC started with applying 8-azido-ATP to BHM [200,446]. It was shown to incorporate primarily to a 30 kDa band in SDS gels identified as AAC by the inhibition with ATR. Also with the more flexible arylazido-ADP, AAC was the preferential target in BHM, but in yeast mitochondria [447] a 37 kDa band, probably porin, was the major target. For crosslinking studies a dual photoaffinity label of ATP was synthesised by introducing both the 8-azido and arylazido group [448]. 2-azido-ADP [449] with a fixed

Table 1
A list of topological studies of the folding of AAC in the mitochondrial membrane

AAC in	Probe	Sequence region	Conditions	Conclusion	Reference
C-, N-terminals					
BHM	N-terminal antibody C-terminal antibody	N-terminal positive C-terminal negative	BKA+	N-terminal at c-side C-terminal masked	[459]
UCP1 as AAC analogue	Trypsin	C-terminal cleavage	(ATP)	C-terminal at c-side	[460]
Yeast-M	2-azido-naphtoyl-ADP	C-terminal	BKA+	C-terminal at c-side	[454]
Matrix loops					
BHM	Azido-ATR	C155–M200	–	Binding site at segment C155–M200 accessible from the c-side	[452]
BHM	Pyridoxal-phosphate	K162	CAT-BKA+	M2 loop accessible from c-side by entering the channel	[444]
BHM	2-azido-ADP	F152-M200 T250-M281	–	Binding site at loops M2 and M3 accessible from c-side	[450]
Yeast-M	2-azido-ATP, 8-azido-ATP	G172 – M210	–	Access from c-side loop M2 protrudes into channel	[453]
BMH-SMP	Trypsin Lys-protease	Cleavage at K42, K146, K244	BKA+ CAT–	Proposal that C-Loops (otherwise M1and M2) intrude channel from c-side	[461]
Yeast-M	2 azido-naphthoyl-ADP	S183-R191 I311-K318	–	M2 loops intrudes channel, additional C-term. contact	[454]
Yeast-M	Trypsin	cleavage at K163 at K178	CAT+ BKA+	Loop M2 protrudes from m-side into membrane	[462]
	Eosin maleimide (EMA) access	S161C	BKA+	M2 protrudes into channel	
BHM-SMP	EMA-access	C159		M2 loop m-side gated by M1 with CAT	[342,343]
BHM	C-C crosslinking (Cu-phenanthroline) (bis-maleimide)	C56–C56	BKA+ CAT–	Inter-subunit contact indicates large M1 loop mobility	[267] [268]
Cytosol loop					
Yeast-M	C-scanning by charged SH reagents	106–132	BKA– CAT+	C1 loop changes on transition m-to c-state	[466]
Transmembrane area					
BHM	Pyridoxal-phosphate (PLP)	K 91, 98, 95 K198	BKA+ CAT-	c-side end of TMH2, TMH4 unmasked in m-state	[444]
Yeast-M	"Cys scanning" EMA access	F98 to F107	BKA+ CAT (−+)	Twisting of TMH2 on transition c→m-state	[445]
BHM SMP	Pyridoxal phosphate	K42, K48 K146	BKA+ CAT (−+)	Access from m-side unmasked in m-state, incorporation only in SMP, not in BHM	[444]

BHM, bovine heart mitochondria; SMP, submitochondrial particles; Yeast M, yeast mitochondria; TMH, transmembrane helices; CAT, BKA+ stimulated, CAT, BKA− inhibited by CAT or BKA.

anti-conformation is a closer analogue of ADP that also has a preferred *anti*-conformation, whereas 8-azido derivatives are in the *syn*-conformation. As a result 2-azido-ADP has a higher binding affinity to the AAC than 8-azido-ADP [201]. In BHM 2-azido-ADP photo-labels again mostly AAC [201], but no incorporation to the AAC is seen in SMP [450] demonstrating the c-side specificity of 2-azido-ADP binding.

After the amino acid sequence of bAAC1 [49] became available the labelled sites were allocated in the sequence. Mapping of the 2-azido-ADP ^{32}P-labelled peptides revealed [450] two target sites in the sequence, the major one at F153-M200 in the second repeat with incorporation at K162, K65, I183 similarly to azido-ATR (see below), and the second in Y250-M281 which include the last TMH and C-terminal peptide. Since both labelled segments were accessible from the c-side a folding pattern was adopted with only 5 TMH placing these segments at the c-side [450]. In previous studies various azido-ATR derivatives had been synthesised and their specific incorporation to bAAC1 was assigned [451,452] to the segment C159–M200 which corresponds to the first region of 2-azido-ADP incorporation. These results indicate that ATR and ADP binding sites are not separate as was suggested by the results with N-ADP binding [8].

Photoaffinity labelling of the yeast AAC-2 was performed with both 2- and 8-azido-ATP [453]. Different from 2-azido-ATP, labelling with 8-azido-ATP required isolated AAC because of its lower affinity. Identification of labelled peptides from yAAC2 was easier because CNBr cleavage sites are more evenly distributed than in b-AAC1. Both with 2- and 8-azido-ATP all the incorporated label was confined to the A175-M210 segment, which encompasses matrix loop M2 and the adjacent TMH4. The absence in yAAC2 of the second site found in bAAC1 [450] suggests that the secondary site results from a side reaction of 2-azido-ADP. This interpretation was supported by the finding that in yAAC2 with 2-azido-naphtoyl-ADP (azido N-ATP) [454] only the C-terminal stretch I311-K318 was labelled. The naphtoyl group may confer less specificity and derange the binding into a less precise orientation. This reasoning is at variance to Dianoux et al. [454] assuming a sort of half site reactivity between the monomers in the dimer AAC in which the substrate alternates between the two different sites in step with the transport and the 2-azido-naphthoyl group reaches out to the C-terminal of the other monomer.

