Previously, the role of residues in the ADP/ATP carrier (AAC) from Saccharomyces cerevisiae has been studied by mutagenesis, but the dependence of mitochondrial biogenesis on functional AAC impedes segregation of the mutational effects on transport and biogenesis. Unlike other mitochondrial carriers, expression of the AAC from yeast or mammals in Escherichia coli encountered difficulties because of disparate codon usage. Here we introduce the AAC from Neurospora crassa in E. coli, where it is accumulated in inclusion bodies and establish the reconstitution conditions. AAC expressed with heat shock vector gave higher activity than with pET-3a. Transport activity was absolutely dependent on cardiolipin. The 10 single mutations of intrahelical positive residues and of the matrix repeat (C10E5) motif resulted in lower activity, except of R245A. R143A had decreased sensitivity toward carboxyatractylate. The ATP-linked exchange is generally more affected than ADP exchange. This reflects a charge network that propagates positive charge defects to ATP\(^{4+}\) more strongly than to ADP\(^{3+}\) transport. Comparison to the homologous mutants of yeast AAC2 permits attribution of the roles of these residues more to ADP/ATP transport or to AAC import into mitochondria.

The adenosine triphosphate carrier (AAC)\(^3\) is involved in the last step of the oxidative phosphorylation system by delivering ATP into the cytosol. Its slow intrinsic turnover is a result of unusually large and highly charged substrates; it is the most strongly expressed member of the mitochondrial carrier family (1). The AAC has been instrumental in understanding certain principles of transport mechanism, such as the “single binding gated pore mechanism” (2–4) and the “induced transition fit” (5, 6) of transport catalysis. The conformational changes linked to the transport are well documented (7, 8). Based on its sequence and topological studies, it is generally assumed that the AAC has six transmembrane helices, with three repeat domains containing two helices each. However, very little is known about the three-dimensional structure of the AAC, as is the situation with all mitochondrial solute transporters.

We have approached the structure-function relationship problem of the AAC in recent years by mutating residues within the AAC2 from Saccharomyces cerevisiae. Most mutations involved neutralizing charged residues and the effects on various functions were determined (9–11). A beneficial spin-off from the yeast system was the occurrence of spontaneous revertants by second-site mutations (12–14). A disadvantage of the yeast expression system is the dependence on mitochondrial growth and biogenesis on the transport performance of AAC. The level of AAC expression varied widely among the mutants and could be drastically suppressed. Therefore, in several mutants that lacked AAC protein, the functional effect could not be accurately determined. Further, it could not be clearly deduced whether the mutated residue was involved primarily in the incorporation or in the transport function of AAC. In fact, it seemed that functional impairment also decreased the level of AAC expression.

To avoid the dilemma of how to interpret the mutational effect, we aimed to express AAC heterologously in an organism that does not depend on AAC such as Escherichia coli. The deposition of the synthesized AAC into inclusion bodies (IB) instead of the E. coli membrane may prevent a possible devitalizing influence on E. coli (15). Previously, the expression of the bovine AAC1 in E. coli (16) was reported; however, the very low levels detected in immunoblots are questionable. In our hands, it was not possible to express marked amounts of yeast AAC2 in E. coli, despite the abundant expression reported for other mitochondrial carriers from yeast (15, 17). This discrepancy might be due to unfavorable codon usage for yeast AAC2 in E. coli. However, the AAC from Neurospora crassa could be expressed in high amounts in E. coli. Here we determined the conditions in which AAC from N. crassa could be renatured and reconstituted from IB into phospholipid vesicles by introducing some additional measures that were not required for the reconstitution of other mitochondrial carriers from inclusion bodies. Single mutational neutralizations of 10 positive charges are introduced in the AAC from N. crassa, and the effects on the transport properties and the interaction with inhibitors were measured.

EXPERIMENTAL PROCEDURES

Materials—The detergents pentaethylene glycol monodecylether (C\(_{10}\)E\(_5\)), octaethylene glycol monodecylether (C\(_{12}\)E\(_8\)), phosphatidylethanolamine (PE), and Dowex I\(_X\)S (200–400-mesh) were obtained from Fluka, Triton X-114 and Triton X-100 from Sigma, the zwitterionic detergents Empigen BB and Sulfabetaine B12 from Marchon Ltd. (France), and Amberlite...
AAC Expressed in E. coli

XAD-2 from Aldrich. Octyl polyoxymethylene, a mixture of C₈H₂₃O₄, to C₁₀H₂₈O₄, was kindly donated by J. Rosenbusch. [¹⁴C]ADP and [¹⁴C]ATP were purchased from Amersham Pharmacia Biotech. Restriction endonucleases and T4-DNA ligase were obtained from Roche Molecular Biochemicals or New England Biolabs and used as recommended by the supplier.

