Probing the Role of Positive Residues in the ADP/ATP Carrier from Yeast. The Effect of Six Arginine Mutations on Oxidative Phosphorylation and AAC Expression†

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ABSTRACT: ADP/ATP transport is the terminal step of oxidative phosphorylation in mitochondria. In this paper seven mutants of AAC2 from *Saccharomyces cerevisiae* are studied on the cellular and mitochondrial level. Six conspicuously located arginines were mutated into mostly neutral residues [Nelson, D. R., Lawson, J. E., Klingenberg, M., & Douglas, M. G. (1993) *J. Mol. Biol. 230,* 1159−1170]. R96A, R96H, R204L, and R294A are located in the second transmembrane helix of each repeat while R252I, R253I, and R254I are in the arginine triplet of the last domain. All six arginine residues are conserved in all known ADP/ATP carrier sequences. At the cellular level, oxidative phosphorylation in R96H and R294A retains 8% of the wild-type rate, but it is virtually zero in the other mutants. However, cytochrome *c*, a parameter of oxidative capacity, remains at 4−42% of wt. The weak coordination of respiratory chain and AAC expression indicates that respiration is needed also for other purposes. In mitochondria the AAC-linked ATP synthesis is measured and segregated by using the AAC inhibitor bongkrekate (BKA). Only the R96H and R294A mutants express a significant rate of AAC-dependent ATP synthesis amounting to 2−18% of the plasmid-borne wild-type AAC2 mitochondria. In all other mutants it is virtually zero. However, respiratory capacity and cytochrome *c* content are reduced only by 20−70%. Whereas in immunoblots the presence of AAC is detected in all mutant mitochondria, by quantitative ELISA no AAC can be measured down to 0.05 μmol of AAC dimer/g of protein in R96A and R204L, whereas in R96H, R252I, R253I, and R254I the content is around 0.2 and in R294A the content is 0.46 as compared to 0.6 in the plasmid wild type. Also the [^{3}H]CAT and [^{3}H]BKA binding is virtually zero in some mutants and closely parallels the ELISA-determined AAC content, indicating that the mutations did not affect the inhibitor binding site. The turnover of AAC [*V*(ATP)/AAC content] in oxidative phosphorylation is reduced to 10% or 20% except for the two intrahelical mutants R96H and R294A. In the three Arg triplet mutants, it is nearly zero. In conclusion, the first two intrahelical arginines R96 and R204, are essential for expression but probably also for the activity of AAC. R294A still retains good transport activity and a remarkably high expression of AAC. All arginines in the triplet 252, 253, 254 are essential. Extrapolation of the *in vitro* phosphorylation rates to the cellular level by the cytochrome *c* factor reveals a large discrepancy to the *in vivo* rates in particular for R294A. This indicates that these mutations render the AAC more sensitive to the regulatory intracellular ATP/ADP ratio than the wt AAC.

The ADP/ATP carrier (AAC)[1] of mitochondria is a key participant in the energy supply of the aerobic eukaryotic cell. It is the last member of the oxidative phosphorylation system, allowing ATP to be exported into the cytosol in exchange for ADP. Since it catalyzes one of the most active transport activities in the eukaryotic cell, it is in many higher organisms the most abundant membrane protein. Studies on the AAC provided for the first time evidence for the translocation mechanism on a molecular level involving a single binding center gated pore [for review see Klingenberg (1976)].

The amino acid sequence of the bovine heart AAC was found to be cluttered with a high percentage of polar residues (Aquila et al., 1982). Out of 297 amino acids 39 are basic and 29 acidic. A similar composition was found in the AAC2 of *Saccharomyces cerevisiae* (Lawson & Douglas, 1988). As a unique feature the primary structure could be divided up into three similar domains of 100 residues each (Saraste & Walker, 1982; Aquila et al., 1985). The sequences of further mitochondrial carriers turned out to be similar and also to have the characteristic three-repeat domain structure (Aquila et al., 1987; Runswick et al., 1990). On the basis of a wide variety of experimental evidence the transmembrane folding of the mitochondrial carriers is assumed to include six transmembrane helices with both the C- and N-terminal protruding to the cytosolic face of the inner membrane (Capobianco et al., 1991; Klingenberg, 1987, 1989, 1993; Bisaccia et al., 1994). In this model most of the charged residues are localized in three regions about 40 residues long, facing the matrix, and connecting the two membrane helices within each domain. Several clusters of

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[1] Abbreviations: AAC, ADP/ATP carrier; BKA, bongkrekate; CAT, carboxyatractylate; PBS, buffer consisting of 5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 150 mM NaCl; wt, wild type.

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charged residues are quite well conserved between the three domains. One might surmise that these conserved polar regions are essential for transport.

In previous publications it was shown that yeast, because of its genetic malleability, provides particularly favorable conditions to examine the AAC (Lawson & Douglas, 1988; Lawson et al., 1990; Nelson et al., 1993). *S. cerevisiae* contains three genes for the AAC, however only one, AAC2, is actively expressed in wild-type cells (Lawson & Douglas, 1988; Lawson et al., 1990; Kolarov et al., 1990). It has been possible also to express AAC1 and AAC2 separately from high-copy plasmids in *S. cerevisiae* strains from which both chromosomal AAC1 and AAC2 have been deleted (Lawson et al., 1990; Gawaz et al., 1990). The kinetic characteristics of the two AAC isoforms were determined after isolation in the reconstituted form (Lawson et al., 1990; Gawaz et al., 1990).

To initiate a systematic analysis of structure-function relationships within the ADP/ATP carrier, *S. cerevisiae* strains were prepared lacking both ADP/ATP carrier isoforms, AAC1 and AAC2, to be used as the recipient for a plasmid carrying selected AAC2 mutation (Lawson et al., 1990; Nelson et al., 1993). The deletion of the third isoform AAC3 (Nelson et al., 1993) proved to be unnecessary, since it is not expressed in any of our mutants under the growth conditions used. AAC2 was chosen for these studies because it is the isoform principally expressed in aerobically grown yeast cells. In this AAC-deleted host system of *S. cerevisiae* site-directed mutants of the AAC2 has been screened by a cell growth assay on glycerol media and discussed in the context of the AAC structure (Nelson et al., 1993). Mutants which replace six intriguingly located arginines are here functionally scrutinized on the cellular and on the mitochondrial level by measuring various parameters of the oxidative phosphorylation system and of the AAC involvement. A number of unexpected findings are obtained by the quantitative determination of the mutational effects. In the accompanying report the kinetic characterization of the isolated mutant AAC in the reconstituted system will be presented.

MATERIALS AND METHODS

Yeast Strains and AAC2 Mutants. DNY1 was described in Nelson et al. (1993). The genotype for DNY1 is MAT<u>a</u>, aac1::LEU2, aac2::HIS3, his 3-11,15, trp 1-1, ura 3-1, leu 2-3,112, ade 2-1, can 1-100. The entire coding region of AAC1 and AAC2 is deleted to prevent recombination events between plasmid-borne mutants and these genes. The generation of the AAC1 deletion was described previously (Lawson et al., 1990). AAC2 mutants except R96A were described in Nelson et al. (1993). R96A was made by PCR using a degenerate oligonucleotide reverse primer (5′ GC GAA ATT CA<u>A AGC TT</u>G AGT GGG GAA ATA T[G,T],-[C,T] GAT AAC G) that is complementary to nucleotides 279−317 in AAC2. The *Hin*dIII site is underlined. This oligo had the potential to make four mutants at R96: K, A, T, or E. The forward primer was the M13(−40) primer. The template was the AAC2 gene in plasmid pSEYc58 (Emr et al., 1986). The PCR product was cut with *Sph*I and *Hin*dIII and ligated into the AAC2 gene in pSEYc58. Mutants were identified by sequencing.

Culture Media. The yeast strains were grown aerobically in a semisynthetic medium (0.3% yeast extract, 0.05% peptone, 3.4 mM $CaCl_2$, 8.55 mM NaCl, 2.4 mM $MgSO_4$, 7.35 mM KH_2PO_4, 18.7 mM NH_4Cl, 11 μM $FeCl_3$). As a major carbon source for the gly^+ strains 2% lactate + 5.55 mM glucose, and for the gly^- strains 1% galactose and 0.55 mM glucose, were used. The cultures were grown in two steps, first a preculture in 500 mL Fernbach flasks for 20−50 h depending on the strain until the absorbance reached 4−6. The main culture was grown in 8 L flasks (New Brunswick) at 30 °C under vigorous aeration. Cells were harvested in the near-end logarithmic phase. For the wild type this required 8−9 h, and for the mutant strains this required up to 50 h. The harvested and washed cells were stored at −80 °C.