It must be noted that the binding of azido-ADP and ATP to the central region (A175-M210 in yAAC2, Y153-M200 in bAAC1), seemed to be at odds with the six TMH folding model where this region is at the matrix side while the azido-nucleotides as well as azido-ATR bind from the c-side. At variance to Dalbon et al. [450] these results were interpreted, by retaining the 6 THM structure, to support the view [444,453] that this region intrudes into the translocation path where it can be reached from the c-side [383]. Analogous hairpin structures were proposed to exist in K^+-channels [455] and confirmed in the crystal structure [456]. The strong mobility of this segment in AAC was further elucidated by SH-probes (see below):

14.3. Immunochemical properties and mapping of the AAC

Polyclonal antibodies raised against native CAT–AAC (bAAC1) complex reacted [457] in immuno-precipitation specifically against the CAT–AAC and not with the unloaded or the ADP and ADP plus BKA loaded AAC. Vice versa antiserum against the BKA–AAC complex did not react with unloaded AAC or CAT–AAC. SDS treated AAC was not recognised by both antisera. Thus these antibodies display conformation specificity reflecting the differences in antigen exposure between the c- and m-state of the AAC. The CAT–AAC antiserum inhibited transport only weakly in mitochondria but fully in reconstituted vesicles, probably due to restricted access in mitochondria [260,457]. Interestingly, this antiserum prevented the replacement of ^{35}S-CAT by BKA (+ADP) but did not influence the exchange of ^{35}S-CAT against CAT. Obviously the antibodies did not "cover" and recognise the CAT-site but "clamped" the AAC in the c-state conformation. These antibodies apparently reacted preferentially with the matrix side of the AAC where most charged residues are located, indicating large structural rearrangements. Applying quantitative immuno-absorption techniques [260] the cross-reactivity between c- and m-state antibodies was determined to about 30%.

In another work antiserum against the CAT–bAAC1 [458] were assigned to react with the C- and N-terminal regions, and antiserum against SDS–AAC to react with the central region of AAC. Among antibodies raised against synthesised short peptides of the N- and C-terminal, only the N-terminal antibody reacted with the AAC in mitochondria [459] which provided evidence that the N-terminal extends to the c-side and that the C-terminal is masked. In the presence of BKA the N-terminal antibody did not react, suggesting a recessed N-terminal in the m-state.

14.4. Limited proteolysis

First indication for the c-side location of the C-terminal came from limited proteolysis of the uncoupling protein (UCP1), a homologue of AAC with the same folding pattern [391]. Limited proteolysis of UCP1 in mitochondria cleaved a C-terminal peptide of 10 residues without affecting the function [460]. In limited proteolysis of bAAC1 by trypsin and C-endoprotease in mitoplasts and SMP [461], cleavage was observed only in SMP loaded with BKA at sites identified as K42 and K244, which are localised in the intrahelical loops of the first and third repeat, suggesting that both sites face the m-side and are retracted in the c-state. These results seemed to contradict a c-side localisation of these loops deduced from 2-azido-ADP labelling [450]. To reconcile these results with a 5 TMH folding model a variable intrusion of the putative c-side loop into the membrane space becoming accessibly to proteases from the m-side was discussed [461]. This folding contrasts to the model, reviewed above, of an intrusion of the matrix loops within a 6 TMH fold. In yeast mitochondria, exposure to trypsin produced selective cleavage at two sites in the central loop; K163 with CAT and K178 with BKA [462]. The reason for this difference between yeast and bovine AAC in mitochondria is not clear. But the yeast results are consistent with the accessibility of this loop from the outside, as concluded from 2- and 8-azido-ADP labelling in yeast mitochondria [453] and in support of the postulated intrusion of this loop [383].

14.5. SH reagents as conformation probes

Three cysteines occur in conserved three repeat positions in the intramembrane matrix loops in AAC from mammalian sources [383]. As reviewed above, the ADP dependent inhibition by NEM of transport and ADP stimulated NEM incorporation were interpreted to require the m-state of the AAC [2]. Further work on SH groups in AAC addressed the identification of the targets of the SH reagents in the sequence and their use in probing conformation changes. NEM was shown [463] to target C56 located in the first repeat of bAAC1. The fluorescent, membrane impermeable maleimide derivative EMA accesses the AAC only in SMP [464] and was shown by micro-sequencing to label primarily C159 [342] located in the second matrix loop of bAAC1. While the reaction of EMA was rapid, NEM labelled C159 much more slowly. C128 remained inaccessible for both reagents and C256 was weakly labelled by EMA. The incorporation of EMA in SMP was inhibited by high concentration of ADP and by BKA, but by CAT only when added before sonication [343]. The results suggest that the central loop M2 moves into the translocation path and exposes in the m-state C159 to EMA. Different from NEM, EMA inhibits transport in SMP, indicating that it shares with BKA and DAN-AMP an overlapping binding site in the m-state. The eosin moiety, which has an analogous structure to adenosine, may bind to the substrate site near C159 and thus enhance the local concentration for the maleimide moiety to rapidly react with C159. Further the two anionic charges may increase affinity to the

cationic sites at the entrance of the M2 loop [342]. Eosin Y (EMA without maleimide) is an effective fluorescent ligand for the binding site in the m-state [465]. The fluorescence response to BKA, CAT and ADP characterise eosin Y as an m-state ligand in line with the results for EMA.

A covalent $(AAC)_2$ dimer of bAAC1 linked by a C56–C56 bridge was generated by oxidative crosslinking with Cu-phenanthroline [267]. The crosslinking was only possible in SMP and was inhibited by loading the mitochondria with CAT before sonication. Using divalent SH group reagents of various spacer length crosslinking exclusively between C56 was also observed [268]. With these agents the flexibility of C56 between the monomers was estimated [268] to allow a maximum movement of 12 Å of the M1 loop. Both types of crosslinking inhibited transport in the SMP [268].

The folding problem of the central loop was further approached in yAAC2 by the S161C mutation [462]. EMA reacted with this SH group in mitochondria treated with BKA but not with CAT. The accessibility of the central loop from the c-side conforms with the results obtained with the lysine reagent PLP [444]. In another probing of the translocation path of yAAC2, in 9 positions of TMH2 close to the c-side single Cys were introduced and exposed to EMA [445]. Whereas in CAT loaded mitochondria nearly all these residues were protected in accordance with the crystal structure, under ATR 3 Cys positions reacted with EMA and under BKA other 4 Cys residues. This is interpreted to reflect a twisting of TMH2 on the transition from the c- to m-state. A similar approach [466] was applied to the first external loop C1 consisting of a small coil, flanked by the TMH2 and TMH3. Differences of EMA labelling between BKA and CAT loaded mitochondria were more pronounced in the N-terminal part of C2 and linked to the twisting described for the C-terminal of TMH2 [445]. By arranging the residues in a helical wheel the observed EMA access could be shown to be concentrated at one side. With the exception of K108C, in the BKA induced m-state the upstream residues were less accessible, whereas in the downstream region of TMH2 the differences between c- and m-state were minor. A role of K108 in binding was suggested since K108C was completely protected by CAT. Further, by attaching positive or negative charges to the SH groups with MTS reagents, transport became inhibited by implanting a negative charge at K108C whereas the response at other residues was relatively weak.

