Probing the Role of Positive Residues in the ADP/ATP Carrier from Yeast. The Effect of Six Arginine Mutations on Transport and the Four ATP versus ADP Exchange Modes†

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ABSTRACT: Mutagenesis of three intrahelical arginines, R96, R204, or R294, and of each member of the arginine triplet R252, R253, R254 into neutral residues had resulted in a strong suppression of oxidative phosphorylation in cells and isolated mitochondria [Müller, V., Basset, G., Nelson, D. R., & Klingenberg, M. (1996) *Biochemistry 35*, 16132−16143]. Here we determine the transport activity of wild-type and mutant AAC in reconstituted proteoliposomes using a new rapid removal−stop method without relying on the inhibitor stop which can be compromised by mutations. The basic electroneutral ADP/ADP exchange activity is strongly or totally suppressed in six out of seven of these mutations, with the exception of R294A, which retains nearly wild-type activity. Carboxyatractylate (CAT) inhibits the ADP/ATP exchange rate only to 3−10% in wild type and R294A and up to 40% in other mutants, whereas bongkrekic acid (BKA) inhibits 50% (wild type and R294A) and 90% (other mutants). Consequently, AAC is preferentially reconstituted with the matrix surface outside. All these mutations drastically change activity distribution among the four exchange modes ADP/ADP, ADP/ATP, ATP/ADP, and ATP/ATP. Whereas in wild-type AAC the homo ATP/ATP exchange is twice as high as the ADP/ADP exchange, in mutants it is 10 to 15 times lower. Similarly, the hetero ATP/ADP exchange in wild-type AAC is higher than the ADP/ATP exchange, but in mutants it is several times lower. Thus, these mutations afflict the ATP-linked modes, in particular those linked to external ATP. The inhibition of oxidative phosphorylation is thus explained by the suppression of ATP export versus ADP import mode. The "extra"-inhibition of oxidative phosphorylation in mutant cells is explained by the extreme shift in mutants in favour of ATP import versus ADP export. Besides structural changes, the mutant effects indicate electrostatic interactions of these arginines with the anionic substrates. The loss of one positive charge raises the translocation barrier the more negative the substrate, i.e. more for ATP^{4-} than for ADP^{3-}. In none of these arginine mutants was the binding of CAT or BKA abolished.

As shown in previous publications, for elucidating structure/function relationships in the ADP/ATP carrier of mitochondria, site-directed mutagenesis of some polar residues has proven to be very rewarding (Nelson et al., 1993; Klingenberg & Nelson, 1994). Several arginines, which are conserved within the family of mitochondrial carriers or which are characteristic for the AAC,[1] were mutagenized into neutral residues. The most prominent targets were the intrahelical arginines occurring in the second transmembrane helix of each repeat within the three-repeat domain structure of the mitochondrial carrier. A further target was the striking arginine triplet which occurs in the third repeat domain on the matrix side and which is a typical member of the AAC [see Figure 1 in Müller et al. (1996)].

To analyze the mutational defects we determined the capacity for oxidative phosphorylation linked to the ADP/ATP transport both on the cellular and mitochondrial level for the wild type and mutants (Müller et al., 1996). Important discrepancies between the oxidative phosphorylation capacity in mutant mitochondria and in cells were found and attributed to regulatory inhibiting factors in the intracellular environment. The binding of the specific radiolabeled inhibitors [^{3}H]CAT and [^{3}H]BKA were determined in mitochondria, and in addition an ELISA competition assay for measuring the AAC content was performed. No major discrepancy between the [^{3}H]CAT, [^{3}H]BKA binding and the quantitative ELISA assay was observed, with the possible exception of R294A. These results indicated that the mutated polar groups were not involved specifically in the binding of these inhibitors. Based both on the binding and ELISA assays large differences in the AAC contents were observed with nearly zero expression in several inactive mutants. Different from these drastic changes in the AAC content, the electron transport activity and the content of respiratory components were relatively less affected even on complete suppression of oxidative phosphorylation. Obviously, in yeast cells there is only a loose regulatory connection between the expression of the respiratory system and the expression of AAC as a limiting factor of oxidative phosphorylation.

Crucial for elucidating the structure/function relationships in a mutation program is a good assay of the transport activity

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[1] Abbreviations: AAC, ADP/ATP carrier; CAT, carboxyatractylate; BKA, bongkrekic acid, PC, egg lecithin.

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of the mutated AAC. ADP/ATP exchange measurements cannot be applied on a routine basis to mitochondria from the manipulated yeast strains. The smallness and fragility of mitochondria from yeast, in particular from mutant cells makes it difficult to procure sufficient amounts of endogenous adenine nucleotides which are necessary for the measurement of meaningful adenine nucleotide exchanges in the mutant mitochondria (Ohnishi et al., 1967). Therefore we concentrated our efforts on determining the functional activity of the AAC in the reconstituted system with the solubilized AAC reintroduced into phospholipid vesicles (Krämer & Klingenberg, 1979). Moreover, in this system we can select at will the exchange components on both sides of the membrane, which turned out to be very important for understanding the mutational effects.

MATERIALS AND METHODS

Reconstitution of Phospholipid Vesicles. The reconstitution method relied essentially on the detergent absorption method with batchwise addition of polystyrene beads for forming the vesicles (Klingenberg & Winkler, 1986; Klingenberg et al., 1995). The methods for measuring the exchange activity with the reconstituted vesicles underwent several changes during the study of these AAC mutants. The specific exchange rates were improved by lowering the protein−phospholipid ratio from about 1:100 to 1:500. Major technical changes were introduced for starting and stopping the transport reaction and the separation of the vesicles. Crucial was the replacement of the inhibitor stop method by the rapid removal stop. Here the nucleotides were separated from the vesicles using an anion exchange column through which the sample was rapidly sucked. The small sample volume was adapted to small columns containing Dowex acetate. In the inhibitor stop method, however, two discrete steps were required (Klingenberg & Pfaff, 1967; Palmieri & Klingenberg, 1979), i.e., the stop by inhibitors and the subsequent removal of the substrate by anion exchange columns. In the new rapid removal stop method both were combined in one step. As a result, the sample volumes can be reduced to 50 μL, whereas the admixing of the inhibitors requires a minimum sample volume of 150 μL to handle the stirring rods. The smaller volumes are advantageous for the low yields of mutant AAC. Although here the stop was not as fast as with the inhibitors, it was sufficient for most applications with the reconstituted yeast AAC. As another advantage, the time interval between the inhibitor stop and the separation of external solutes by the Dowex column was avoided. A careful comparison of the inhibitor stop method, using the same device and preparation, with the removal stop method gave no difference at intermediate sampling times (Figure 1).