The identity of the strains was continuously controlled over the several years of collecting the data. From the main culture samples were assayed on agar plates with a synthetic medium, which was made selective for the plasmid containing strains by the omission of uracil and histidine. In some cases for the cultures the plasmid was reisolated and the identity of the mutant AAC confirmed by sequencing the AAC insert.

Preparation of Mitochondria. Protoplasts were formed by enzymatic digestion of the cell wall (Daum et al., 1982). The procedure was followed essentially as described by Gawaz et al. (1990).

Respiration. Oxygen consumption was determined by polarography with the platinum electrode at 25 °C. Isolated mitochondria were suspended in the "standard buffer" consisting of 0.65 M mannitol, 2 mM $MgCl_2$, 10 mM KH_2PO_4, 10 mM Hepes, 0.2% bovine serum albumin (0.5−1 mg/mL) 5 mM KCl, pH 7.4. Substrate was 5 mM L-α-glycerophosphate.

Determination of Cytochrome Content. The cytochrome content of yeast cells and mitochondria was measured using a Cary model 117A spectrophotometer. The reduced minus oxidized visible spectrum was recorded. Cells were diluted in a weight ratio of 1:10 in 10 mM NaH_2PO_4, 20 mM Tris, pH 7.5 (0.01−0.05 g dry weight/mL). The oxidized state was established by aerating the cells (and also the mitochondria) of the reference cuvette with oxygen. The reduced state was obtained by letting the cells become anaerobic with 2% ethanol. Mitochondria (5−10 mg/mL) were incubated in a standard buffer and the sample cuvette was made anaerobic by the addition of 10 mM L-α-glycerophosphate as a substrate for respiration.

Oxidative Phosphorylation in Cells. Cells at a 50 mg wet weight/mL were incubated in a medium containing 20 mM Tris, 5 M $NaPO_4$, pH 7.4, and aerated overnight in order to deplete endogenous substrates. The starved cells were centrifuged at 4500*g* for 10 min at 4 °C. The pellet was washed in 40 mM citric acid and 40 mM $NaPO_4$, pH 4.3, centrifuged and resuspended at 100−150 mg/μL wet weight in the same buffer. To deplete the ATP, this suspension was flushed with N_2 and kept anaerobic by the addition of 2% ethanol for 30 min at 25 °C. In a typical experiment the total volume was 10 mL from which 8 mL were sucked into a 10 mL gaslight glass syringe which had prior been flushed with N_2. The syringe was brought to 10 °C in a water bath, and to start oxidative phosphorylation the content was injected into a vessel flushed with O_2 which was also thermostated at 10 °C. Under stirring, 0.5 mL aliquots were withdrawn at increasing time intervals and injected into 0.2 mL of 52% $HClO_4$ for extraction of ADP. The time intervals

depended on the cell type. For wild type they were after 10, 20, 30, 60, and 120 s, and for mutants the intervals were 30, 60, 120, and 300 s. For the control of AAC-independent ATP synthesis a 2 mL aliquot of the cell suspension was incubated with 10 μM BKA for 10 min under N_2 and sucked into a 4 mL glass syringe. The solute was brought to 10 °C and to start oxidative phosphorylation injected into a O_2 flushed vessel. After 150 s 0.5 mL was withdrawn for the extraction of ATP. The denatured samples were centrifuged, and the supernatants were neutralized with KOH. $KClO_4$ was removed by centrifugation. ATP was measured by the hexokinase-glucose-6-dehydrogenase assay. From the time dependent increase of ATP content the initial rate of oxidative phosphorylation V(ATP) was calculated. The BKA insensitive phosphorylation rate, *V*(ATP)-corrected, is calculated using ATP content with and without BKA measured at 150 s or at other times when BKA preincubated cells were quenched.

$$V(\text{ATP})\text{-corrected} = \alpha \cdot V(\text{ATP})$$

with

$$\alpha = 1 - \text{ATP}(+\text{BKA})/\text{ATP}$$

Oxidative Phosphorylation in Mitochondria. Isolated mitochondria were suspended at 1 mg of protein/mL in 2.0 mL of a medium containing 0.25 M sucrose, 20 mM KCl, 20 mM Tris, 0.5 mM EDTA, 10 mM glucose, 4 mM $NaPO_4$, and 3 mM $MgCl_2$ at pH 7.2. Further additives were 0.5 mg of bovine serum albumin, 10 μM AP_5A, 50 μM ADP, 3 units/mL hexokinase. The suspension was aerated with O_2 for 2−3 min at 25 °C. The "zero sample" was withdrawn, and the oxidative phosphorylation was started by the addition of 5 mM L-α-glycerophosphate and 50 mM ethanol. As a control value for AAC-dependent oxidative phosphorylation, one aliquot of 0.5 mL was withdrawn and incubated with 20 μM CAT and 10 μM BKA prior to the addition of the substrates. This sample was then also incubated with oxygen and oxidative phosphorylation started with the substrates and after 2 min quenched by $HClO_4$. From the main suspension aliquots of 0.25 mL were withdrawn and injected into 0.02 mL of 45% $HClO_4$ at the following time intervals: wild-type mitochondria at 10, 20, 40, 60, and 120 s, and mutant mitochondria at 0.5, 1, 2, 4, 8, and 12 min. After neutralization with KOH and removal of $KClO_4$ by centrifugation, ATP was assayed by the hexokinase−glucose-6-phosphate-dehydrogenase system. The initial phosphorylation rate *V*(ATP) was calculated from the increase of ATP content. For obtaining the AAC dependent rate, *V*(ATP) corrected, a correction factor was calculated from the ATP content with and without CAT + BKA taken 2 min after the start.

$$V(\text{ATP})\text{-corrected} = \alpha V(\text{ATP}) \text{ with } (\alpha = 1 - [\text{ATP}]_{(+\text{CAT}+\text{BKA})}/[\text{ATP}])$$

$$[\text{ATP}]_{(+\text{CAT}+\text{BKA})} = \text{ATP content in the presence of CAT and BKA}$$

$$[\text{ATP}] = \text{ATP content without inhibitors}$$

Binding of [^{3}H]CAT and [^{3}H]BKA. For binding measurements mitochondria were incubated with 3 mg of protein in 1 mL of a medium containing 0.25 mM sucrose, 20 mM Tris, 1 mM EDTA, and 50 μM ADP, pH 6.8, at 25 °C. For the blank value aliquots of 450 μL were preincubated with 20 μM unlabeled CAT. To this and the other aliquot of 450 μL each 0.6−1.2 nmol/mg of protein was added [^{3}H]CAT with a specific activity of 3000 dpm/nmol and incubated for 20 min. The mitochondria were sedimented at 30000*g*, and the pellets were solubilized with 200 μL of 5% SDS solution. The [^{3}H]CAT was measured by scintillation counting both the solubilized sediments and the supernatants. To measure [^{3}H]BKA binding the equivalent procedure was applied. For the blank value the mitochondria were preincubated with 20 μM unlabeled BKA instead. The specific activity of [^{3}H]-BKA was 4500 dpm/nmol. [^{3}H]BKA and [^{3}H]CAT were prepared as described (Babel, 1975; Babel et al., 1976).

Generation of Polyclonal Antibodies. Mitochondria isolated from yeast were loaded with CAT (2 μmol/mg of protein). AAC2 was isolated by solubilization with TX-100 and passage through hydroxyapatite. Protein was precipitated from the eluate with 4.5% trichloroacetic acid, 34% acetone, and dissolved in 4% SDS, 12% glycerol, 1% mercaptoethanol, 50 mM Tris, pH 6.8. The solute was electrophoresed in a 1.6 mm thick, 12% polyacrylamide gel, with a minimal amount of Coomassie Brilliant Blue. The AAC2 band was excised and homogenized by sonication. This suspension was injected into rabbits.