15. The crystal structure of AAC

For obtaining the long awaited crystal structure of the AAC [297] the bovine heart CAT–bAAC1 complex was the most favourable candidate because of its high stability in non-ionic detergents [254,256,259]. The early introduced [185] but otherwise little applied LAPAO was obviously superior to dodecyl-maltoside, the most popular detergent for crystallisation of membrane proteins. Independently, two-dimensional crystals of yeast ATR–yAAC3 complex have been resolved [277] to 8 Å by electron diffraction. Both structures agree showing a six helix barrel fold with no dimer formation. The 2.2 Å structure has been the principal source for understanding the architecture of the AAC and the discussion of a possible mechanism.

With the crystal structure some striking features pointed out in the amino acid sequence [49,383] and modelled into transmembrane structures [382,391] assume a new more precise and definite meaning [9,278,297,467] (Fig. 8A and B). While the structure contains many features which agree with the conclusions drawn from mapping the AAC folding by biochemical and physical methods, as reviewed in Chapter 6, there are a number of features at variance to those predictions. The basic fold (Fig. 9) of a barrel of 6 transmembrane helices (TMH) consisting of three repeat domains containing each two TMH [382] around a pseudo-threefold axis agrees with the predictions. But the TMH are longer than modelled by the hydrophobic algorithms and are tilted, thus accommodating the long TMH within the membrane area. They form a quite

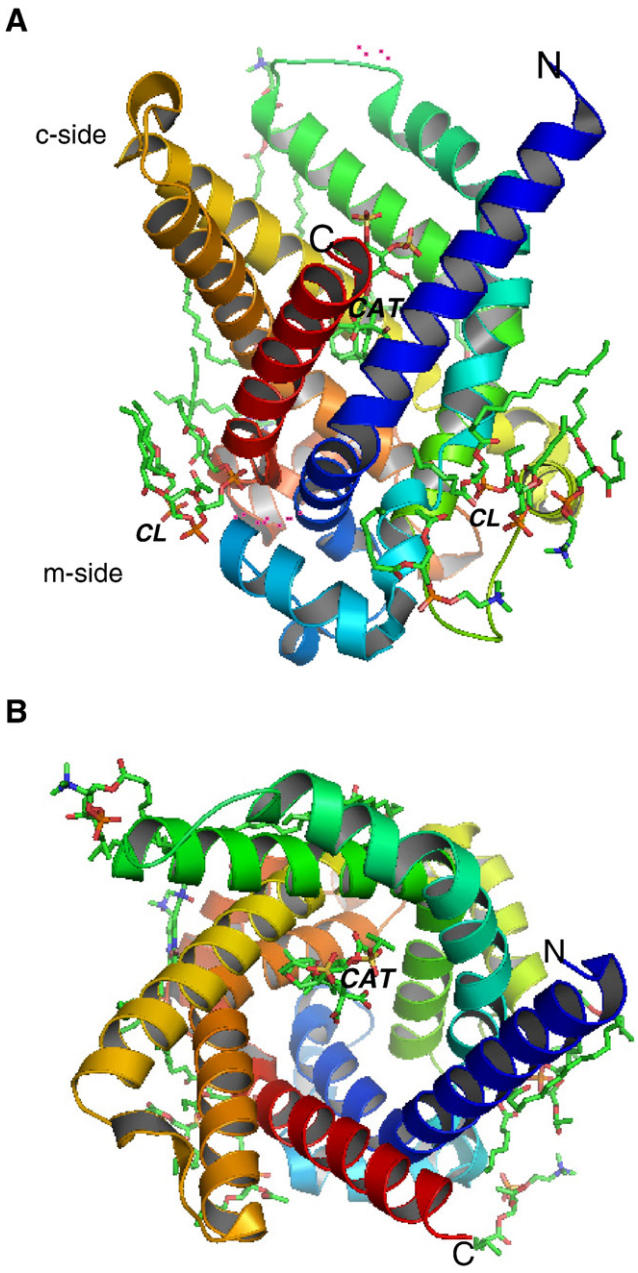

Fig. 8. The 2.2 Å crystal structure of the CAT–AAC complex (bAAC1) as a ribbon diagram. A. View from the membrane plane with 6 transmembrane helices (TMH) surrounding the c-side cavity. The positions of CAT bound in the cavity and of cardiolipin (CL) at the membrane interface are indicated. B. View vertical to the membrane from the c-side into the open cavity walled by 6 TMH. CAT bound in the cavity and some surrounding CL are shown. The TMH converge to close the cavity at the m-side. Adapted from [297].

regular wall surrounding a basket type cavity extending into the center of the membrane. Near the m-side in each repeat domain, the odd numbered TMH1, 3 and 5 are bent at the threefold conserved prolines, interrupting the TMH with a brief coil. The continuing short helix contains the mitochondrial carrier motif PhD/EXXK/RhK/R (see above) including the AAC signature motif RRRMMM in the third repeat domain. The following matrix side segments M of each repeat turn the chain towards the center axis and thus contribute to the closure at the matrix side. Small, 11 residues long helices in the M segments run nearly parallel to the membrane. They also contain conserved charge clusters, forming at the end the second less stringent motif D/**EGXXXXΦK/RG** (Φ aromatic residue) (Fig. 9). A threefold circular salt bridge network of intrahelical charged residues (E29-R137, D134-R234, and D231-K32) locks the base of the cavity by tying together the m-sides of the odd