To heat shock vectors carrying the AAC gene from *N. crassa* (18) was obtained from W. Neupert (Institute of Physiological Chemistry, The University of Munich, Germany). The shuttle vector pSEYc58 (19) containing the gene AAC2 from *S. cerevisiae* (20) was obtained from D. Nelson (Dept. of Biochemistry, University of Tennessee, Memphis, TN). The human gene AAC1 was supplied by N. Neckelmann (21). The three wild type (wt) AAC genes were cloned for use in the shuttle vector used for protein expression into the vector pET-3a (Novagen). DNA isolation, restriction, cloning, and transformation into the E. coli strain cells DH1 and BL21(DE3)plysS were performed as described in Ref. 22. All mutants of the AAC gene from *N. crassa* were generated by using an oligonucleotide-directed system (U.S.E. mutagenesis kit, Amersham Pharmacia Biotech). The AAG codon for the mutant K28A was changed to GCA, and for the mutant K28R, codons CGT and CGC were changed to GCA. The mutated AAC gene from *N. crassa* contains the restriction sites for Ndel, BamHI, and EcoRI, which allowed the recloning of the 1207-base pair DNA fragment from the p7/Blue shuttle vector into the heat shock vector pLAJ503 vector (23). All the cloned and mutated AAC genes were sequenced by dideoxy chain termination using a Thermo Sequenase kit (Amersham Pharmacia Biotech). Expression of the different AAC proteins (wt and mutants) was carried out in the E. coli strain DH1 with the heat shock vector pLAJ503 or in the strain BL21(DE3)plysS with the pET-3a vector.

Expression of AAC in E. coli—A total of 500 ml of Luria-Bertani (LB) medium plus 50 mg of ampicillin was inoculated with an overnight culture of transformed DH1 cells, starting with an A₆₀₀ in ml of 0.1. Under vigorous shaking at 30 °C (about 3.5 h), cells were grown to an A₆₀₀ of 1.2–1.4. For the expression vector pLAJ503, AAC expression was induced with the addition of 500 ml of 54°C LB medium to the cell suspension. Growth was continued for 1 h at 43°C. For induction by isopropyl-β-D-thiogalactopyranoside, 600 ml of LB medium plus ampicillin were inoculated with an overnight cell culture of transformed BL21(DE3)plysS cells containing the vector pET-3a-AAC. Under vigorous shaking at 30 °C, expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside, and growth was continued for 3 h at 37°C. The cells were harvested and stored at 20°C.

Purification of AAC-containing Inclusion Bodies—The cells were pelleted by centrifugation and resuspended in 10 ml of solution 1 (50 mM Tris base, 25% sucrose, pH 7.0) and incubated with lysozyme (20 mg/g of cells) for 10 min. All the following steps were performed at 4 °C. After 10 min of solution 1 was added, with further additions of 18.5 mM EDTA, 1.5% Triton X-100, and 1 mM phenylmethanesulfonyl fluoride. Cells were disrupted by sonification and centrifuged at 30,000 × g at 4°C for 30 min. The pellet containing IB was resuspended in 20 ml of solution 2 (1 M urea, 1% Triton X-100, 0.1% β-mercaptoethanol), sonified, and centrifuged. Further enrichment of IB was achieved by additional washing steps including centrifugation and resuspension period, using 2(1M urea, 1% Triton X-100, 0.1% β-mercaptoethanol), sonified, and centrifuged. For the purpose of washing, 400–500 mg of IB/1 liter of E. coli suspension was stored in liquid nitrogen.

Phospholipid Preparation—Phospholipid from turkey egg yolk (PC) was isolated from fresh turkey eggs as described (24) and was used as standard PC for developing the standard reconstitution procedure. The extraction step with diethylether was omitted. To remove phosphatidylethanolamine (PE), the standard PC was dissolved in a mixture of chloroform/methanol (2:3) and treated with Al₂O₃. To remove residual cholesterol, the standard PC was dissolved in methanol and centrifuged at 25,000 × g for 10 min. The supernatant was depleted by a vigorous stream of nitrogen, resulting in the final PC product.