Competitive ELISA Assay. The mitochondria (0.3−0.5 mg) were precipitated with 50% trichloroacetic acid, and the sediments were dissolved in 3% SDS solution. After determination of the protein content, a dilution series was generated using PBS containing 0.04% TX-100 with 0.004% SDS, such that 1.000, 750, 500, 375, 250, 125, 62.5, 31, 16, and 0 ng of AAC2 protein reacted with a constant amount of AAC2 antiserum, diluted at 1:8000 with PBS-Tween. After 2 h at 25 °C, the solution was ready for application to the ELISA assay.

For the competitive ELISA assay a MaxiSorp (NUNC) ELISA plate (8 × 12 wells) was coated overnight with 50 ng of AAC per well. The plate was washed 3 times with PBS-Tween. Subsequently it was blocked for 1 h with 200 μL/well of Boehringer blocking reagent and again washed 3 times with PBS-Tween. From the mitochondria dilution antibody series each 100 μL/well were incubated overnight at 4 °C. The plate was washed 3 times with PBS-Tween and then incubated with 50 μL/well of anti rabbit IgG peroxidase conjugate, diluted 1:4000 with PBS-Tween for 1 h at 25 °C. After being washed 3 times with PBS-Tween in a darkened room, 200 μL of the *o*-phenylenediamine hydrochloride H_2O_2 solution was added. The solution was prepared by dissolving 100 mg of *o*-phenylenediamine hydrochloride 3 μL of 3% H_2O_2 in 25 mL of a buffer containing 0.1 M Na_2 HCl, 0.05 M citric acid, adjusted to pH 5.0. After 10 min, the reaction was stopped with 50 μL/well of 4 N H_2SO_4 and the absorbance was measured at 492 nm in an ELISA plate reader.

RESULTS

First, we measured the oxidative phosphorylation rates in the mutant and control cells. Further, on isolated mitochondria AAC-related parameters, such as the ATP-synthesis rate and the effect of AAC inhibitors, are studied. The content of AAC in mitochondria is determined by quantitative ELISA and matched with the capability for binding the AAC-specific inhibitors bongkrekate (BKA) and carboxyatractylate (CAT).

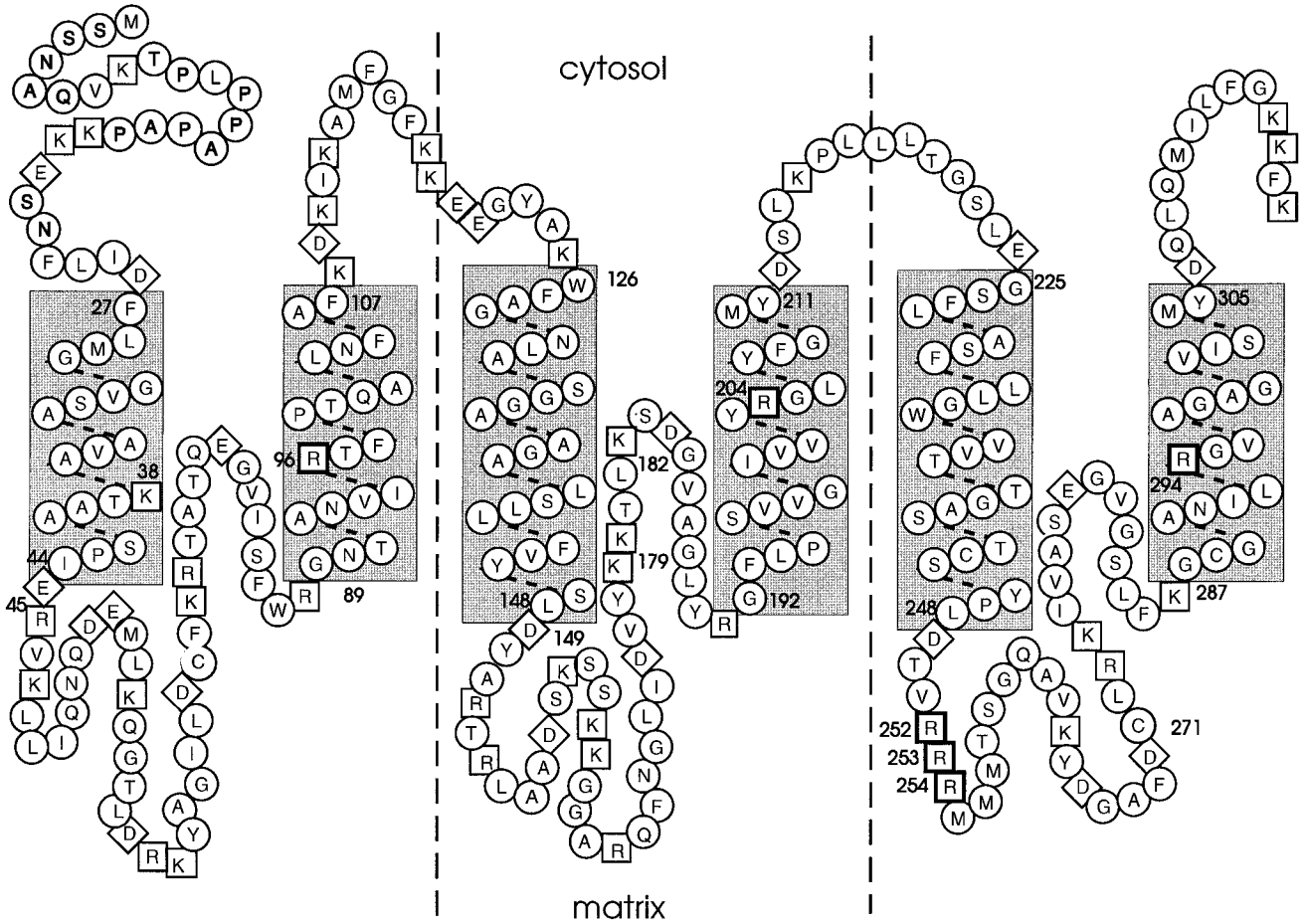

FIGURE 1: Folding diagram of the AAC2 from *S. cerevisiae*. The three-repeat structure of the AAC with the transmembrane helices in shaded blocks. The mutated residues are in bold frames. The frames around the residue symbols signify (◇) acidic; (□) basic; (○) neutral residues.

The cytochrome content of cells and mitochondria serves to correlate the cellular and mitochondrial AAC activities during oxidative phosphorylation.

The first target were the three arginines in the second helix of each repeat domain, R96, R204, and R294 (Figure 1), which were converted into neutral residues, R96A, R204L, and R294A. In addition, the R96H mutant was generated since the same mutation occurs in the pet9 mutant, which is the only naturally occurring AAC mutant known (Kolarov et al., 1990; Lawson et al., 1994). Out of the abundant polar residues in the matrix directed regions we have selected the arginine triplet in the third domain. Different from the intrahelical arginines, this triplet R252, R253, R254 is nearly specific for the AAC and is found in all 34 AAC sequences known so far.

Cells. In a previous publication growth on glycerol was used to screen mutations of the AAC as to whether they cause a defect in oxidative phosphorylation (Nelson et al., 1993). All mutational neutralization of the intrahelical arginines in helix 2 of each repeat, R96A, R96H, R204L, and R294A, produced gly$^-$ strains. The same is true for mutations in the arginine triplet R252, R253, R254 converting each of the arginines to isoleucine.

To correlate quantitatively the mutational effects on the yeast cell metabolism, the rate of intracellular oxidative-phosphorylation was determined. For this purpose the oxygen dependent ATP production in these mutant cells was measured. In the substrate-depleted and anaerobic cells, the ATP production is initiated by flushing the suspension with 100% oxygen with 50 mM ethanol as a substrate. A typical time dependence of ATP synthesis in wild-type and R294A mutant yeast cells is given in Figure 2A. To discern the AAC-mediated ATP synthesis, the inhibitor BKA is added in control samples. BKA is membrane permeant in contrast to CAT (Scherer & Klingenberg, 1974). However, for BKA to penetrate into yeast cells and to accumulate at sufficiently high concentration, the cells have to be incubated at a low pH of 4.5 (Kolarov, 1972). The effectiveness of BKA is shown in wild-type cells where in the presence of BKA the ATP production rate is down to only a few percent.