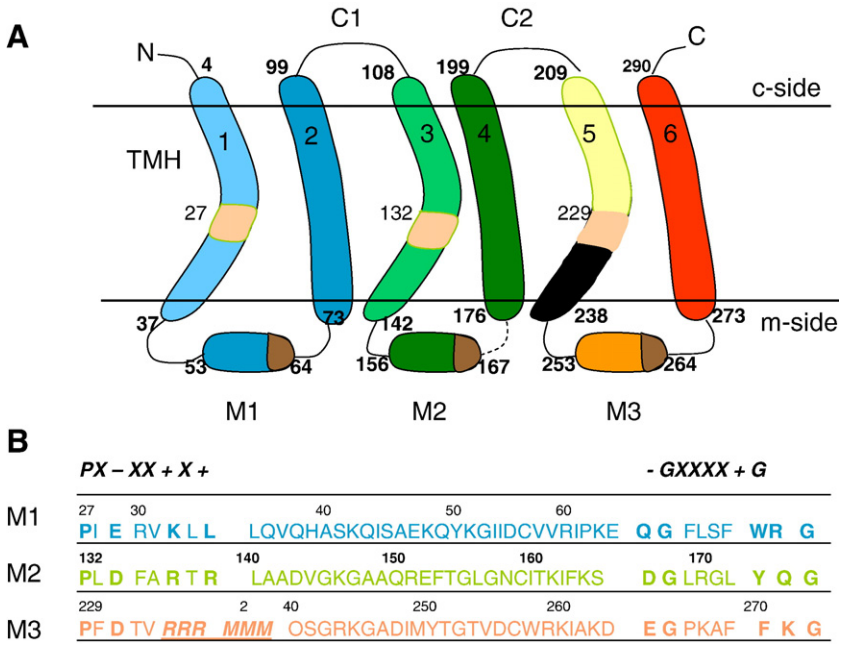

Fig. 9. A. Principle folding diagram of bAAC1 based on the crystal structure of the CAT–AAC complex. Each of the three repeat domains contains a TMH which is bent at an intrahelical proline. The pink areas contain MCF motifs given in part B. The AAC specific signature in TMH5 is enhanced. The matrix regions contain short helixes. B. Parallel alignment for similarity of the 3 m-side sequences (M-loop) including two MCF motifs. The AAC specific signature in M3 is highlighted. Adapted from [9].

numbered TMH. These salt bridges have been predicted by second site revertants [396]. CAT sits in the cavity with the aglycon carboxyatractylogenin near the bottom and the glucose moiety near the c-side. The two carboxyl groups form salt bridges with R79 of the triad intrahelical Arg and hydrogen bonds. The two $-SO^{4-}$ groups form salt bridges to K91 and R187 and hydrogen bonds to N87 in the cavity wall near the c-side. The numerous positive residues lining the basket may steer ADP^{3-} and ATP^{4-} into the binding site, as well as strip Mg^{++} from the entering nucleotide complexes. Further a striking ladder of tyrosines on TMH4 has been suggested [9] contributing to a "non-sticking" steering of the substrate in the cavity.

16. Mechanism

16.1. Induced transition fit

Before reviewing the mechanism of the AAC mediated transport, a brief summary of the catalytic principles governing the carrier transport will be given that are important for the interpretation of function–structure relations of the AAC. The results obtained with the AAC have been a prime reason for conceiving a new description of transport catalysis called the "induced transition fit" (ITF) [187,212,468]. The most striking result leading to the single site reorientation mechanism was the intriguing "mobilisation" of the AAC by the substrate. It comprises not only the net translocation but a mobilisation of the whole structure with many intermediate steps. This conformational transition within a well defined trajectory of the structural coordinates is integrated with Brownian movements within the AAC, enhanced by ADP or ATP that might reach a maximum in the transition state.

To illustrate the ITF, the purely catalytic role of the AAC without external electrical forces as in the homo-exchange modes D–D and T–T will be considered. Being large hydrophilic substrates, ADP and ATP require large conformation changes in the AAC during the translocations entailing high energy barriers which can be compensated only by a strong substrate–protein binding energy provided by the transportable substrates ATP and ADP. On binding to the "ground" c- or m-state they mobilise the carrier to switch into the opposite ground state. Why would these substrates activate the translocation process, whereas other analogue nucleotides are "stuck" in the carrier? The ITF of transport catalysis rationalises these observations and is best explained by comparison to enzyme catalysis. In enzyme catalysis the substrate changes, in carrier catalysis it is the protein which changes and by this token uses the substrate as a catalyst. In both cases the substrate–catalyst interaction must be maximised in the transition state for efficient catalysis. For this reason the substrates initially should not fit perfectly to the binding site of the catalyst (Fig. 10). For optimising the fit in the transition state, opposite interactions occur for enzymes and carriers: whereas in enzymes the binding site has evolved to optimally bind the transition state configuration of the substrate, in carriers the substrate remains unchanged but induces a conformational change of the carrier to attain an optimum fit for the substrate. Thus not only is a maximum fit of the substrate to the binding center attained, but also global changes in the carrier are facilitated leading to the closure and opening of the translocation channel at the opposite sides. The increased binding energy in the transition state drives the transfer of the carrier complexes $(CS)_i$ or $(CS)_e$ to the transition state $(CS)_T$. It should be added that the induction of substrate change in enzymes versus induction of protein change in carrier, does not exclude in the transition state local change in enzyme [469,470] and reversible changes in the substrate configuration during the translocation.

The comparatively loose binding of ADP and ATP (Fig. 10) and the high selectivity may seem to be a paradox, but actually were an essential guide to the ITF: the loose fit in the ground state contrasts to the perfect fit in the transition state where a precise match of substrate and binding site encompassing all parts of the molecule is required, explaining the high specificity. High intrinsic binding energy is needed to overcome the large conformation energies and facilitate the translocation. The energetic constraints of the ITF result in a preference of exchange versus unidirectional transport where the "return" branch of the carrier cycle is not facilitated by binding energy. Therefore, dependent on the size of the substrate and concomitant conformation changes, the obligatory exchange of AAC rationalises the unidirectional mode of P_i transport in mitochondria handling a small substrate requiring small conformation changes. In unidirectional transport of large substrates, the lack of intrinsic binding energy can be compensated by external energy such as in ATP dependent transport [471].