For protection against proteolysis of the AAC, the atractyloside (ATR−AAC) complex was formed prior to solubilization by incubating the mitochondria with ATR. 10 nmol of ATR and 10 nmol of ADP were added to the 200 μL suspension of yeast mitochondria containing 4 mg of protein and incubated for 10 min at 0 °C. The suspension was solubilized by addition of 60 μL of 20% $C_{12}E_8$, 100 μL of 4M NH_4Cl, 4 μL of 50 mM EDTA, 4 μL of 50 mM PMSF to a total volume of 400 μL, corresponding to 10 mg of protein/mL. For the isolation of the AAC to about 50% purity the solution was thoroughly mixed with a paste of 0.72 g of hydroxyapatite and centrifuged 2 times for 3 min

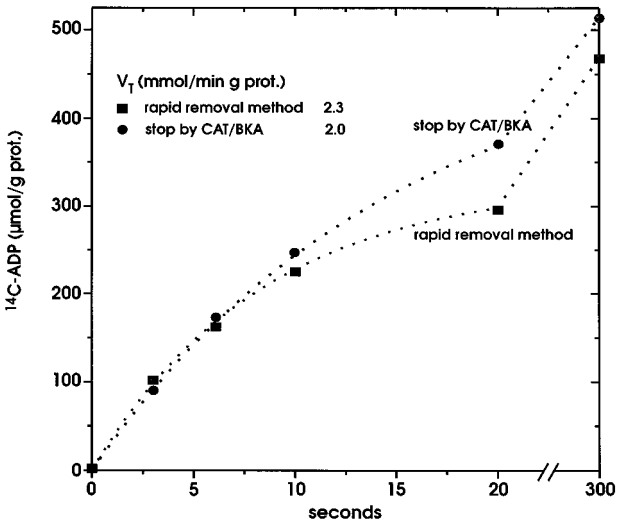

FIGURE 1: Comparison between the rapid removal and the inhibitor stop method for following the time course of the [^{14}C]ADP/ADP exchange. Wt AAC2 from *Saccharomyces cerevisiae* was reconstituted into phospholipid vesicles. Parallel experiments were done with the same vesicles loaded with ADP under identical incubation conditions and additions of 100 μM [^{14}C]ADP. In both cases the same device for the starting of the transport and for the removal of the external nucleotides was applied, with the only difference that in the inhibitor stop case the samples were injected first into a mixture of a final concentration of 10 μM CAT and 4 μM BKA before being applied to the anion exchange columns, whereas in the rapid removal case each sample was directly injected into the columns for rapid passage by suction. The rates given in the figure are evaluated as described in Materials and Methods.

at 15 000 rpm. The supernatant of 400 μL was diluted with 1.4 mL of the detergent medium used for the solubilization, resulting in a concentration of $\approx$ 0.05 mg of protein/mL.

For the reconstitution a phospholipid stock suspension was prepared as follows. 400 mg of egg lecithin (PC) from turkey (Sigma) was sonicated in 1 mL of 0.4 M Na_2SO_4 and 0.5 M Tricine, pH 7.5, until the suspension became clear. From this PC suspension typically 55 μL was added dropwise to a detergent solution with a final volume of 200 μL containing 21 mg of $C_{12}E_8$, 1 mM EDTA, 0.6 M KCl, and in most cases 0.5 mg of cardiolipin. 600 μL of the AAC containing about 30 μg of protein was added dropwise to the detergent−PC solution. Further, 20 mM ADP or 20 mM ATP were added for loading of the vesicles with internal ADP or ATP. For the formation of vesicles to this detergent PC-AAC solution a total of 640 mg BioBeads (BioRad) were added in eight portions at 20 min intervals, 4 × 40 mg, 3 × 80 mg, and 1 × 240 mg with gentle shaking in the cold. The vesicles in the supernatant were separated from external ADP or ATP by passage over a Sephadex G 75 column of 0.6 × 28 cm. The elution buffer contained 80 mM sucrose, 20 mM Tricine, 30 mM Na_2SO_4, 1 mM EDTA, pH 7.5.

Measurements of Transport Rates with Reconstituted AAC Proteoliposomes. The transport measurements are based on the counterexchange of external [^{14}C]ADP or [^{14}C]ATP with internal ADP or ATP. A newly developed device was used, which allowed following the time dependence of the exchange with small sample volumes of 50 μL at a time resolution of 3 s. The stop of the transport was achieved by the rapid removal of the external [^{14}C]ADP or [^{14}C]ATP by rapid passage through a small anion exchange column. This procedure does not rely on the stop of the transport by

inhibitors, such as CAT and BKA, since in some mutants those inhibitors can be expected to be ineffective.

The rapid removal procedure worked as follows: 355 μL of the vesicle suspension were sucked into a 500 μL syringe (Multipette Eppendorf) which was kept at 10 °C using a thermostated water jacket. If required, to the vesicles 14 μL 4 M KCl were added to obtain external 150 mM KCl. Further, if required 2 μM valinomycin was added. After 1 min temperature adjustment in the syringe, the suspension was rapidly injected into a cup which contained 12 μL of 3.2 mM [^{14}C]ADP or [^{14}C]ATP for a final concentration of 100 μM. The mixture was immediately sucked back into the multipette syringe to cause high turbulence and rapid mixing. After predetermined time intervals, aliquots of 50 μL were injected stepwise onto small anion exchange columns (5 × 30 mm) containing 650 mg of Dowex 1×8 (200−400 mesh, Fluka) which have been converted into the acetate form and which were preequilibrated with 200 μL of a solution containing 100 mM sodium acetate, 1% PC and 0.5% bovine serum albumin. The injected aliquots were rapidly sucked through the column by vacuum for removal of external [^{14}C]ADP or [^{14}C]ATP. The eluate of 400 μL which contained the [^{14}C]ADP or [^{14}C]ATP taken up into the vesicles was collected and counted. The time intervals after start of the reaction in a typical experiment were t = 4, 6, 8, 10, 20, and 300 s. The comparison of both methods for a typical exchange rate measurement in Figure 1 shows the virtual identity of the values measured.