The oxidative phosphorylation rates are plotted (Figure 3) with two different scales in order to account for the large differences between the gly$^+$ and gly$^-$ strains. The host strain from which the nuclear AAC2 and AAC1 genes are deleted and in which wild-type AAC2 is expressed via the plasmid carrying gene is the appropriate reference for the mutants. In this plasmid AAC2 strain the oxidative phosphorylation is only 30% of the nuclear encoded AAC2 strain. As a negative control the host strain from which the AAC2 plasmid is deleted shows no oxidative phosphorylation. Among the three intrahelical arginine mutants the ATP production is completely abolished in R96A and R204L. Still significant phosphorylation rates of 8% and 7.5% are found in the R96H and R294A mutants. In all three single mutants of the arginine triplet (R252I, R253I, R254I) no intracellular oxidative ATP production can be detected. These results show that mutation of R96 and R294 does not completely annihilate the intracellular ATP production but that each

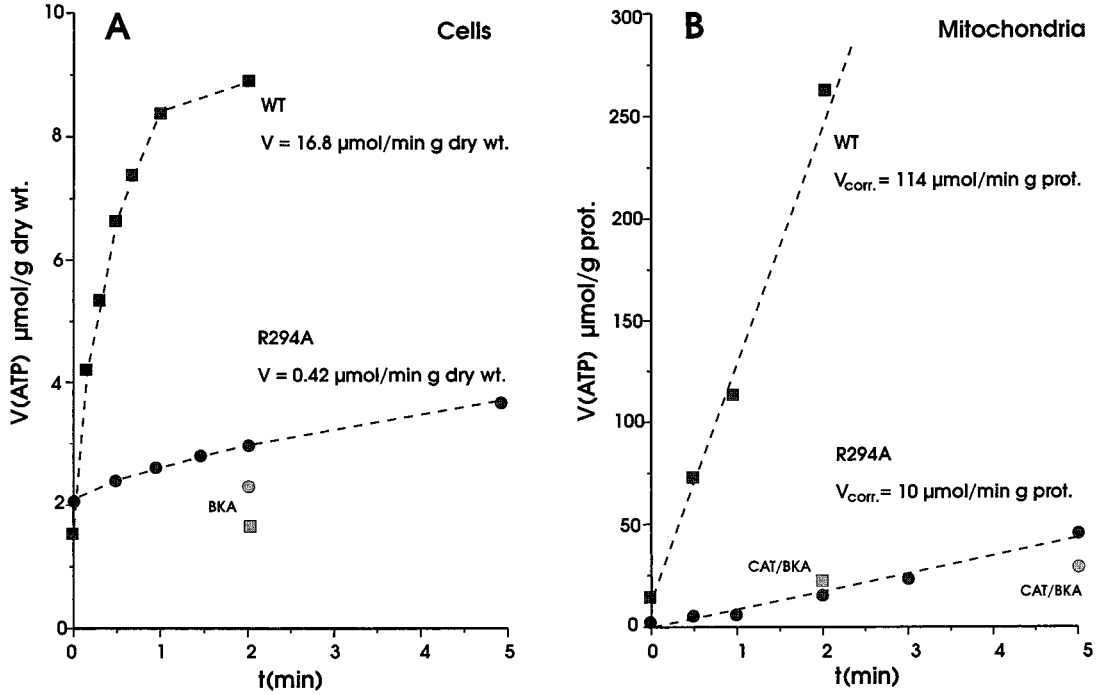

FIGURE 2: Oxidative phosphorylation in cells and mitochondria. Time dependence of ATP formation in the wild type (wt) and the R294A mutant. By preincubation with the inhibitors BKA and CAT the AAC-independent ATP formation is measured and used to calculate from the total ATP synthesis rate the AAC dependent corrected rate. In cells only BKA and in mitochondria a combination of CAT and BKA is added. For details see Materials and Methods.

Table 1: Cytochrome *c* Content in Cells with Wild-Type (wt) AAC, Plasmid Wild-Type AAC2 (p-AAC2), AAC2-Deficient (p-AAC2(−)), and Mutated AAC2[a]

	cytochrome *c* (nmol/g dry weight)		cytochrome *c* (nmol/g dry weight)
wt	180	R96H	30
p-AAC2	48	R204L	19
p-AAC2(−)	28	R294A	76
R96A	8	R252I	26

[a] The cytochrome content was measured by anaerobic minus aerobic cells, using the absorbance difference at 550 nm and the extinction coefficient $\Delta\epsilon^{mM}_{550nM} = 19.5\ cm^{-1}$.

arginine of the triplet is vital for intracellular oxidative phosphorylation.

BKA completely inhibited the oxidative ATP synthesis in the two wild-type strains. BKA was unable to inhibit phosphorylation of the R96H strain but completely inhibited R294A. The marginal ability of BKA to inhibit the low phosphorylation rates of the mutants is not easily explained. One possibility might be a poor ability of the mutant cells to take up BKA since the isolated mitochondria do bind [^{3}H]-BKA.

The oxidative phosphorylation system of mitochondria extends from the respiratory chain to the AAC. Partial or total inactivation of the AAC by mutation should also influence the capacity for electron transport, as measured by the cytochrome content. We determined the content of cytochrome *c* since it is the only cytochrome that can be measured with some accuracy in the yeast cells, even at the low content of some mutant strains. As shown in Table 1, the cytochrome *c* content varies much less than the oxidative phosphorylation rates. It does not reach zero even when oxidative phosphorylation is completely suppressed, as in some mutants and in the AAC2-deleted host strain. The residual content of the respiratory chain may serve other purposes, such as maintaining the membrane potential for substrate transport of these mitochondria.

Mitochondria. The isolation of sufficient mitochondria for studying the mutational effects in the AAC required large scale preparation of yeast cells. In particular from the gly$^-$ strains the yield of mitochondria was low. The mitochondria were used to study a number of AAC-linked parameters, such as the rate of oxidative phosphorylation, the content of AAC by quantitative ELISA, the binding of CAT and of BKA. Further, the respiratory capacities of the mitochondria isolated from these mutant strains were investigated.

The rate of ATP synthesis in the isolated mitochondria from the various yeast strains was determined as a parameter linked to the activity of the ATP/ADP exchange. For technical reasons these measurements had to be performed with frozen−thawed mitochondria. These contain a low portion of broken mitochondria and inverted inner membrane vesicles which should not contribute to the ATP synthesis via the AAC transport. The AAC-linked portion of ATP synthesis is determined by control experiments in which the AAC transport is inhibited with a combined addition of CAT and BKA. In most mitochondria preparations from wt and mutants even with CAT alone the inhibition reached 90−95% indicating that they mostly retained the mitochondrial configuration after freeze−thawing. A typical example for the time progress of ATP formation is given in Figure 2B. The total rate of ATP formation is corrected using the CAT and BKA resistant ATP synthesis to obtain the AAC-dependent ATP synthesis rate. As shown in Figure 4, in wild-type mitochondria the total ATP synthesis is nearly identical to the corrected rate because it was almost fully suppressed by CAT and BKA. This allows the conclusion that all ATP formed has to pass through the AAC. A small part of ATP could be due to a residual adenylate kinase-type reaction although adenylate kinase is largely inhibited

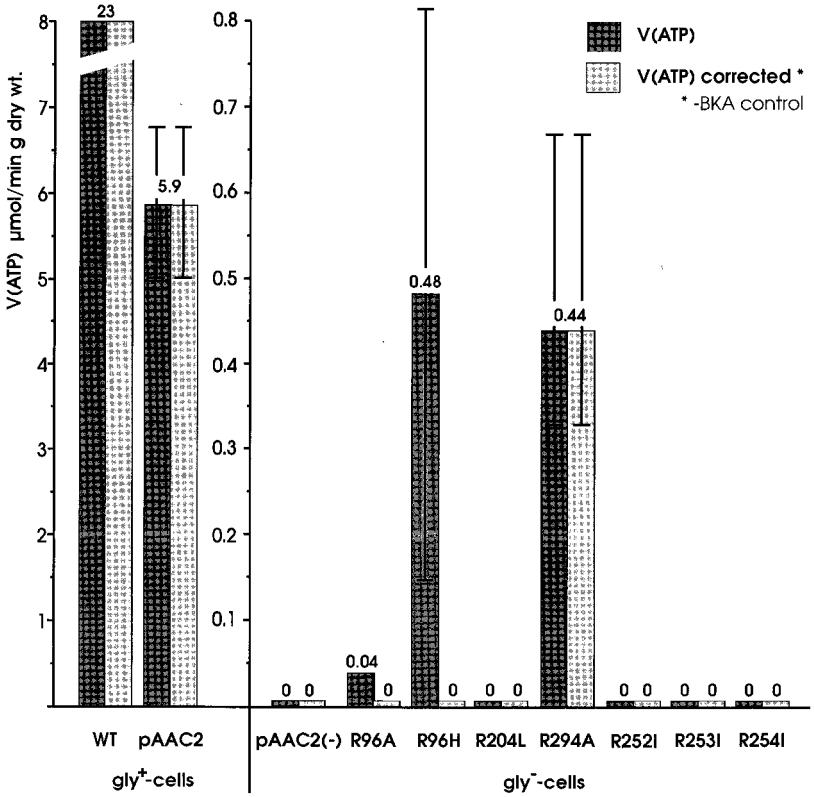

FIGURE 3: Oxidative phosphorylation in cells. The rates of ATP formation are given without and with correction for the BKA resistant rates. Note the difference in scales for the gly$^+$ and gly$^-$ cells. The ATP synthesis rates V(ATP) is evaluated from the time dependence of ATP formation as shown in Figure 2. For details see Materials and Methods.

by the addition of AP_5A. This portion becomes more prominent in mutant mitochondria with a low ATP synthesis rate.