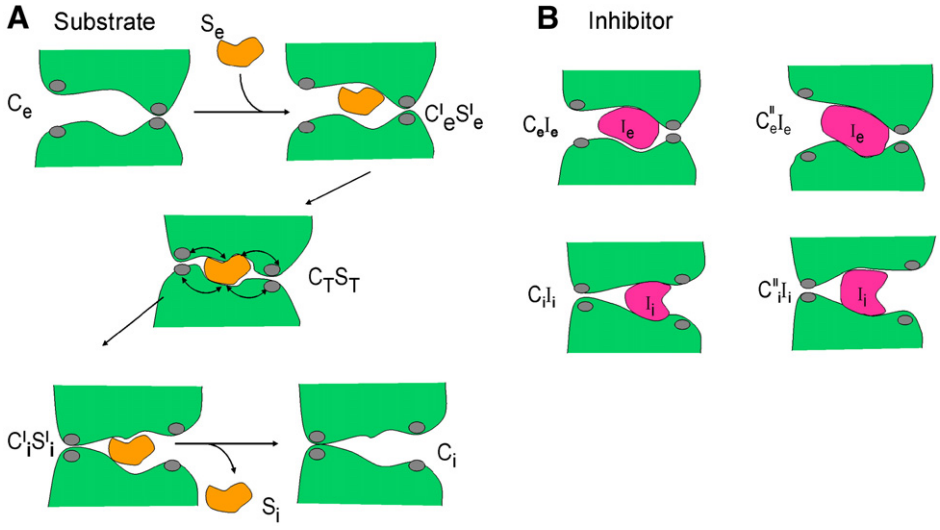

Fig. 10. Induced transition fit. The interaction of the carrier binding center with the substrate. A. Poor fit of substrate to the binding site in the ground states C_e and C_i of the carrier. The transition state is induced by an optimised fit of the substrate producing a high intrinsic binding energy. The changes of the binding center trigger global changes in the carrier leading to closing and opening of the opposite gate. B. Inhibitors are well adapted to the ground states for tight binding. The different interfaces of the opposite ground states require side specific inhibitors. Inhibitors can induce the abortive ground state conformations c′ or m′ to further increase the binding interaction. From [187].

Another important consequence of the ITF is the position of inhibitors in the transport cycle (Fig. 10). In contrast to enzymes where substrate analogues type inhibitors mimic the transition state configuration for tight binding, in carriers inhibitors must bind to the ground states and not to the transition state configuration. Transport inhibitors should be designed to better fit the binding site in the ground state than transportable substrates and thus produce high binding energy without an incentive to go into the transition state. Two types of inhibitors can exist, whether binding to the out- or in-ground state. For the AAC these inhibitors are represented by some nucleotide analogues (see above). Inhibitors using an enlarged binding surface, proposed to induce the ground states into "abortive" states, are seen to be represented by ATR, CAT and by BKA.

16.2. The single binding center reorienting mechanism of AAC

Transport mechanisms of carriers have relied for many years on kinetic studies [37]. With the ADP/ATP exchange in mitochondria it was possible for the first time to penetrate to the molecular level and to define the carrier sites and their binding dynamics [2]. The reasons for this paradigm role of the AAC are twofold: the high density of AAC sites in the inner mitochondrial membrane and the existence of exclusive high affinity inhibitors for binding to the outer or inner aspect of the carrier binding site. At first the non-linear binding of ADP and ATP and the effects of ATR on binding to depleted mitochondria provided an insight into the switching of the AAC binding site between the out- and inside [166]. This view treated the binding measurements not as an open equilibrium of the substrates with all carrier sites but interjected a translocation step by which a part of the binding sites, once loaded with external substrate, becomes oriented inside thus establishing an indirect equilibrium with the added substrates. With the arrival of BKA, the translocation of carrier sites from the intramembrane (cytosolic) to the matrix side, henceforth called the c- to the m-state (see [2,3]) could be demonstrated more directly, by conceiving and experimentally verifying that the BKA induced binding of ADP or ATP reflected in fact trapping of translocated ADP or ATP inside [153,171] where BKA displaces ADP and ATP from the binding site [172]. The complex binding phenomena of ADP and ATP involving ATR and BKA, the cooperative effects, slow binding kinetics, temperature and pH effects caused by the rate limiting permeation of BKA could all be reasonably rationalised [3,266,472–474] by the single reorienting binding center-gated pore mechanism (SBCGP). The possibility to fix all carrier molecules in mitochondria on the out- or inside or in the c- and m-states gave a unique insight into the operation of a carrier.

This view implied that the inhibitors ATR, CAT and BKA as well as ADP and ATP bind to the same site. However in another work the single site concept was not accepted and separate sites for ADP and BKA [168] or, on the basis of ATR spin labels, "α and β" subunits on the outer and inner membrane leaflet were proposed [218]. Based on amino acid probes different binding sites for CAT and BKA and ADP were postulated in a series of papers [8,237,238]. On the basis of fluorescent ligands [196,205,208,211] BKA was proposed to act as "allosteric" effector from a separate site. Further the "BA-state" was concluded not to represent the inside orientation [208] On the basis of BKA binding studies the existence of a stable ternary complex was conceived [168] and based on intrinsic fluorescence studies, a transitory BKA–ADP–AAC complex was proposed [169,216].

An essential consequence of the single site reorienting mechanism is the change in the aspect of the binding site between the external and internal state (for AAC c-state and m-state) engaging partially different residues [2,473]. Whereas the substrates are bivalent, the inhibitors are specialised for the c- and m-states. In fact CAT, ATR and BKA are proposed to engage more residues than the substrates and induce a conformation change of the "ground" c- or m-states into the "abortive ground" c′- and m′-states [212,473] (Fig. 10) moving the AAC structure further away from the transition state. This difference is monitored by various signals, such as by CAT induced resistance to proteolysis [184,256], intrinsic fluorescence change [216], spin label immobilisation [220]. In view of this difference between the abortive and ground states, the term "CAT-" and "BA-state" for the c- and m-state can be misleading [8,9].

16.3. Structural changes on c- to m-state transition

The following is a distillate of the various types of evidence reviewed above for the structural changes involved in the translocation. With the isolation of the AAC large differences in the stability of the protein in the detergent micelles towards proteolysis were noted, indicating an overall more flexible structure in the m- and m′-state with BKA as compared to the maximally rigid c′-state with CAT [184,256]. By the same criteria the AAC attains maximum flexibility

with ADP. Within the tripartite structure mobility seems to be especially high in the three loops, connecting the transmembrane helix pairs on the matrix side. This was first noticed probing lysine residues [444], showing that the matrix M2 loop is accessible from the c-side, leading to the proposal that part of this loop intrudes the translocation channel in the m-state. Further support came from labelling M2 in BHM with 2-azido-ADP [201] and M2 in yeast mitochondria with 2- and 8-azido-ATP [453] as well as with azido-ATR [210,452], although the results were interpreted differently [201,210,452]. In yAAC2 S161C located in M2 and adjacent to TMH3 was labelled from the c-side in the c-state [462]. The variable accessibility to three similarly positioned cysteines in M1, M2 and M3 of each repeat domain in bAAC1 provided strong and specific evidence for configuration changes. NEM reacted with M1 C56 only in the m-state [2,342] whereas the polar EMA reacted with C159 in M2 from the m-side only in the c-state. A gating role was assigned to the matrix loops: M1 closing in the c-state and M2 closing in the m-state [343]. Large fluctuations in the m-state of M1 were deduced from C56-C56 crosslinking studies in the bAAC1 dimer [268]. The external N- and C-terminal segments changed their exposure from c- to m-state as shown by immuno-assays [459] and proteolysis [462]. All these results underlined the c- to m-state rearrangement of the M-loops in the channel. Configuration changes in TMH2 probed in yAAC2 [445] by "Cys scanning" were interpreted as a 180° twisting of TMH2 going from the c- to m-state. In hindsight the lysine residues access data [444] are compatible with these changes of both TMH2 and TMH4 in bAAC1. The homologous repeat helices apparently move in a coordinated fashion rendering these charged groups inaccessible on transition to the c-state.