Solubilization and Reconstitution of AAC from Inclusion Bodies—AAC from inclusion bodies (IB-AAC) was solubilized at 0 °C in 100 ml of 1 ml of 1.67% (w/v) N-lauroylsarcosine (sarkosyl), 0.1 mM EDTA, 1 mM dithioerythrol, 10 mM Tris base, pH 7.0 (15), and 0.05% polyethylene glycol 4000 (PEG) was added to prevent protein aggregation (25). After 15 min the solution was diluted 5-fold with 10 ml Tris base to give a final detergent concentration of 0.56% and centrifuged at 12,000 × g for 4 min at 4°C.

For reconstitution the phospholipid was solubilized by sonication 200 mg of PC in 800 ml of 0.1 mM PIPES, pH 7.5, until the suspension became clear. Typically, 100 ml of this PC suspension was added dropwise to a detergent (D) solution (290 mg/ml C₈E₄) to a final volume of 240 ml and stored on ice. C₈E₄ was found to be very useful for the reconstitution of the uncoupling protein (26). The PC-detergent solution contained 20 ml of DPC and 28 mg of C₈E₄ (D/PC = 1.4). 80 ml of this suspension was mixed with 1.6 mg of cardiolipin (CL). To a final volume of 1 ml, a solution of 1 mol phenylmethanesulfonyl fluoride, 20 mM Tris buffer, and 20 ml ADP or ATP were added. Then 100 μl of the solubilized IB-AAC protein were added, followed by 100 mg of Amberlite XAD-2 (Aldrich). 80 ml of the PC-detergent mixture and 100 mg of Amberlite were added twice in 30-min intervals. In subsequent intervals of 30 min, 2 × 100 mg and 1 × 200 mg Amberlite were added with gentle shaking overnight. The Amberlite was separated from the proteoliposomes by centrifugation, and the external ADP or ATP were removed by passage over a Sephadex G75 column of 0.6 × 28 cm. The elution buffer contained 50 mM NaCl and 10 mM PIPES, pH 7.4.

Exchange Measurements with Reconstituted IB-AAC or mt-AAC Proteoliposomes—The transport measurements were based on the counter-exchange of external [¹⁴C]ADP or [¹⁴C]ATP with internal ADP or ATP. A newly developed automated sampling and separation device was used for the time course of the exchange process using mixing samples from 2 to 300 s as described previously (11). Additionally, the equation for the calculation of the transport rates is given here. For inhibition studies, 50 ml of IB-AAC proteoliposomes or 35 ml of mt-AAC proteoliposomes were incubated with 10 μM carboxyatractylate (CAT), 10 μM bongkrekic acid (BKA), or a combination of 10 μM CAT and 4 μM BKA. After 10 min, 100 μM [¹⁴C]ADP was added for 5 min. An aliquot of 50 μl of this mixture was injected on a Dowex column and rapidly processed as described above.

Estimation of Protein Content—The protein concentration of the proteoliposomes was estimated using the “Amido Black method” described in Refs. 27 and 28. Protein was precipitated from 200 ml of proteoliposomes of IB-AAC or 400 ml of mt-AAC with trichloroacetic acid (104 g/100 ml) and filtered. (Millipore HAWP 020 000, 0.45-μm pore size, 24-mm diameter). Filters were stained, dried, and then eluted. The absorbance of the eluate was read at 630 nm in a 1-m1 cuvette against water. The blank containing all components except protein was subtracted, and a standard curve was calculated with 2–24 μg of bovine serum albumin.

RESULTS

AAC2 (S. cerevisiae) Versus AAC (N. crassa) Expression in E. coli—To compare AAC mutations expressed in E. coli with those previously obtained in mitochondria, we first attempted to express the AAC2 from *S. cerevisiae* in E. coli. The cDNAs for yeast AAC2 and human AAC1 were incorporated into the heat shock vector pLAJ503 and into the T7 RNA polymerase pET-3a vector. In both cases, virtually no expression of AAC protein was observed, as deduced from the lack of IB formation and documented by immunoblots of extracts from whole cells. We noted that the codons for several residues are very unfavorable for E. coli expression. In particular, the arginine codons AGG and AGA, which have a very low expression incidence in E. coli (29) are present in the AAC2 sequences. We also tried to express human AAC1 in E. coli. Again, no measurable synthesis of human AAC1 was obtained, probably due to a disparate codon usage. In particular, the arginine triplet Arg-252/Arg-253/Arg-254 may make translation in E. coli very difficult. Therefore, the DNA sequence of AAC2 (S. cerevisiae) for this triplet was changed from AGAAGAAGA to CGTCGTAGA, which has a high translation probability. Additionally, there was no detectable expression of the modified AAC2 in E. coli. We then converted to the AAC from *S. cerevisiae*, which has been shown to be abundantly expressed in E. coli (30). The codons for arginine in AAC (N. crassa) are mostly CGT or CCG, which provide a high translation probability.