The ATP synthesis rate of wild-type mitochondria is V(ATP) = 180 μmol/min/g of protein but only 63 μmol/min/g of protein in the plasmid AAC2 mitochondria. There is virtually no BKA-sensitive ATP synthesis rate (note 10 times smaller scale) in the plasmid AAC2 less mitochondria. These control values prove that there is no AAC3 which can compensate for the loss of AAC1 and AAC2. It also confirms the validity of our assumption to use the ATP synthesis rate as an indicator of AAC activity. Also in the intrahelical R96A and R204L mutants as well as in the triplet arginine mutants R252I, R253I, R254I virtually no CAT and BKA sensitive ATP synthesis is measured. This agrees with the absence of measurable ATP synthesis of these mutants in the cells. But in the intrahelical R96H mutant distinct phosphorylation rates amounting to 2.7% and in the R294A mutant even 17.5% of the wild-type activity are found. Again this accords approximately with the oxidative phosphorylation activities seen in the cells where the same hierarchy of activity, R294A = R96H > R96A > R204L, is measured.

The respiratory activity can be expected to be linked to the capacity for oxidative phosphorylation, which again is dependent on the AAC activity. Also, the cytochrome content was determined by difference absorption spectra of mitochondria. The respiration of the mitochondria was measured using as substrates a mixture of glycerol-1-phosphate, glutamate, and malate (Table 2). The respiratory rate of wild-type mitochondria, as well as that of the plasmid wild-type AAC2, is 200−300 μatoms/min/g of protein. The gly$^-$ mutants including the AAC2 deficient mitochondria still have a remarkably high respiration between 60 and 200 μatoms/min/g of protein despite the complete suppression of oxidative phosphorylation in some mutant cells. This discrepancy is strongest with mitochondria of the R294A mutant.

Table 2: Oxidative Capacity of Mitochondria in Various AAC Mutants, Measured by the Respiratory Rate and Cytochrome *c* Content[a]

	respiration (μA of O/ min g of protein)	cytochrome *c* content (μmol/g of protein)	respiration cytochrome *c* $(\text{min})^{-1}$
wt	206 (133−301)	0.69 (0.60−1.11)	281
p-AAC2	245 (202−293)	0.50 (0.30−0.65)	490
p-AAC2(−)	74	0.25	302
R96A	57 (39−75)	0.19 (0.12−0.25)	308
R96H	98 (81−106)	0.32 (0.19−0.43)	303
R204L	68	0.28 (0.18−0.39)	243
R294A	138 (103−197)	0.33 0.27−0.46)	422
R252I	54 (43−65)	0.65	83
R253I	100 (80−114)	0.54 (0.47−0.60)	186
R254I	109	0.51	215

[a] The respiration is measured polarographically as described in Materials and Methods. For measuring the cytochrome *c* content see Materials and Methods.

As in cells, also in mitochondria only the absorption difference of cytochrome *c* could be evaluated with sufficient accuracy since the cytochrome *a* content is quite low in the mutants as to be expected from the strongly decreased requirement for oxidative phosphorylation. In AAC2 deficient mutants the cytochrome *c* content of mitochondria is half of the wild-type (pAAC2) level. In the mutant mitochondria R96A and R204L from cells, which are completely devoid of oxidative phosphorylation, the cytochrome *c* content is still half as high as in the pAAC2 wild type.

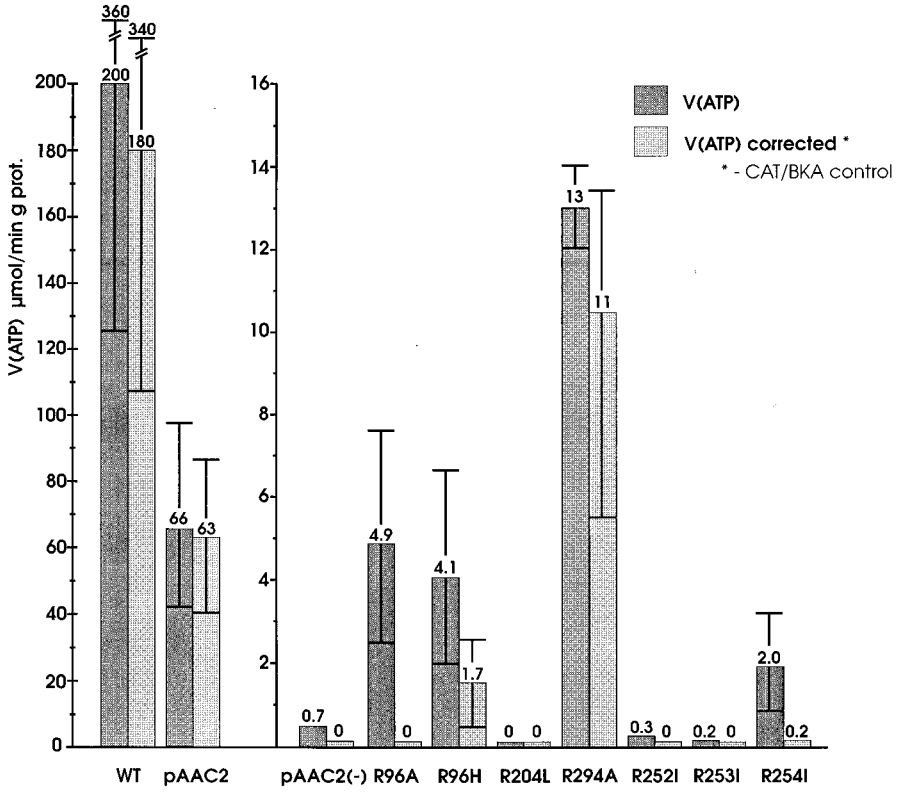

FIGURE 4: Oxidative phosphorylation in isolated mitochondria of various AAC mutants. The ATP synthesis rates are evaluated from the time dependence shown in Figure 2. The rates are given before and after correction for the BKA + CAT resistant rates. The range encompasses three to five measurements, and the mean value is given. For details see Materials and Methods.

Particularly in the triplet arginine mutants R252I to R254I the discrepancy between the oxidative phosphorylation measured in cells and the cytochrome content is striking since the cytochrome *c* content is not decreased, although oxidative phosphorylation is virtually zero. The high cytochrome *c*content in the triple R mutants is also responsible for the low ratio of respiration to cytochrome *c*. Probably here cytochrome *c* is present in a higher stoichiometry to cytochrome aa_3, for unknown reasons. Not being a fixed member of any respirating complex, the content of cytochrome *c* can have varying stoichiometric ratios to cytochrome aa_3.

Determination of AAC Content by Quantitative ELISA. The question can be asked to what extent the low oxidative phosphorylation activity observed in mutant cells and in isolated mitochondria is due to low expression levels and/or due to the low activity of the mutant AAC. The classical method for the determination of the AAC content in mitochondria was the binding of [^{3}H]CAT (Klingenberg et al., 1975). Since mutation may affect the binding of this highly specific ligand to the AAC the quantitative ELISA was applied. Using polyclonal antibodies specific for the AAC2 from yeast, a quantitative determination of the AAC content in isolated mitochondria was performed by the competitive ELISA with purified AAC2 for calibration. The linearity of the method over a relatively large range provides the most accurate immunological approach to measure the AAC content. A comparison of the immunological determination of the AAC content with the [^{3}H]CAT and [^{3}H]-BKA binding data might reveal discrepancies and a possible mutational effect on the ability to bind these ligands. The quantitative ELISA determination requires that the influence of the detergents used for solubilization of the mitochondria and which accompany the purified antigen are carefully controlled since they can interfere with the antibody reaction.