16.4. Crystal structure and mechanism

Any considerations about relating the transport mechanism to the crystal structure must take into account that according to the ITF the AAC–CAT complex represents an extreme c-state of the highly mobile carrier machinery frozen by the binding of CAT [187,383]. In this abortive c′-state the carrier configuration is probably somewhat distorted to a more extreme c-side opening than in the "empty" c-state which is the starting state for the translocation cycle where the cavity may be narrower with still enough space to permit passage of the substrate and CAT to the binding center. Without CAT the matrix area appears less tightly connected as suggested by easier access to proteases and reagents in the c- than in the c′-state [256]. An important conclusion to be drawn from the crystal structure is that ADP and ATP may share the binding site with CAT, in agreement with the single binding center gated pore (SBCGP) mechanism [2]. Thus the crystal structure does not support the tenet [7,8,214,475] as reviewed above, that there are two binding sites for CAT and for BKA and, according to some models, there are segregated outer and inner binding sites for ADP or ATP [475]. In other versions inhibitor and nucleotide binding sites overlap so that there remain two binding sites for simultaneous ADP and BKA binding [7].

When trying to fit the nucleotide into the binding site, a central issue of the ITF must be considered: a poor binding of the substrates in the ground state and optimum binding in the transition state. Candidate residues for ADP, ATP binding are the basic residues located, according to the derived and the crystal structure, within the TMH and that are partially involved in CAT binding: R79, R187, R279, and K22 in bAAC1 and, by analogy, R 96, R204, R294, and K38 in yAAC2. Whereas the intrahelical Arg are variably conserved in the MCF, in TMH1 bK22/yK38 are conserved in all AAC known [476]. The yAAC2 mutants, K38A, R96A have strongly reduced, R204L no transport activity, whereas R294A is nearly fully active [66,477]. Importantly, in all mutants the transport modes with ATP are more affected than with ADP, this effect being most dramatic in R294A on a background of a normal D/D transport. This may suggest that the γ-phosphate of ATP is close to the discriminating R294 in yAAC2 or homologue R279 in bAAC1. In the CAT–bAAC1 structure these residues together with Y186 form a "bottleneck" of about 8 Å diameter above the bottom of the cavity. This structural element was called a selection filter [9], a term used for ion channels but less useful for carriers, unless one may associate this term with the high selectivity of the transition state required by the ITF. In an "*in silico*" study based on the crystal structure [467] this configuration on omission of CAT is assumed to be a general binding site for MCF substrates including ADP or ATP. However, it must be cautioned as emphasised above that according to the ITF, the CAT–AAC structure may represent a distorted ground state, where binding elements may be moved out of their normal positions by CAT.

Pointing out [159] that the common 3 or 4 negative charges are segregated in the inhibitors (ATR, CAT, BKA, acyl-CoA) but clustered in ADP and ATP, it was speculated [159] that inhibitors by pulling the positive residue apart cause a change in the binding center. Accordingly in the ligand less ground c-state, K22, R79, R279 may be in an intermediary position and initially not well adapted to the di- or tri-phosphate moiety. In *in silico* extrapolations of the AAC crystal structure [478], ADP is projected to interact with the three cationic residues but the distances of the phosphate groups to R79 and K22 are large, allowing for only weak bonding. This seems to agree with the concept that the CAT–AAC structure represents an "abortive ground" state where the binding center should be wide open and offers only a poor fit to the substrate. Without CAT these residues might be somewhat differently arrange. At any rate, after initial lose binding of ADP the positive residues might be pulled closer to the phosphates, and thus the binding center driven into the transition state. In addition other residues of the AAC contribute to binding, including the well positioned Y186 [9]. These and further residues will engulf ADP or ATP in the transition state resulting in a maximum intrinsic binding energy. The energy released from those local changes propagates to global shifts of the TMH and the matrix regions going into the m-state.

Coming from the c-state, the translocation steps require removal of the barriers constituting the m-gate. One step is pivoting the C-terminal parts of the kinked helices away from the center. The intrahelical prolines have been proposed to serve as hinges for that movement [297]. In the third repeat domain this C-terminal part of TMH5 is connected to neighbouring TMH6 by a salt bridge R236-E264, which has to be broken. It is unclear why specifically ATP with its additional negative charge, has been suggested to cleave this bridge [9] as if ADP takes another passage. Another critical step should be the opening of the three circular salt bridges linking these helices at the m-side. Here the oscillating charge model [479] may come into play specifying that the translocation path contains a mobile electrostatic system that responds to the arriving ADP^{3-} and ATP^{4-}. Strategic negative residues in salt bridges are assumed to be mobile and weakly bonded and can be pushed by the negative substrate clusters to find other electrostatic compensation. In this way the substrate may cleave the salt bridge circle for opening the m-gate. Simultaneously the c-gate will be closed and a cluster of positive and negative charges at the C-terminals of the even numbered TMH seems to invite for a similar circular salt bridge closure. The twist of TMH2 and also of TMH4 between the c- and m-state [445] may contribute to this gating. The masking of the adjacent upstream half of loop C1 on transition into the m-state [466] suggests a participation of these loops in the c-gate. The charged residues K108, D109 and K112 may be involved by switching their ion bonds. In particular K108 may play an active role in the c-gating.