The evaluation of the transport rates from the measured exchange kinetics makes use of the first order equilibration between the externally labeled and internally unlabeled ADP or ATP which follows the equation [from Klingenberg et al. (1995)]:

$$N_i^*(t) - N_i^*(0) = \frac{N_e N_i}{N_e + N_i}(1 - e^{-kt}) \qquad (1)$$

where N_e and N_i are the external and internal amounts of nucleotides, $N_i^*(t)$ is the labeled nucleotide taken up at time t, k is the first-order rate constant. The unspecific uptake is accounted for by subtracting the control value $N_i^*(0)$, obtained during preincubation with CAT + BKA. The absolute exchange rate is $V = kN_e$ generally expressed in μmol/min·g of protein. The rates are related to the protein content measured in the detergent extract after passing through the hydroxyapatite, but before it is mixed with the phospholipid for the vesicle formation. The exchange constant and transport rates are evaluated from the measurement uptake values by a graphic fitting program, which also contained a plotting routine (see Figure 1 and Table 2).

RESULTS

Reconstitution. Measurements of adenine nucleotide exchange in mitochondria from yeast are difficult and the results are mostly questionable because of the low endogenous adenine nucleotide content (unpublished results). Yeast mitochondria are smaller and more fragile than the paradigm rat liver mitochondria and may have lost adenine nucleotides to a varying degree on isolation. In wild-type mitochondria we formerly succeeded in obtaining representative exchange rates (Ohnishi et al., 1967). However, the mutant mitochondria are still smaller and contain an endogenous pool of only 0.5−1 μmol of ADP + ATP + AMP/g of protein, whereas in intact wild-type yeast this content can reach 6 μmol/g of protein (Ohnishi et al., 1967). In the mutants the low content may not only be due to loss of nucleotides during the isolation but also reflect the reduced need for adenine nucleotides. Therefore we isolated and reconstituted the AAC into vesicles in order to measure the exchange activity. Another advantage is the possibility to vary inside and outside the nucleotides both in concentration and composition. Also, the membrane potential can be adjusted which is important for the ATP-linked exchange types (Krämer & Klingenberg, 1982).

For this purpose the AAC was extracted with non-ionic detergents from the mitochondria followed by partial purification through hydroxyapatite passage (Krämer & Klingenberg, 1979). As a standard for this procedure the wild-type AAC was used which yields about a 60% pure AAC preparation (Knirsch et al., 1989; Gawaz et al., 1990). The exchange rates are related to the protein content of the partially purified extract before the reconstitution. To assure that the efficiency of reconstitution is the same with wt and mutant extracts, in several experiments, parallel to the mutants, also wt was reconstituted. Further, in the case of mutants with low transport activity a mixture of equal amounts of protein from mutants and wt extracts was coreconstituted into vesicles which yielded the expected activity from the wt portion.

Exchange Rates. First with these methods a large amount of transport rate data were collected in the reconstituted AAC system, using the "classical" inhibition stop method and are in part published (Klingenberg et al., 1992, 1995; Klingenberg & Nelson, 1995). Although the formerly measured rates are lower, the relation between the rate of the wild type and mutant AAC are the same as with the improved conditions used here. We present here for consistency only data with the new rapid removal method (see Materials and Methods for details). The first measurements are confined to the "basic" ADP/ADP exchange rate which is not complicated by charge differences. It should be largely independent of the membrane potential and the associated supplements such as K^+ ions and valinomycin. This "homo"-exchange of external ADP against internal ADP is taken as basic transport activity of the AAC since the ADP−AAC complex is thought to be electroneutral in contrast to the ATP−AAC complex and should not be influenced by a membrane potential (Krämer & Klingenberg, 1980, 1982). This was concluded from studies with the reconstituted bovine heart AAC in the reconstituted system, where the ADP/ADP exchange was not influenced by $\Delta\psi$, whereas the ATP/ATP exchange was inhibited (Krämer & Klingenberg, 1982).

In Figure 2 the results of the ADP/ADP exchange in the reconstituted system for the various mutant strains are assembled. The wild type and the plasmid AAC2 strains have similar ADP/ADP exchange activity in the reconstituted system. The negative control value with the plasmid-less mutant confirms the validity of the methods since no external radioactive nucleotide penetrates into the vesicles. Among the intrahelical arginine mutant AACs the ADP/ADP exchange is virtually zero with R96A and R204L whereas the R96H mutant retains a significant exchange activity, and R294A reaches nearly wild-type activity. The surprisingly high exchange rate in particular of the R294A mutant contrasts with the previously reported low rate of oxidative