The results of the ELISA determination of the AAC content in several mitochondrial preparations from each mutant are assembled in Figure 5A. In wt and in mitochondria isolated from the plasmid AAC2 strain, the AAC content amounts on average to 0.7 μmol/g of protein. A control value without plasmid is zero. A virtual absence of AAC was also determined in the two intrahelical R96A and R204L mutants. The highest AAC content for these gly$^-$ mutants is found in the R294A mutant. A lower but still remarkable content of AAC amounting to 20% of the wild type is measured in each of the three triple Arg mutant mitochondria.

Immunoblots (Figure 5B) of the same mitochondria preparation as used for the ELISA assay and [^{3}H]CAT and [^{3}H]BKA binding determinations produce positive signals for the AAC content in all mutants. The stains between wild type and several mutants differ, but much less as to be expected from the ELISA assay. This demonstrates that the convenient and rapidly performed immunoblots can give a misleading impression of the AAC content [see Nelson et al. (1993)] since its signal is quite insensitive to the at least 50-fold variation of the AAC content between wt and some mutants, such as R96A and R204L. The immunoblots also illustrate that these mitochondria do not contain any AAC3. This isoform with a 1.2 kDa smaller molecular weight reacts also with antiserum against AAC2 (Drgon et al., 1992) and can be clearly segregated on the SDS gels at a lower position (Drgon et al., 1991). The expression of AAC3 cannot be expected to compensate for the AAC2 defects, since it requires strict anaerobiosis, whereas here the cells are grown with ample oxygen supply.

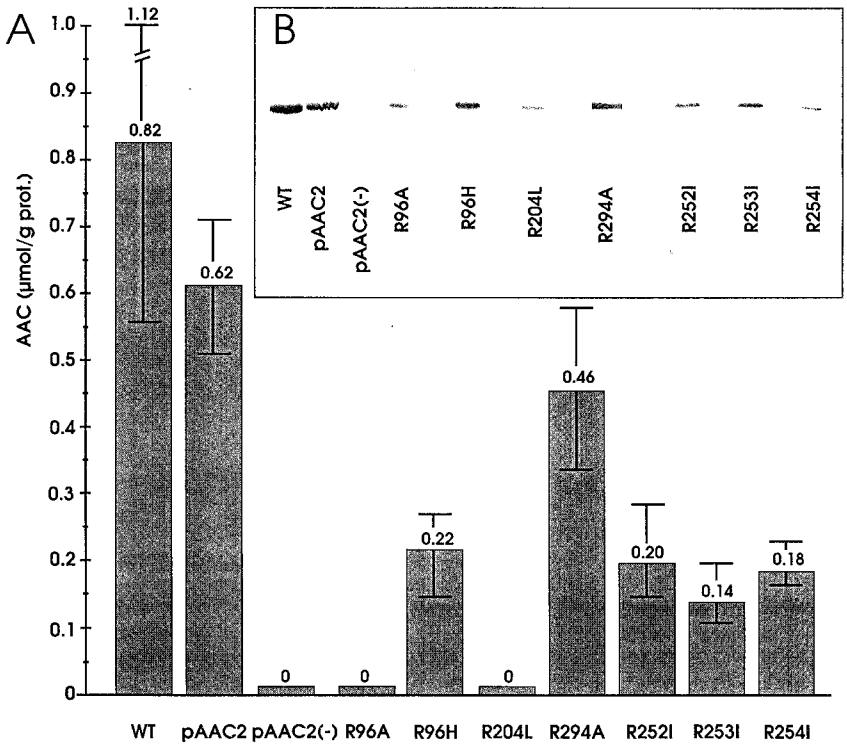

FIGURE 5: (A) The content of AAC in the mitochondria from various AAC mutants as determined by competitive ELISA. The range encompasses three to eight values from different mitochondrial preparations. The content refers to the AAC dimer. For details see Materials and Methods. (B) Immunoblots of mitochondria. Proteins from 20 μg of mitochondria were separated in "Laemmli" gels, blotted, and stained with AAC2-specific antiserum peroxidase conjugated antibodies using 3,3′-diamino benzidine.

[^{3}H]CAT and [^{3}H]BKA Binding to Mitochondria. The interest to determine the binding of the highly specific inhibitory ligand CAT to AAC is 2-fold: to use the binding as an accurate method to measure the AAC content and/or to determine on the basis of the immunological measurements whether the mutation might have influenced the binding of this ligand. The measurements of [^{3}H]CAT are technically more precise than the ELISA determination. A possibly moderate decrease in the affinity of CAT binding in the mutants is compensated by using excess of [^{3}H]CAT for measuring the binding capacity. As shown in Figure 6A, in wild-type mitochondria [^{3}H]CAT binding amounts on the average to 0.8 μmol/g of protein. A similar high binding is measured in mitochondria from the plasmid wild-type AAC2 containing cells, whereas [^{3}H]CAT binding is zero in the AAC2-less strain. There is also no binding in the two intrahelical R96A and R204L mutants. However, in the R96H mutant [^{3}H]CAT binds to 25% of the wild-type level. Surprisingly, in the intrahelical mutant R294A [^{3}H]CAT binding of mitochondria is only reduced by 25%. Also in the triplet arginine mutants R252I, R253I, and R254I, [^{3}H]-CAT binding reaches 20−25% of the wild-type level. There is a good reason to assume that the [^{3}H]CAT binding, at least in those mutants with good binding, accurately measures the content of the AAC.

We also determined the binding of bongkrekate (BKA) to mitochondria. BKA has been shown to bind to the AAC of bovine heart mitochondria in a near 1:1 molar ratio with CAT (Klingenberg et al., 1983). Both inhibitors bind to the AAC in different states, CAT binding requires the binding site of the AAC to be in the c-state and BKA binding in the m-state (Klingenberg, 1976). Therefore and because of the largely different chemical structures of BKA and CAT, different residues of the AAC could be involved in the binding of both inhibitors. Consequently, a mutation could be expected to affect the binding of the inhibitors differently.

In Figure 6B the binding of [^{3}H]BKA to the various mutant mitochondria is plotted. Only the specific binding is given, after subtraction of the unspecific [^{3}H]BKA uptake. No binding of BKA is found in the two intrahelical R96A and R204L mutant mitochondria. The R96H mutant mitochondria bind some and the R294A mutant mitochondria bind a comparatively high amount of BKA. Also the three R triplet mutants retain considerable BKA binding. In general, the [^{3}H]BKA binding parallels quite well that of [^{3}H]CAT. In agreement with earlier experiences the [^{3}H]BKA binding is always somewhat higher than the [^{3}H]CAT binding (Klingenberg et al., 1983). The binding pattern of [^{3}H]CAT and [^{3}H]BKA also agrees with the AAC content measured by ELISA. This allows the conclusion that none of the mutated residues is essential for the binding of either of the two inhibitors.