For comparison it should be noted that in the homologue oxoglutarate carrier (OGC) the TMH were probed by Cys scanning [480,481] and the influence of MTS type SH reagents on transport was determined. The effects were evaluated on the basis of the OGC structure extrapolated from the AAC crystal structure. It was pointed out that in OGC one of three salt brides of AAC is missing and thus, in

Table 2
A list of research highlights on the AAC in chronological order

Year	Result	Details	References
1964–65	Discovery of ADP, ATP exchange.	Differentiation of uptake into intermembrane and matrix space. Identification of transport of ADP and ATP through inner membrane, excluding AMP. 1:1 exchange with endogenous pool.	[20,26,34]
1965–66	Atractyloside (ATR) inhibitor of ADP/ATP exchange	Direct evidence that ATR inhibits exchange with endogenous pool but not uptake into intermembrane space.	[26,33]
		Indirect evidence: ATR inhibits only phosphorylation of exogenous but not of endogenous pool.	[30,22,23]
1966–71	Membrane energisation ($\Delta\mu H^+$) controls ADP/ATP exchange	In "energised" mitochondria: preferred ADP uptake and ATP release.	[34,42]
		In de-energised state (uncoupling, K^+-valinomycin) no difference between ADP and ATP transport.	
1970	Identification and dynamics of carrier binding sites with ADP and ATP.	Titration of ADP and ATP binding to AAC carrier sites in membranes. Differentiation with ATR. Biphasic concentration dependence explained by ADP, ATP driven reorientation of binding sites.	[166]
1970–72	Electrophoretic ATP^{4-}–ADP^{3-} exchange.	Membrane potential $\Delta\psi$ identified as driving force in ATP^{4-}–ADP^{3-} exchange by ionophores.	[89,90]
1970–72	BKA inhibits exchange but has other opposite effects to ATR	BKA increases apparent ADP, ATP binding.	[151,170]
		BKA blocks, ATR stimulates efflux.	[246]
1970–75	Molecular demonstration of a single binding center gated pore mechanism.	ADP, ATP mobilises the binding site transition between out-(c) and in (m) states: ATR, CAT fix the carrier sites in the c- and BKA in the m-state.	[153,171]
	Experimental definition of "c and m-state"	The apparent BKA induced increase of ADP, ATP binding is explained as trapping of ADP, ATP inside.	
1972	$(ATP/ADP)_{cyt} > (ATP/ADP)_{mito}$ $(\Delta G_{ATP})_{cyt} > (\Delta G_{ATP})_{mito}$.	$\Delta\psi$ driven ATP/ADP gradient causes higher energy level of ATP in cytosol than in mitochondria.	[98,99,100]
1972	Decrease of P/O by $n/n+1 (n=3)$	Export of ATP lowers yield of oxidative phosphorylation by 25%, by diverting 1 out of 4 H+ pumped by electron transport.	[82,81,89,102]
1972–76	Strategic SH groups.	ADP induced NEM unmasking.	[69,70]
	Indicators of c–m conformation changes.	Evidence for NEM unmasking reflects m-state transition.	[2]
1975	Isolation of CAT–AAC complex.	Solubilisation and purification of CAT–AAC from bovine heart using Triton X100. First isolation of a biomembrane transporter in intact state.	[254,183]
1977	Reconstitution of transport with isolated AAC.	First reconstitution of transport with an isolated carrier protein. Acidic lipid requirements.	[279]
	$\Delta\psi$ controls V_{max} not K_M	Demonstration with reconstituted AAC.	[107]
1982	Complete amino acid sequence of the AAC.	Sequence of AAC from bovine heart. First primary structure of a mammalian transporter.	[49]
	6 transmembrane helix 3 repeat domain model	Hydropathy and repeat search evaluations of amino acid sequence.	[382]
1982	Dimer structure of isolated AAC.	Hydrodynamic measurements of AAC-detergent micelle in Triton X100.	[263]
1983	Matrix side (M) loop protrudes into translocation path accessible from c-side.	Pyridoxal phosphate accesses to lysines, located in matrix (M) loops from the c-side.	[444]
		8- and 2-azido-ATP incorporation to M-loops from "c" side.	[453]
1985	High level tight association of cardiolipin to AAC.	6 mol CL oer AAC dimer tightly bound and dissociated only by SDS at >30°, as detected by ^{3}1P-NMR.	[252]
1988	AAC role in mitochondrial pore transition	Ca^{++} induced pore transition has opposite response to CAT and BKA.	[313]
		Dependence on cyclophilin.	[320,321]
1991	"Induced transition fit" of transport catalysis	ITF is evident in the mobilisation by of ADP, ATP of AAC transition from c- to m-dtate and the paradox of low binding affinity and high specificity, the stabilisation of AAC by CAT or BKA.	[212,187]
1993	Hydrophilic EMA accesses central M2-loop	Conversely to M1, the SH group in the central loop M2 is accessed by EMA but blocked by BKA indicating direct role in translocation path.	[342,343]
1995–98	Charge pair network between odd numbered TMH at the matrix side of AAC	Spontaneous second site mutations in yAAC2 compensate primary charge defects by opposite charge deletion at odd numbered TMH.	[396]
1996	Large Ca^{++} activated channel in isolated AAC.	Single channel patch clamp with reconstituted AAC, voltage gated channel closed by BKA not by ATR.	[292]
		Direct support for role of AAC in "mitochondrial pore transition".	
1996	Electrical currents measured with ATP^{4-}, ADP^{3-} in reconstituted AAC	Flash photolysis of caged ATP, ADP with AAC proteoliposomes adsorbed to planar membranes.	[93,94]
		Measurement of currents caused by charge movements of ADP and ATP and of the reorienting AAC binding center	
2003	High resolution 2.2 Å crystal structure of CAT–AAC complex.	c-side open cavity with CAT bound in pseudo 3$^-$ fold symmetry. The 6 transmembrane bundle is skewed to the membrane. Odd numbered TMH are kinked. Evidence for single binding site.	[297,9]

accordance with the ITF [187,212], that the smaller substrate binding energy requires smaller rearrangement energies.

The unusual high level of cardiolipin (CL) binding to bAAC1 [252] has been implicated in balancing the high conformation energy requirements during the translocation [272,337]. Most notably, CL molecules are also present in the crystals of the bAAC1–CAT complex [297]. The special structure of CL sporting two phosphate groups seems to be used to pull together two TMH between adjacent repeat domains. The hydrogen bonds of CL extend close to the proline of the neighbouring uneven TMH. The position of the CL head groups at the region in AAC, where strong rearrangements are to be expected, suggests a significant role of CL in transport catalysis of AAC. For example, in the transition state the dual grip of CL on even numbered TMH and the m-helix might be relieved concomitant with the opening of the m-gate. Thus CL may change to other binding positions in the m-state.

In a second crystal form C222, generated at low salt concentration, three CL per monomer are visible [278], showing that all tightly bound CL molecules [252] are retained. CL is positioned at each repeat domain with their head group towards the m-side. In the C222 crystals contacts between two monomers can be discerned. The interface is prominently filled with two CL around the twofold dimer axis, which seems to bond the subunits [278] in addition to contacts between the C- and N-terminals. At the dimer interface CL forms an additional salt bridge to K267 in TMH6 of the facing monomer.