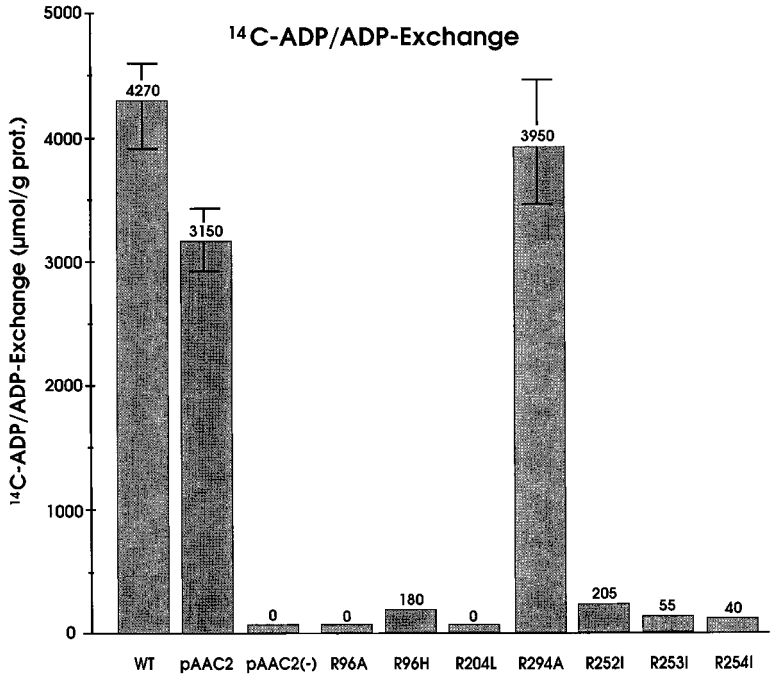

FIGURE 2: "Basic" ADP/ADP exchange rates in the reconstituted system with wt AAC and AAC2 mutants. Measurements of up to five different preparations by the rapid removal stop method at 10 °C. The reconstituted AAC are extracted from wild type *S. cerevisiae* (wt), plasmid-wt AAC2 cells (p AAC2), from AAC2, AAC1 deficient cells (no AAC), or from plasmid mutant AAC2 cells. For technical details see Materials and Methods section.

phosphorylation. The arginine triplet mutants R252I, R253I, and R254I have low ADP/ADP exchange rates amounting to between 1 and 6% of wild-type activity, which agrees with what one should expect from the very low oxidative phosphorylation rates measured in these cells and isolated mitochondria (Müller et al., 1996).

Effect of Inhibitors. Of great interest is the question to what extent the inhibitors of the AAC, CAT, and BKA will affect the transport activity of the mutant AAC. In the previous studies of the oxidative phosphorylation of cells and mitochondria of AAC mutants, these inhibitors were applied to discern the involvement of AAC. It remained unresolved whether an incomplete response to these ligands was due to mutational effects, to AAC-independent phosphorylation or, in the case of cells, due to incomplete penetration. The binding studies of [^{3}H]CAT and [^{3}H]BKA to the mitochondria indicated that the mutations did not impair the binding.

The reconstituted system again offers opportunities to check the influence of mutations on the interaction of CAT and BKA with AAC. For this purpose we chose the "basic" ADP/ADP exchange system and assayed the inhibition by either CAT and BKA alone or in combination (Table 1). Each inhibitor was applied at what are normally saturating concentrations. Both inhibitors require that the AAC assume specific conformational states, i.e., for CAT binding the cytosolic-orientated state (c-state) and for BKA binding the matrix-oriented state (m-state) (Erdelt et al., 1972). A further factor to be reckoned with is that CAT is membrane impermeant (Klingenberg et al., 1975). It will bind only to cytosolic side-out oriented AAC molecules, whereas the permeant BKA can also bind to both orientations (Scherer & Klingenberg, 1974; Klingenberg et al., 1983). For all AAC types the inhibition by CAT is weaker than with BKA (Table 1). In the two wt as well as in R294A, i.e., in AAC with high activity, CAT inhibits less than 10%, and in the "slow" mutants inhibits between 30 and 50%. BKA inhibits the ADP/ADP exchange more efficiently to between 50 and 75%. The combined additions of CAT and BKA inhibit to about 90% with the notable exception of R294A. Here the exchange cannot be inhibited to more than 50% by these ligands. The fact that the inhibition of CAT and BKA alone does not add up to the nearly complete inhibition by the combined additions of CAT and BKA, as observed here for the wt, is a known phenomenon and used for the inhibition stop in reconstituted AAC liposomes (Krämer & Klingenberg, 1979). The reasons for this cooperative effect of CAT and BKA seem to contradict the classic picture of mutually exclusive binding of CAT and BKA and are not clear at this point.

Table 1: Percent Inhibition of ADP/ADP Exchange by CAT and BKA in Isolated AAC Reconstituted Vesicles[a]

strain	10 μM CAT	10 μM BKA	10 μM CAT, 4 μM BKA
wt	7	54	92
p-AAC2	11	83	90
R96H	49	74	97
R294A	3	43	50
R252I	38	56	88
R253I	48	84	96
R254I	26	91	99

[a] The additions of the inhibitors to the vesicles were made 2 min before the start of the reaction. Three types of additions were made, 10 μM CAT, 10 μM BKA, and 10 μM CAT plus 4 μM BKA combined. The experiments were performed with the rapid removal technique at 10 °C.

These results indicate that the AAC is incorporated into vesicles mainly in the inside-out orientation, barring CAT from accessing the AAC. In the case of higher amounts of AAC incorporated into vesicles, such as in wt and R294A, the orientation is at least 90% inside-out, that means highly ordered. If low amounts of AAC are incorporated as in the mutants, the orientation becomes less ordered although still

preferential inside-out. Obviously there is a mutual ordering influence when more AAC molecules are incorporated. Another conclusion is that the mutations have not affected the sensitivity to CAT and BKA. Only the R294A mutant with its relatively high transport activity is less sensitive to BKA and CAT. Since the binding of [^{3}H]CAT and [^{3}H]-BKA to the mitochondria from R294A is still high, we must conclude that here the interaction of CAT and BKA does not fit the usual pattern, where binding corresponds to inhibition.

Exchange Modes. For the ability of yeast cells to grow on non-fermentable substrates, not only the absolute transport activity of the AAC is important, but the distribution of this activity into the productive mode of ADP import versus ATP export.

With the view that mutations may affect the transport of ADP and ATP differently, we embarked on elucidating the different "exchange modes", which are the homo-exchange modes ADP/ADP (D/D) and ATP/ATP (T/T) and the hetero-exchange modes ADP/ATP (D/T) or ATP/ADP (T/D). Apart from important consequences for the intracellular efficiency of the AAC, differential impairments of ADP and ATP transport by mutations would allow important insights into how critical residues within the carrier structure deal with the charge differences between ADP^{3-} and ADP^{4-} which are of utmost importance for the electrophoretic function of AAC.

Because of the supposed electrical nature of the ATP involving exchange modes, we first explored the influence of the membrane potential and of charge compensating cation movements on the various exchange modes in order to establish standard conditions for all measurements. KCl and valinomycin were used for setting the membrane potential in the reconstituted vesicles containing the wild-type AAC (Krämer & Klingenberg, 1980). Apart from its role in generating the diffusion potential, KCl was also found to have a general stimulating effect on the transport activity of AAC in mitochondria (Pfaff & Klingenberg, 1968). Therefore KCl was not only placed either inside or outside but also on both sides of the vesicle membrane. In this case valinomycin would not induce a diffusion potential but would facilitate a charge compensating K^+ flux for the hetero-exchange modes.