Expression Systems in E. coli—Two systems were used to express AAC (N. crassa) in E. coli: the T7 RNA polymerase promoter system with the expression vector pET-3a and the
different solubilization and reconstitution conditions were used to develop the standard reconstitution procedure. Phospholipid from fresh turkey egg yolk (PC) was isolated as described in Ref. 24 and was used as standard PC. The estimated D/D exchange activity of these exchange measurements were related to the exchange activity of the basic D/D exchange $V_{T} = 1280$ (μmol/min g protein) of the heat-shock-induced, reconstituted wt IB-AAC.

**Table I**

<table>
<thead>
<tr>
<th>Detergents used for reconstitution</th>
<th>0</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100 (D/PC = 1.1)</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Triton X-114 (D/PC = 0.8)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Octyl polyoxyethylene (D/PC = 1.4)</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>C$<em>{8}$E$</em>{4}$ (D/PC = 1.4)</td>
<td>16</td>
<td>27</td>
</tr>
<tr>
<td>C$<em>{10}$E$</em>{5}$ (D/PC = 1.4)</td>
<td>10</td>
<td>36</td>
</tr>
<tr>
<td>C$<em>{12}$E$</em>{8}$ (D/PC = 1.4)</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

**Phospholipids from turkey egg yolk (PC)**
- PC (standard) 39
- PC purified with Al$_{2}$O$_{3}$ 25
- PC addition of PE (10%) 26
- PC purified with Al$_{2}$O$_{3}$ addition of PE (10%) 21
- PC extracted with methanol 100
- Detergent adsorbers
  - Biobeads 28
  - Amberlite XAD-2 80

*The influence of various detergents on the reconstitution. IB-AAC solubilization with and without addition of PEG 4000. PEG 4000 was added at a concentration of 0.1 mg/ml to the PC-detergent suspension. Detergent was removed as described under “Experimental Procedures.”

**Different phospholipid compositions were used for the reconstitution.** Detergent was removed by stepwise addition of Biobeads (350 mg) at 30-min intervals. For details of further purification steps with Al$_{2}$O$_{3}$ and methanol of the PC preparation (24), see “Experimental Procedures.” IB-AAC was solubilized in the presence of PEG.

*Equal amounts of Biobeads and Amberlite XAD-2 were added stepwise (see “Experimental Procedures”).

heat shock promoter system with the expression vector pJLA503. The IB-AAC expressed in the two different systems had different abilities to reconstitute transport activity. With the pET-3a expression system, the transport activity reached only about 40% of the activity obtained with the heat shock promoted vector (data not shown). This difference was observed throughout the variations of the reconstitution parameters. For this reason, all of the following solubilization and renaturation experiments were performed with heat shock-induced IB-AAC using the pJLA503 vector expression system.

**Solubilization-Renaturation-Reconstitution**—The previously reported procedure for the solubilization and renaturation of the phosphate and citrate carrier from inclusion bodies did not produce active vesicles with the IB-AAC. In the pursuit of an active AAC, several modifications had to be introduced, which are briefly highlighted here. Among the various solubilizers of IB-AAC tried, sarkosyl also proved to provide the basis for the reconstitution. However, some subsequent steps had to be modified, as monitored by the exchange rate (Table 1). These included addition of PEG during the solubilization of IB and during reconstitution. Further, the nonionic detergent required for the solubilization of the phospholipid and subsequent proteoliposome formation proved to be quite critical. The best reconstitution was obtained with C$_{8}$E$_{4}$ and C$_{10}$E$_{5}$, whereas the use of Triton X-100 or X-114 resulted in low exchange rates, probably due to leaky proteoliposomes. Several other factors were tested to improve the reconstitution yield (data not shown). For example, the addition of salts such as KCl, ammonium acetate, or Na$_{2}$SO$_{4}$ decreased the activity of reconstituted E. coli IB-AAC (see also Fig. 3). This is in contrast to the conditions applied to reconstitute AAC from mitochondria in which salts increased the activity (31).

**Phospholipids**—The choice of appropriate phospholipids was an important factor for reconstitution. (Table 1). Phospholipids from egg yolk (PC) have been shown to be superior for reconstitution of yeast AAC, as compared with phospholipids from other sources such as asolectin from soybean or from E. coli (32). The additional extraction step of the standard PC with methanol, which removed residual cholesterol improved activity more than 3-fold. In contrast, purification using Al$_{2}$O$_{3}$, which removes PE, resulted in less active proteoliposomes. On the other hand, further addition of PE to the standard PC did not enhance activity.