16.5. Dimer mechanism

A much discussed issue has been the role of the AAC dimer in transport. Although the single binding gated pore mechanism and the overriding induced transition fit mechanism are noncommittal whether a dimer or monomer are the functional unit, various types of evidence and the implication for a functional dimer role are reviewed here. Originally it was speculated, in view of one CAT molecule binding per dimer, that the translocation channel passes between the two monomers [2,472]. However the asymmetry of the binding site would be incompatible with a channel at the two fold axis [3]. With the much preferred model of two separate translocation channels in each subunit, the question arose whether they act independently or cooperate by inter-subunit contact. Kinetic studies, although they may be flawed (see above), claimed a simultaneous mechanism of ADP–ATP transport [71,72], requiring two binding sites which might operate by a reciprocating exchange between both units in a

dimer. This mechanism implies that equal numbers of subunits are in the c- and m-state, contrary to the evidence that all sites can be trapped in one state.

Based on the results of fluorescent substrate probes and the stoichiometry of CAT binding the formation of AAC tetramers in the mitochondria was proposed [7,8,475,482], containing 2 sites for CAT and for BKA and a total of 4 nucleotide sites, 2 in the c-state and 2 in the m-state. Whereas a tetramer or dimer may have essentially compatible mechanisms, the stoichiometry of binding sites differs. From fluorescence titrations with FTP, two nucleotide sites per CAT binding site were deduced [205], while with N-ADP and other nucleotide derivatives approximately 1:1 binding ratio was reported [482]. Direct measurements with radio-labelled ligands resulted in about equal amounts of binding sites for DAN-ADP and CAT or BKA [207]. Binding studies showing equal amounts of high and low affinity sites were interpreted in terms of negative cooperativity and functional interdependence in the oligomeric structure [8,450,482]. At variance to the single binding center reorientation (SBCG) mechanism, the effects of BKA on nucleotide binding, such as abolishing the non-linearity and negative cooperativity of nucleotide binding, were taken to show that BKA acts from a separate site [8,214].

Engineered covalent dimers of the AAC support the existence and function of a dimer AAC. A tandem dimer with the C-terminal covalently linked to the N-terminal of yAAC2 was expressed reaching the same level as wt yAAC2 [483]. The fusion homodimer had similar transport activity as the wt AAC. In another report on yAAC2 tandem constructs a slower cell growth was noted [484]. The fact that wt–wt tandem fusion products are well expressed and show transport activity equivalent to wt in mitochondria and in proteoliposomes argues for a functional dimer but does not specify independent or cooperating channels. A chimer fusion between wt and op1 mutant [484] had no negative influence on aerobic growth. The transport rates of the yAAC2 chimer construct wt-mutants and wt–wt were compared [485] in mitochondria and reconstituted vesicles. R294A was chosen for generating the chimer wt-294A, because this mutant is basically as active as wt but has an opposite preference for ADP versus ATP (see above). Further wt-R204L was constructed to match wt with an inactive mutant. With wt–wt and wt-R294A the growth on non-fermentable source was half as fast as with the control strain, but it was strongly delayed with wt-R204L. Whereas wt–wt and wt-R294R were well expressed in isolated mitochondria, wt-R204L was not expressed but instead only non-fused wt was found in mitochondria. Obviously genetic splicing to wt-plasmid occurred under the aerobic growth pressure. After isolation and reconstitution, the tandem wt–wt had the same pattern of ATP versus ADP selectivity as wt. The wt-R294A chimer transport modes activities were only slightly dissimilar to wt, indicating an adaptation to the wt and interpreted as a partial interdependence. The non-survival of wt-R204L and R204L-wt seemed to indicate that these chimers are inactive, and that the wt segment was not able to compensate inactive R204L due to cooperativity between the units.

For reasons of analogy, a functional dimer interdependence in the phosphate carrier (PiC) may be quoted [486]. In a sophisticated design PiC was expressed with two different tags. One form was inactivated by NEM and mixed with the active form under various proportions and conditions. The formation of heterodimers was controlled by the two different tag signals. Reconstituted transport clearly depended on the formation of homodimers, while heterodimers were inactive. Recently this question was again approached [487] by co-expressing in yeast wt yAAC2 and the Cys-less mutant in various proportions. Before reconstitution the wt portion was inactivated by the SH reagent MTSES. The activity was linearly correlated to the active Cys-less portion and thus did not show the non-linearity expected from a functional dimer. However, the transport rates were measured at unusually low substrate concentration and thus were much lower than those reported in the previous reconstitution work of yAAC2 [66]. It is not clear how these extreme conditions may have influenced the results.

17. Conclusion

A summary of research highlights on the ADP/ATP transport and its carrier is assembled in Table 2. The major perspectives emerging from the research on the ADP/ATP exchange and its carrier are as follows. The ADP/ATP transport has to cope with a high and widely varying demand for ATP in the cytosol of the eukaryotic cell. An elementary regulation has evolved which tightly couples the ATP export to the cytosolic energy requirement and the activity of oxidative phosphorylation, via the $\Delta\mu H^+$. Within the transport capacity of the underlying carrier AAC the "productive" mode is modulated by $\Delta\psi$ creating an asymmetric activation energy profile which favours export of ATP. No other regulation by protein contact e.g. with ATP synthase seems to be involved. Besides this short term regulation the AAC expression levels can adapt over a wide range to changing cellular circumstances by regulation mechanisms not covered in this review. Dealing with large substrates ATP and ADP implicating large conformation changes, the AAC can be expected to be a "slow" carrier. In fact the turnover of the AAC is moderate but higher than that of other alternate nucleotide carriers of the MCF. The exceptional use of the fully charged ATP^{4-} and ADP^{3-} by the AAC assists in lowering the energy barrier and raising the transport rate. But still a high expression level is required, making the AAC the most abundant inner membrane protein of mitochondria or in some cases of the whole organ. With a comparatively small size and only 6 TMH, the AAC is obviously reduced to its basic transport function making it a foremost object for uncovering the essence of carrier catalysed transport. Passing a large substrate through a small protein should entail unusually large conformation changes. Therefore it is not surprising that the general "induced transition fit" of carrier catalysis has been inspired by the AAC. The elucidation of the molecular mechanism by various probes, has gained a solid structural basis in the crystal structure of the CAT–AAC complex, showing the apparent simplicity of the carrier. However, based on a structure in one extreme state, extrapolation of the changes during the translocation and the substrate protein interactions can only be approximate and the molecular mechanism must await structures which are more representative of the active AAC.

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