The influence of these conditions on the transport activities of wild-type AAC are shown in Figure 3. The results are assembled in two groups according to whether the vesicles were loaded with internal KCl. In vesicles without internal KCl the transport was stimulated by external KCl when they contain ADP but not ATP. Valinomycin further stimulated only the T/D exchange, as expected from a diffusion potential. In the other modes, valinomycin has the tendency to slightly inhibit the exchange. When KCl was present inside, external KCl stimulated the activities in all exchange modes. Valinomycin increased the rates in particular of the T/D exchange. In this case the charge compensation is facilitated by valinomycin catalyzed K^+ influx. However, for the opposite D/T exchange stimulation by valinomycin was only small. An inhibition by valinomycin can be expected in the D/T mode with external KCl only and in the T/D mode with internal KCl only. This is experimentally confirmed in both cases. However, it is unexplained why valinomycin also slightly inhibits the exchange in the absence of any KCl in all exchange modes. Thus, for obtaining the

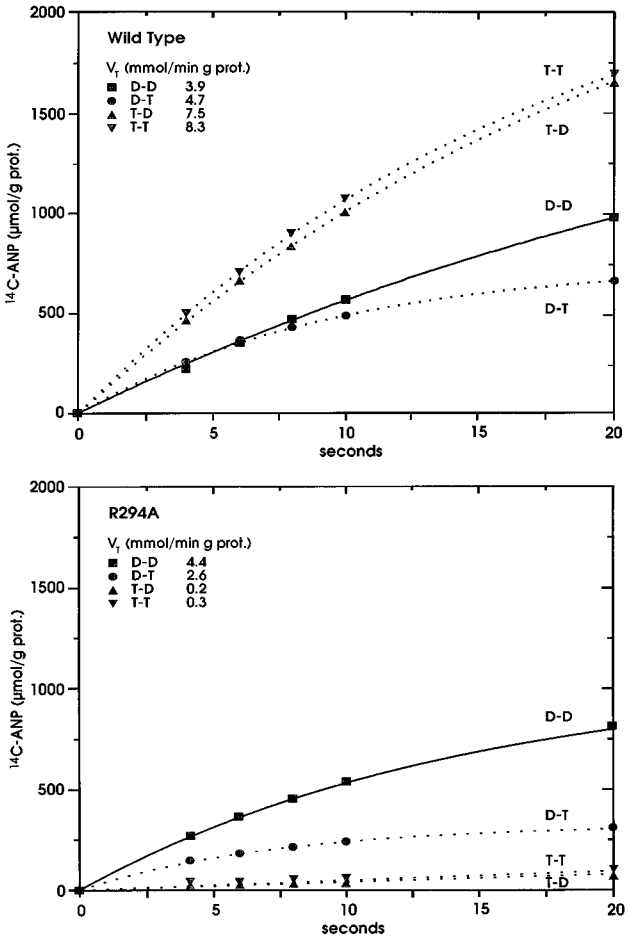

FIGURE 3: Comparison of the exchange kinetics between wt AAC and R294A AAC. The evaluated exchange rates are given. A long time (300 s) value is also measured for checking the vesicle intactness and capacity.

true stimulation by valinomycin, this valinomycin inhibitory effect has to be subtracted from the KCl-dependent stimulation, rendering its effect still larger.

On the basis of these results, for the systematic assays of the reconstituted mutant AAC activity we chose to place KCl on both sides of the vesicle membranes. Examples of typical time progress curves for the four exchange modes are given in Table 2. The dramatic shifts in the pattern of the various exchange modes between the two AAC with about equal "basic" ADP/ADP exchange rates are clearly illustrated. In the wild type the T/T and T/D exchange rates are higher than the D/D and D/T exchange rates; in the mutant R294A the opposite situation is seen, i.e., the exchange rates involving ATP are all smaller than the "basic" D/D rate.

The results of the rate measurements for all mutants are summarized in Figure 4. In the plasmid wt AAC, as in the chromosomal wt AAC, the T/T exchange is nearly twice as high as the D/D exchange mode. Also, among the two hetero exchange modes, the T/D exchange rates are higher than the D/T rates. Although this might seem to be paradoxical to the situation in the cell where the D/T exchange corresponds to the mode which is operative in oxidative phosphorylation, we must consider that in the vesicles the orientation of the carrier is different from the mitochondria, e.g., inserted largely inside-out as was suggested above by the low sensitivity to the membrane impermeant inhibitor CAT.

Among the mutants no activity is measured with R96A and R204L in any of the four exchange modes. Except for