The clearly defined levels of AAC in the R96H mutant and in the R254I, R253I, R254I mutants reaching an even higher level in the R294A mutant indicate, in face of the low phosphorylation rates of the mitochondria, that the specific transport activity is much more reduced by these mutations than the level of expression. This is quantitatively stated in terms of the molecular activity or turnover of the AAC in mitochondria, calculated as the ratio of *V*(ATP) to [^{3}H]CAT binding as given in Figure 7. Both the uncorrected and exclusively AAC-dependent corrected rates of oxidative phosphorylation are shown. The molecular activity of the R294A mutant is reduced to about 10% of the wild type. For the R96H mutant the molecular activity is about 5%. The decrease of the molecular activity is still more pronounced in the poorly expressed triple Arg mutants where it amounts to about 2−5% of the wild-type activity. No

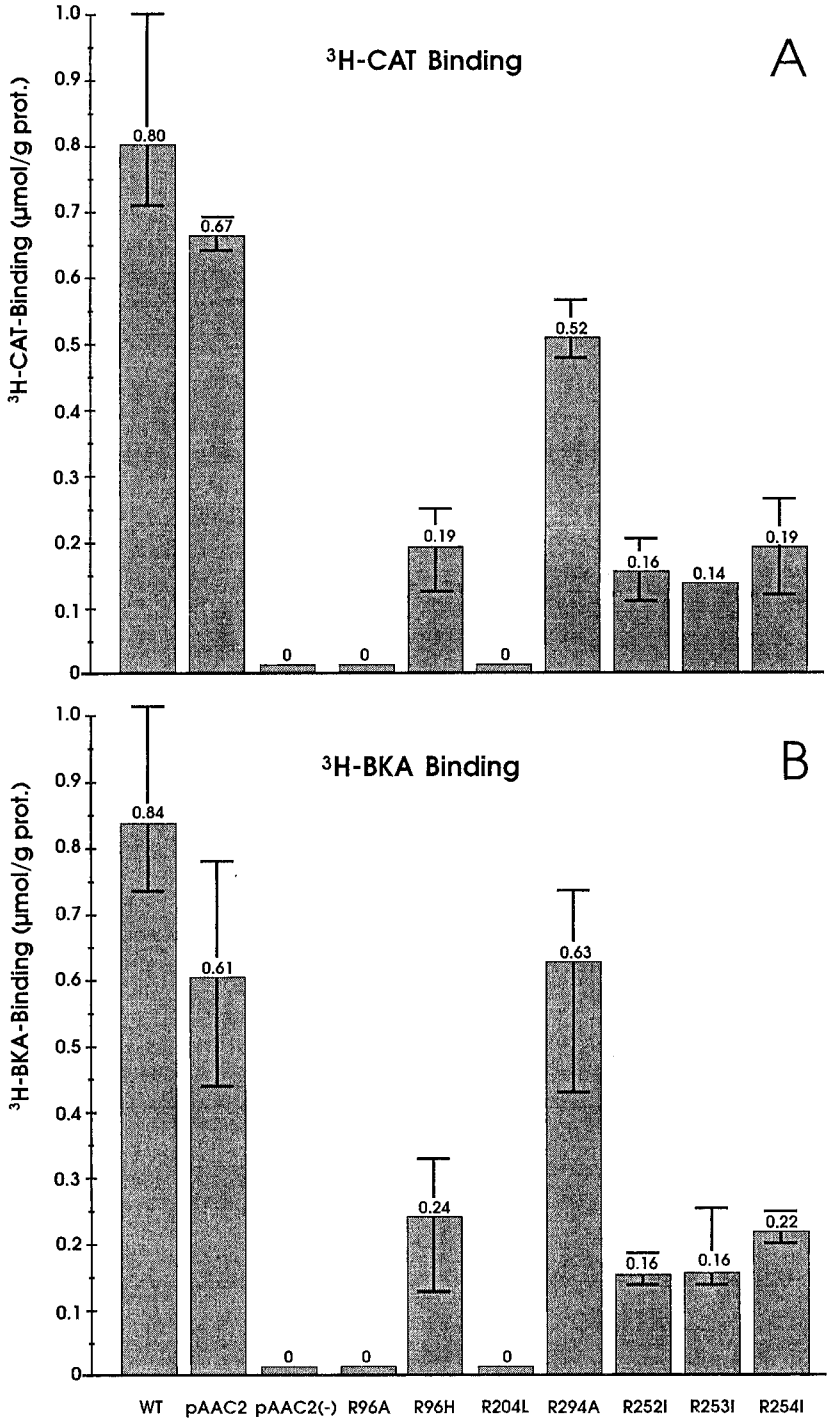

FIGURE 6: (A) [^{3}H]CAT binding to mitochondria from various AAC mutants. (B) [^{3}H]BKA binding to mitochondria from various AAC mutants. The range encompasses three to five values from different mitochondrial preparations.

molecular activity can be calculated for the two intrahelical R96A and R204L mutants with no measurable CAT binding.

DISCUSSION

Study of mutations of the ADP/ATP carrier in yeast has the advantage that one can trace the mutational effects on all levels of integration from the cells to isolated mitochondria, to the isolated AAC protein in the detergent micelle, and finally reconstituted into proteoliposomes. Since the AAC is the last component in the oxidative phosphorylation system, we included in our studies the mutational effects on related parameters, such as the content of cytochromes, the respiratory rates and the phosphorylation rate both at the cellular and mitochondrial level. In mutants the comparison and the extrapolation of mitochondrial phosphorylation rates with the cellular rates reveals striking intracellular control elements of ATP synthesis. Further, the level of expression of the AAC was determined with quantitative ELISA and indirectly, through the binding capacity for CAT and BKA.

In this first set of site-directed mutations exclusively arginines were changed to neutral or nearly neutral residues. The regular three-repeat occurrence of arginine in the second helices of each domain and the striking arginine triplet, were strong candidates for an essential role in the AAC (Nelson et al., 1993; Klingenberg & Nelson, 1994). Whereas the occurrence of three intrahelical arginines is typical for the

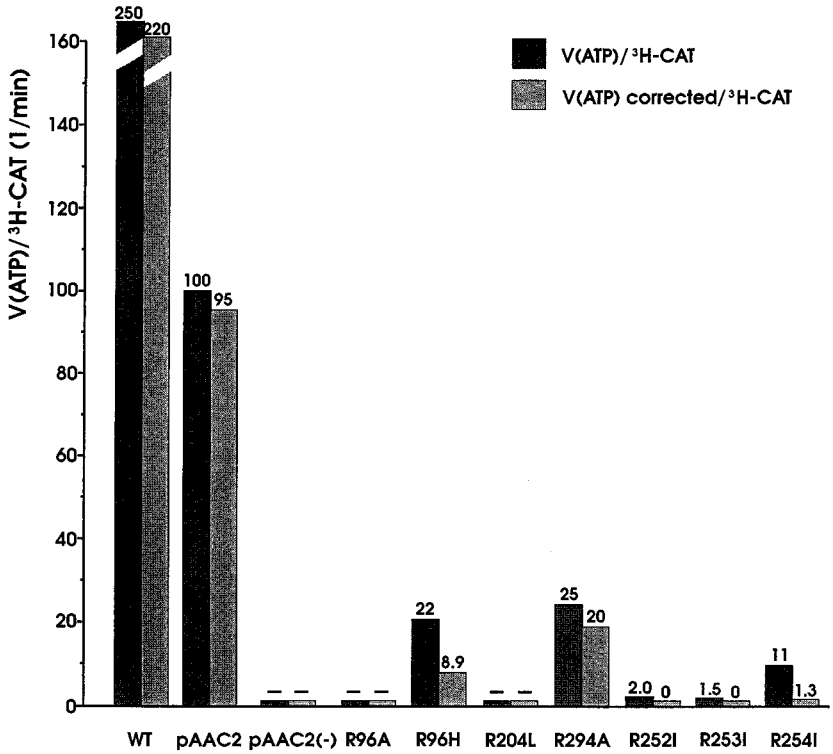

FIGURE 7: Turnover of the AAC from different mutants. The turnover is calculated with the average values of V(ATP) and [^{3}H]CAT binding given in Figures 4 and 6.

mitochondrial superfamily, the arginine triplet is characteristic for all known AAC. Also typical for all known AAC, the second intrahelical arginine R204L is shifted to the helix five amino acids to the N-terminal as compared to the other two intrahelical arginines. A similar rudiment motif KXR or RXR is found also in the other two repeats in the matrix domains at homologous positions. From the present results it is obvious that the six mutated arginine residues play an important role for the AAC activity, however, probably for mechanistically quite different reasons.

Cells. Although the ability of the cells to grow on glycerol is an important criterion for the mutational effect, only the quantitative assay of the oxidative phosphorylation capacity of the cells can be compared with the measurements on the mitochondrial and isolated protein levels. We find a dramatic decrease of oxidative phosphorylation to less than 2% or even zero. As judged from the present mutant selection, the cutoff point for sustaining growth on glycerol is at a cellular oxidative phosphorylation rate above 0.5 μmol of ATP/min/g of protein. For differentiating that portion of ATP synthesis which has to pass through the AAC, the inhibition of the intracellular AAC by BKA instead of CAT was applied since BKA should be permeant to the cell membrane. In the gly$^+$ cells and in the R294A mutant most of the ATP synthesis can be blocked by BKA while in the R96H cells the ATP formation seems to be BKA-insensitive. At face value this could mean that in this mutant no phosphorylation is passing through the AAC, however, this seems unlikely. One possibility, as mentioned above, is that BKA does not sufficiently accumulate in these mutant cells because of energy deficiency. This possibility finds some support from the fact that the isolated mitochondria from these three gly$^-$ mutants have a definite though low CAT and BKA-sensitive oxidative phosphorylation. Another factor is that BKA has to compete with the intracellular ADP and ATP which probably have higher concentrations than in the experiments with isolated mitochondria.