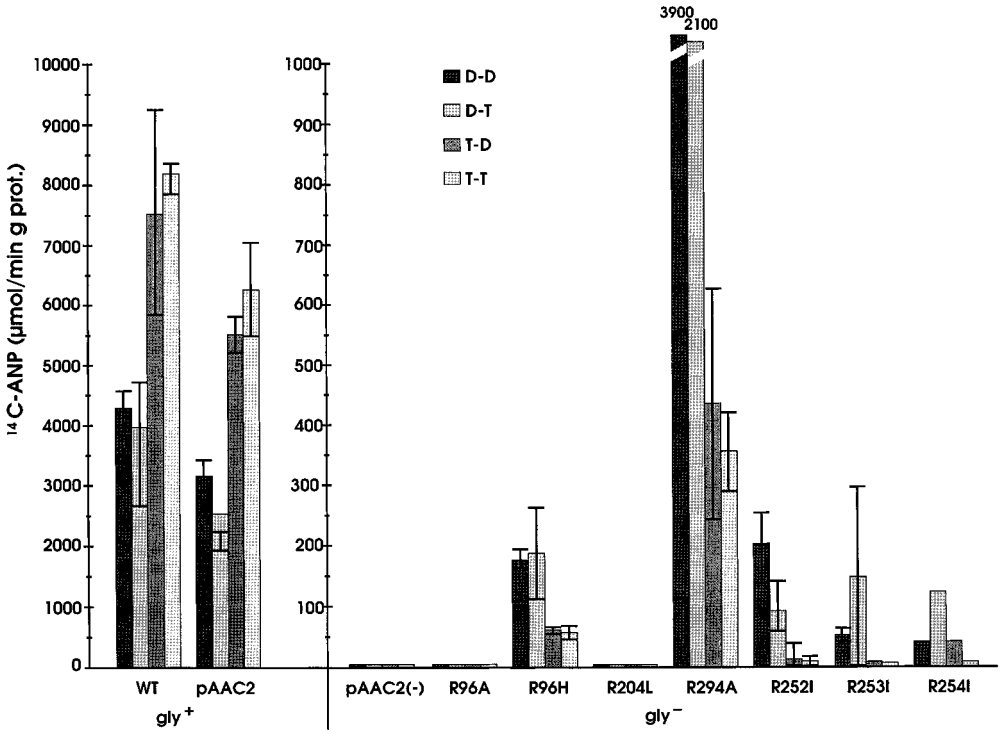

FIGURE 4: The influence of mutations on the four exchange modes of the AAC. Exchange measurements with reconstituted vesicles. Experiments from up to five different AAC preparations per strain. D/D = [^{14}C]ADP external/ADP internal exchange, D/T = [^{14}C]ADP/ATP exchange, T/D = [^{14}C]ATP/ADP exchange, and T/T = [^{14}C]ATP/ATP exchange. Measurements with the rapid removal technique at 10 °C. The rates are evaluated from the time course according the first-order eq 1.

Table 2: The Influence of a K^+ Diffusion Gradient on the Four Exchange Modes of the ADP/ATP Exchange[a]

addition		D/D	D/T	T/D	T/T
^{14}C-ANP Exchange Rate (mmol/min g of protein)					
−	−	2.6	2.8	2.8	8.2
−	+ Val	2.1	2.2	1.9	5.9
KCl_e	−	4.5	2.9	5.6	7.1
KCl_e	+ Val	5.0	2.6	9.1	7.2
		KCl_{intern}			
−	−	1.4	2.0	2.0	2.8
−	+ Val	1.5	1.6	1.0	2.7
KCl_e	−	3.4	2.3	2.8	6.7
KCl_e	+ Val	4.4	2.7	5.8	7.8

[a] Experiments with wt AAC reconstituted into vesicles using the rapid removal technique. KCl concentration throughout 150 mM KCl. KCl_{intern} = vesicles are preloaded with 150 mM KCl. KCl_e = 150 mM (external) KCl is added to the incubation medium for the exchange. Val = Valinomycin (2 µM) is added prior to the start of the transport. D/D = [^{14}C]ADP external/ADP internal exchange, D/T = ADP/ATP exchange, T/D = ATP/ADP exchange, and T/T = ATP/ATP exchange. Results from different vesicle preparations loaded with KCl or not, with ADP, or with ATP.

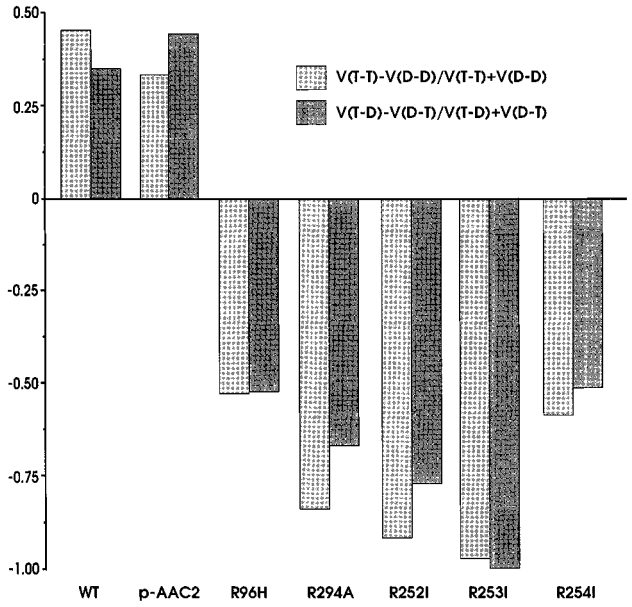

FIGURE 5: Exchange mode pattern designed to illustrate the "inversions" caused by the mutations. For this purpose relative rates are given to suppress the changes of the absolute rates. The difference between the two homo-exchange mode rates T/T minus D/D divided by the sum of both rates are plotted and in the same manner the differences between the two hetero exchange modes T/D minus D/T. The average values from Figure 6 are used.

R294A, the exchange activity in the other five mutant AACs, R96H, R294A, R252I, R253I, and R254I, is much lower in all modes than in the wild type. Most interestingly, the patterns of exchange activity are strikingly reversed as compared to the wild-type. In all mutants the activity of the homo exchanges D/D is much higher than the T/T exchange. In the R294A AAC this difference is extreme, the T/T exchange reaches only 7% of the D/D exchange activity. At a much lower level the same extreme inhibition of the T/T versus D/D exchange rate is observed for the R252I AAC. Also, the activities of the hetero modes T/D versus D/T are opposite in the mutants to the wt AAC. The T/D activity is throughout smaller than the D/T activity. In R294A AAC the T/D activity amounts to only 9%, whereas in the wt AAC it reaches twice the D/T rate.

The characteristic shifts of the exchange modes by the mutations can be illustrated by the difference between the T/T and D/D modes, which is reported as the ratio to the sum of both modes in Figure 5. It also contains the difference between the D/T and T/D modes as the ratio to

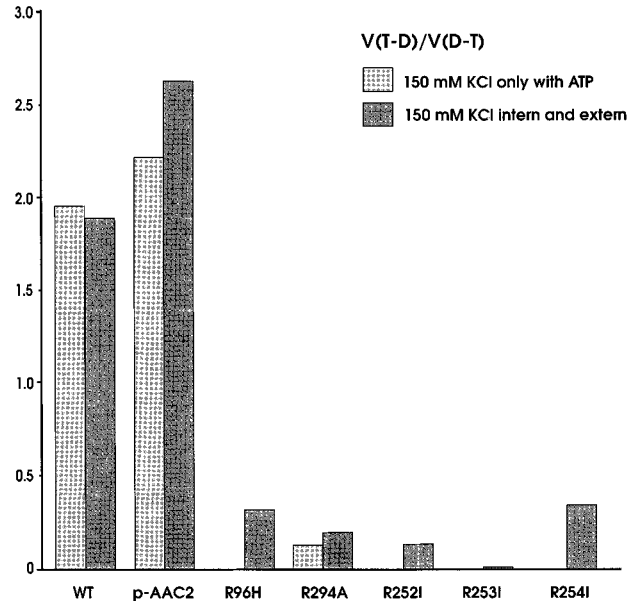

FIGURE 6: The influence of mutation on the ratio of the two hetero exchange modes. This ratio determines the efficiency of the AAC in oxidative phosphorylation in the cell. The dramatic decrease of *V*(T/D) versus the *V*(D/T) is illustrated. In addition to the average values taken from this figure, exchange rates obtained under influence of a K^+ diffusion potential are incorporated, i.e., when KCl is present only at the ATP containing side. All measurements were made in the presence of 2 μM valinomycin.

the sum of both of these modes. By forming these ratios, the characteristic mode shifts by mutation are visualized independently of the strong variation of the absolute rates. The two classes of exchange modes are clearly illustrated; for the wt AAC the differences T/T−D/D and D/T−T/D are positive. In the mutants throughout these different ratios are negative and nearly reach −1. Of particular interest for understanding the intracellular inhibition of oxidative phosphorylation is the ratio of the D/T versus T/D modes since it is an indicator for the competition of the productive versus the counterproductive modes for the AAC transport capacity. As shown in Figure 6, in the two wt AACs the D/T mode is 2−3 times higher than the T/D mode. For the mutant AAC this ratio is only 0.15−0.35, i.e., the T/D mode is 3−8 times higher than the D/T mode. This ratio is even magnified by a K^+ diffusion gradient potential as shown for the R294A AAC. The consequences of these mode pattern shifts for the ATP export in oxidative phosphorylation will be elucidated in the Discussion.

DISCUSSION

In the preceding paper the effects on oxidative phosphorylation of seven arginine mutations in the AAC2 had been measured in cells and in mitochondria (Müller et al., 1996). Several mutations, such as the intrahelical R96A, R204L as well as the mutations in the arginine triplet R252I, R253I, and R254I had a knock-out effect on oxidative phosphorylation, whereas R96H and R294A still retained some activity. In R96A and R204L also the AAC content was virtually zero whereas in R294A the nearly normal content of AAC contrasts to the decreased activity of oxidative phosphorylation. In mutant cells oxidative phosphorylation activity was found to be low as compared to the free running conditions in isolated mitochondria. It was suggested that the normal control of oxidative phosphorylation by cytosolic ATP is amplified by these mutations of the AAC into a counterproductive mode. With these results as a challenge and with the primary goal being to elucidate the structure−function relationships in the AAC by site-directed mutagenesis, direct measurements of the transport activity become mandatory. Since ADP/ATP exchange measurements in mitochondria, because of their fragility, do not give meaningful and reproducible data, we concentrated here on the isolation and reconstitution of the AAC. During this extensive work the methods of reconstitution and transport measurements were greatly improved.

The Four Exchange Modes. As we have pointed out, the D/D exchange is used as a basic exchange rate, uncomplicated by presumed extra charges as conceived to exist when ATP is translocated through the carrier binding center, i.e. as $C^{3+}\cdot ADP^{3-}$ versus $C^{3+}\cdot ATP^{4-}$ (Klingenberg, 1980). This basic exchange is strongly suppressed in all our arginine mutants with the dramatic exception of R294A. Here the activity retains wild-type level, i.e., is 20-fold or more higher than in the other gly$^-$ mutants. This discrepancy called for analyzing in detail the other exchange modes, in particular the influence of the mutations on the ATP versus ADP transport capacities. In fact, besides affecting the absolute rates, the mutations cause drastic changes of the various exchange modes, such as the neutral or unproductive homo exchange modes ADP/ADP (D/D) and ATP/ATP (T/T), and the hetero exchange modes ADP/ATP (D/T) and ATP/ADP (T/D). In the wt AAC of yeast the T/T exchange is higher than the D/D exchange, which is opposite to the findings with bovine heart AAC (Krämer & Klingenberg, 1980). However, in the still active mutants the situation is generally reversed, the D/D rate being larger than the T/T rate so that the mutations nearly abolish the T/T rate. This 15-fold discrepancy is largest in R294A. The argument that the reversal of exchange mode activity is due to a different orientation of the mutant than the wt AAC in the vesicles (see Table 1 and Figure 7) can be ruled out. Wt and R294A AAC incorporate both to more than 90% inside-out, and yet they show the most dramatic contrast of the exchange mode patterns.

The suppression of the T/T exchange mode may suggest a disability of mutant AAC to transport specifically ATP. However, this simple explanation is not confirmed by the observation that a similar inversion of exchange modes by mutation occurs in the D/T versus T/D exchange. In the wt AAC we find that the D/T exchange is always higher than the T/D exchange. In the mutants the situation is reversed, the D/T exchange is larger than the T/D mode. In other words, the mutation affects primarily the interaction of the carrier with the external rather than the internal ATP. Since the external concentrations are much lower, 100 μM versus about 10 mM inside, these data suggest that mutations strongly increase the K_M of ATP but not of ADP above the external concentration of 100 μM ATP.