Previously BKA was shown to inhibit oxidative phosphorylation only at a low pH 4.5 in yeast cells (Subík et al., 1972), and a low pH was applied also in our experiment. For these reasons early attempts to screen yeast cells for BKA resistant mutants (Perkins et al., 1972; Haslam et al., 1973) through growth on BKA-containing media have produced wrong signals for AAC mutations (Subík et al., 1974).

Mitochondria. In isolated mitochondria the ATP synthesis rate conforms with the difference between gly$^+$ and gly$^-$ mutants but reveals striking variations within the gly$^-$ mutants. This, however, can be excluded by the actual [^{3}H]-CAT and [^{3}H]BKA binding measurements. The mutation of the three intrahelical arginine mutants has quite different effects although they are located in the second transmembrane segment in the three-repeat domains. Whereas the R294A mutant still retains 8% of the wild-type phosphorylation rate, the charge removal at R204A and R96A completely blocks oxidative phosphorylation. The arginine R294 in the third domain obviously has a less essential role than its counterparts in the first and second domain. However, if R96 is replaced by histidine as in the natural pet9 mutant (Kolarov et al., 1990), the residual marginal positive charge of histidine still permits some AAC dependent phosphorylative activity. In the AAC-typical arginine triplet R252 to R254 each of the three arginines appears to be essential for oxidative phosphorylation. Nearly the same low values are observed as in the AAC free strain which contains no plasmid. This confirms the early suggestion that the AAC triplet is important for transport (Babel et al., 1981).

AAC Content and Inhibitor Binding. In immunoblots of mitochondria the presence of AAC2 was detected in all mutants. The enormous variation of the AAC content, however, is only revealed by the quantitative assays. The

Table 3: Measured and Calculated Oxidative Phosphorylation Rates in Yeast Cells[a]

strains	cytochrome *c* factor[b] (g of protein/g dry weight)	*V*(ATP) measured		*V*(ATP) calculated[c]	
		−	corrected[d]	−	corrected[d]
wt	0.26	18	18	37	35
p-AAC2	0.095	5.9	5.9	5.0	3.8
p-AAC2(−)	0.17	0	0	0.07	0
R96A	0.043	0.04	0	0	0
R96H	0.09	0.13	0	0.38	0.16
R294A	0.23	0.44	0.44	3.0	1.16
R252I	0.04	0	0	0.013	0

[a] *V*(ATP) values in μmol/min/g dry weight. [b] Corresponds to mitochondrial protein share of the dry weight of yeast cells and is calculated as the ratio of cytochrome *c* content in mitochondria to cytochrome *c* content in cells. [c] Calculated from the mean value of *V*(ATP) of mitochondria given in Figure 4 according to the formula *V*(ATP)(mitochondria) × cytochrome *c* factor. [d] These values refer to the *V*(ATP) corrected from the BKA-insensitive *V*(ATP).

binding of [^{3}H]CAT is a reliable method unless the mutagenesis suppresses the [^{3}H]CAT binding. We thus applied the competitive ELISA method to determine the AAC content in order not to miss those cases in which the CAT binding is abolished by the mutation. Both the [^{3}H]CAT binding and the competitive ELISA give for most of the gly$^+$ and gly$^-$ strains similar values for the AAC content. In the mutants R96A and R204L with no or nearly no detectable [^{3}H]CAT binding also the ELISA method finds no or very low AAC contents. Also the [^{3}H]BKA binding follows a similar pattern as the ELISA and [^{3}H]CAT binding values. However, in agreement with earlier binding measurements in mitochondria from bovine heart, [^{3}H]BKA binding is about 1.2 times higher than [^{3}H]CAT binding (Klingenberg et al., 1983). This consistent excess is not understood but could result from difficulties of determining the exact molecular extinction coefficient of BKA (Welling et al., 1960). Notwithstanding, the data clearly show that both [^{3}H]CAT and [^{3}H]BKA binding change in parallel. We must conclude that the mutations do not specifically affect the CAT or BKA binding site under the conditions used in these assays. This may be surprising since CAT and BKA binding should require cationic residues which are major targets of all mutations. The virtual absence of [^{3}H]CAT and [^{3}H]BKA binding in some mutants shows also in agreement with the immunoblots that AAC3 is not expressed and cannot compensate for the defects in AAC2 mutants.

Role of Mutations. From the comparison of activity and content in mitochondria, we can differentiate three groups of mutants; (a) virtually no AAC content and no activity in R96A and R204L; (b) low content and very low activity in R252I, 253I, and 254I; and (c) low to medium content and activity in R96H and R294A. The question can be raised whether the mutation inhibits the expression, e.g., the biogenesis of AAC including the insertion into mitochondria, but that these AAC are intrinsically active, or whether a mutational inactivation of AAC also prevents the expression in the mitochondria of an unusable AAC. In a heterologous expression system, such as in *E. coli*, it would be possible to decide whether these mutations inactivate the transport of AAC. When the positive charge R96 is still partially retained as in R96H a low expression of AAC is still possible. Only with the R294A mutation is nearly wild-type level expression of AAC possible. A possible role of R96 in the import of the AAC to mitochondria is suggested by the import studies of recombinant fragments of AAC1. An AAC1 peptide fusion with β-galactosidase that contained the first 115 residues of AAC1 was inserted into the mitochondrial membrane (Adrian et al., 1986). Because ADP/ATP carriers do not have an N-terminal signal sequence, the targeting signal must be internal and within the first 115 amino acids. In the Arg-triplet mutants still a definite level of AAC remains, although the phosphorylation activity is reduced to zero or to a mere trickle. Presumably, these arginines are not that important for the processing expression of AAC, but they are vital for the transport activity.

Respiratory Capacity in AAC Mutants. The measurements of the cytochrome content in cells and mitochondria serve various purposes. First, to determine the influence of the mutational changes of the AAC-linked oxidative phosphorylation of the respiratory capacity, second to use the cytochrome content as a measure of the purity of the mitochondrial preparation which is particularly important for low yield gly$^-$ strains, and third to extrapolate the content of AAC and oxidative phosphorylation rates, as measured in the isolated mitochondria, to the intact cell using the "cytochrome factor". The relative insensitivity of the cytochrome content to mutational inhibition of the AAC exchange in the mitochondria is surprising. The respiratory activity of the mutant mitochondria is still remarkably high in view of the completely suppressed or strongly reduced oxidative phosphorylation rate. In the absence of an AAC mediated ATP supply to the cytosol it seems that this high respiratory capacity is largely unused. The ratio of the cytochrome content to AAC-linked parameters, such as [^{3}H]-CAT binding and oxidative phosphorylation (Table 2), is a clear indicator of the cytochrome content insensitivity to the drastic changes of the AAC content. The lack of coordination of the expression of the components of the oxidative phosphorylation system must be seen in the light of the mutational defect which cannot produce a viable strain with an effective expression control.

The "cytochrome factor", i.e., the ratio of cytochrome contents of cells to that of isolated mitochondria (Klingenberg, 1964) was introduced early to extrapolate the content of mitochondrial components to the whole organ or cell. With this factor the mitochondrial phosphorylation rates are extrapolated to the cell for comparison with the measured rates in the cell (Table 3). The calculated phosphorylation rates are always higher than the *in vivo* rates. In the wild type the measured *in vivo* rates are about half of the calculated rates, but in the mutants R96H and R294A the discrepancy is still more important since the *in vivo* rates reach only $^1/_3$ or $^1/_5$ of the calculated rates. This intriguing finding suggests that by these mutations the AAC becomes more sensitive to an intracellular inhibitory component. A

probable candidate is the intracellular ADP/ATP ratio, which controls the usage of the AAC transport capacity for the oxidative phosphorylation. Whereas in the isolated mitochondria no external ATP is present due to the hexokinase−glucose trap, *in vivo* the ATP may influence the AAC by competition with ADP. This sensitivity may be enhanced by the mutation in a manner to be elucidated with the reconstituted AAC.

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