Mechanisms of Mutant Effects. What is the role of the mutated arginines in transport and by which mechanism do the mutations modify these exchange mode patterns? In all mutants a positive charge is replaced by a neutral or nearly neutral one. Among the three repeated intrahelical arginines, R96 and R204 appear to be absolutely essential, but because of the three-repeat similarity the exceptional tolerance towards neutralization of R294 is unexpected. Obviously, the intrahelical charge in the third domain is not as essential

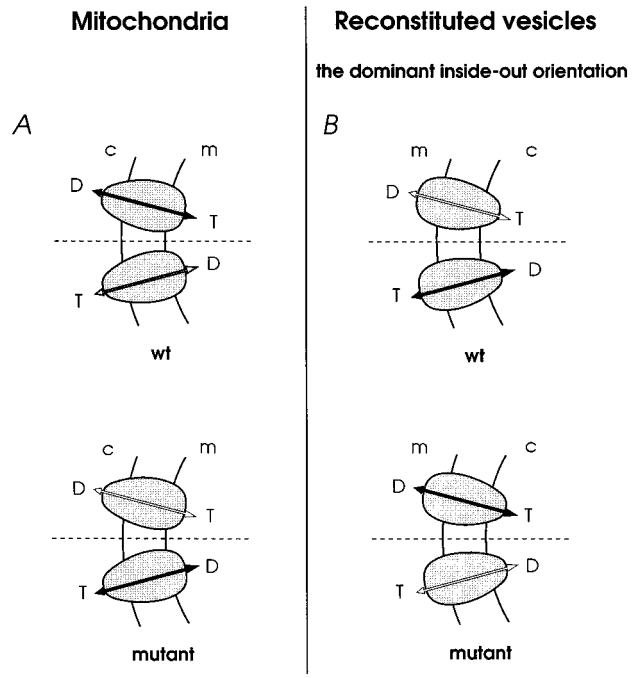

FIGURE 7: Illustration of the partially opposite orientation of the AAC in mitochondria and reconstituted vesicles and the inversion of the ADP/ATP versus ATP/ADP exchange modes by mutation exemplified in the R294A mutant. c = cytosolic side; m = matrix side; D = ADP; T = ATP. The heavy arrows signify the higher activity. (A)The hetero exchange modes T/D and D/T in mitochondria of wild type and mutant. (B) The hetero-exchange modes T/D and D/T in reconstituted vesicles. Only the dominant inside-out orientation of AAC in these vesicles is depicted.

for transport as those in the first and second domain. In this context it is interesting to recall that a different role of the second and third repeat domain was suggested by photoaffinity labeling the AAC in bovine heart and yeast mitochondria. 2-Azido- and 8-Azido-ATP are exclusively incorporated in the second domain of yeast AAC (Mayinger et al., 1989) or more in the second than in the third domain of bovine heart AAC (Dalbon et al., 1988). Although the covalent substitution with the base section occurred in the hydrophilic matrix domain, an interaction of the phosphate and ribose moieties with the helix region is well possible. A survey of other carriers with known functions shows that arginine in positions homologous to R96 are conserved also in the ketoglutarate/malate carrier and the uncoupling protein. Arginines homologous to R294A are present in even five of the six different carriers known, including the carrier for citrate (Kaplan et al., 1995) and for FAD (Tzagaloff et al., 1996). In view of these facts, we cannot assign a specific role to these groups for the transport of ADP and ATP. Another case for the importance of the three domain's repeated arginine groups can be made by the mutations within the R252, 253, 254-triplet. This group belongs to repeated positive charge clusters found as (+ × +) in the other two domains. They are located in the hydrophilic matrix sections. Although the R-triplet is specific for the AAC from all sources known so far, in other carriers it is replaced by the (+ × +) motif. Structural changes can be expected to be caused by these mutations which would impair the transport capability of AAC. The strong discrimination against ATP^{4-} versus ADP^{3-} transport resulting from all these mutations indicates that the positive charges are also involved in handling the negative charges of the substrate. Only thus it is understandable that the transport of ATP^{4-} is more sensitive to the disappearance of a positive charge than that of ADP^{3-}.

The electrical nature of the ATP versus ADP transport was formerly demonstrated in mitochondria (LaNoue et al., 1978; Wulf et al., 1978) and in reconstituted vesicles with various types of experiments (Krämer & Klingenberg, 1980; 1982). Recently the existence of electrical currents associated with the ADP/ATP exchange was directly visualized (Brustovetsky et al., 1995). In an early model (Krämer & Klingenberg, 1982; Klingenberg, 1980) the carrier center was proposed to provide three positive charges which can "neutralize" ADP^{3-} ($AAC^{3+}\cdot ADP^{3-}$) but leave one negative charge excess with ATP^{4-}($AAC^{3+}\cdot ATP^{4-}$). However, the model of a rigid charge interaction has to be modified in view of the present data (Klingenberg, 1992). Since probably all six presently mutated arginines and also further positive residues influence the ATP versus ADP translocation, no fixed stoichiometry for the interaction of positive charges in AAC with ADP or ATP can be formulated. A flexible interaction along the translocation pathway is in line with the concept that ADP and ATP convert the AAC into a more mobile state which oscillates between the two extremes, the c- and m-states, with openings to opposite sides of the membrane (Klingenberg, 1980, 1991). Around the translocation channel due to the three-repeat structure rings of positive charges, partially compensated by mobile negative charges, accompany the translocation of the negative solutes (Klingenberg, 1992). Therefore, arginine groups not involved directly in binding, may strongly influence the translocational barriers. In contrast, the inhibitors CAT and BKA are conceived to be localized in a more rigid binding site. None of these arginines is involved in the fixed binding centers as defined by the inhibitors, since their removal does not markedly influence the binding.

Regulation of Oxidative Phosphorylation by AAC and Its Mutants. How does the inversion of the exchange mode pattern explain the "extra"-inhibition of cellular oxidative phosphorylation, reported in the previous paper (Müller et al., 1996)? Whereas in isolated mitochondria with the hexokinase−glucose system the external ATP concentration is virtually zero, in cells a high ATP/ADP ratio prevails in the cytosol. For efficient ATP export during oxidative phosphorylation from the matrix side in the mitochondria the ATP_{out}/ADP_{in} mode should be faster than the ATP_{out}/ADP_{in} mode. This is observed with the reconstituted wt AAC if one considers that in the reconstituted vesicles the AAC is inserted largely inside-out (see Figure 7). However, in the mutant cells the high ATP/ADP ratio forces the limited AAC capacity even into the counterproductive mode of ATP import versus ADP export because this is strongly favored by the low T/D versus high D/T mode activities measured in proteoliposomes. In wt cells, because of the ATP export favouring mode distribution, the productive mode is never fully suppressed by high external ATP/ADP ratios. So the mutations, in particular the R294A, due to the increase of AAC sensitivity to the cytosolic ATP/ADP ratio, unravel the intracellular regulation of AAC activity in oxidative phosphorylation.

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