The slow addition of the detergent phospholipid mixture to the sarcosyl-solubilized IB-AAC was essential for successful reconstitution. Three equal portions of the detergent phospholipid mixture were added every 30 min. The formation of the proteoliposomes was initiated by the stepwise removal of the detergent by polystyrene beads. As shown in Table I, removal of detergent for the reconstitution with Biobeads was less effective than with Amberlite XAD-2. This procedure is similar to those used for the reconstitution of uncoupling protein from brown adipose tissue (33) and of AAC from yeast and bovine heart mitochondria (34).

**Cardiolipin**—The dependence of AAC activity on CL has been documented for the AAC from bovine heart and yeast (32, 35, 36). Typically, AAC isolated from mitochondria carries some CL; however, this may not be sufficient for full activity. CL is not expected to be present in IB-AAC and this was experimentally confirmed (data not shown). Therefore, it was not surprising to find an absolute dependence on CL addition. As shown in Fig. 1, this dependence is nonlinear. Marked translocation activity is obtained only when the phospholipids contain more than 4% CL and maximum is reached at 12%. The nonlinear dependence on the CL content in the present system differs from the linear dependence observed with reconstituted AAC2 isolated from yeast mitochondria (36).

**Protein/Phospholipid Ratio**—One problem when comparing reported transport activity is the widely different protein/phospholipid ratio used (see “Discussion”). We therefore reexamined the relationship of transport activity to the protein/phospholipid ratio to find optimum conditions for accurately measuring the transport rates in the reconstituted proteoliposomes (Fig. 2). While the $^{14}$C uptake measured at 20 s decreased with the protein amount, the exchange rate $V_{T}$ related to the protein content increased surprisingly 5-fold when changing the phospholipid/protein ratio from $10^{-2}$ to $10^{-3}$. In a compromise for accurate measurements, which are required for mutants with
decreased activity, a PC/protein ratio of 200 was employed.

Charge Compensation by $K^+$—Transport by AAC is electrical in the case of an exchange between ADP$^{3-}$ and ATP$^{4+}$ (37). To compensate for these charge differences, $K^+$ and valinomycin were added to the proteoliposomes. $K^+$ ions were usually added in the form of KCl for reconstitution of mitochondrial AAC. To assess the effect of $K^+$ ions on the transport activity, the two electroneutral (homo) exchange modes D/D and T/T and the electrical (hetero) exchanges D/T and T/D were measured. To better characterize the influence on the exchange modes of IB-AAC, AAC from \textit{N. crassa} mitochondria (mt-AAC) was also isolated and reconstituted. With mt-AAC (\textit{N. crassa}), high activity was observed for the two hetero exchange modes in the presence of KCl, whereas potassium gluconate at high concentrations reduced the homo exchange modes to about 80% without enhancing the hetero exchange modes (Fig. 3A). This is in agreement with results previously obtained with AAC from bovine heart and \textit{S. cerevisiae} (37, 11). Surprisingly, when IB-AAC was reconstituted in the presence of 150 mM KCl, the transport activity was suppressed to a few percentage of that observed in the absence of KCl (Fig. 3B), whereas with the mt-AAC (\textit{N. crassa}), high activity was observed in the presence of KCl. Potassium gluconate instead of KCl was much less inhibitory. At 20 mM decreased the D/D exchange to about 28%, but actually increased the activity of the hetero exchange modes of IB-AAC. The exchange mode pattern, \textit{i.e.} the relative distribution of activity in the four exchange modes, differed in the IB-AAC from that of the mt-AAC. $K^+$ at 150 mM either as KCl or potassium gluconate caused a decrease of the homo exchange modes D/D and T/T, whereas at 20 mM $K^+$ the hetero exchange modes D/T and T/D were increased. With IB-AAC the highest activity was observed in the D/D exchange, whereas with mt-AAC the T/T exchange was the highest.

AAC Mutants—To elucidate structure-function relationships in the AAC by mutagenesis, we concentrated here as previously for the yeast AAC2, on the mutagenesis of charged residues. In the \textit{N. crassa} AAC as in most mitochondrial carriers a frequent repeat positioning of charged residues occurs in the three repeat structure. In this paper we target only positively charged residues. The triad of intrahelical arginines located in the second helix of each repeat domain, Arg-86, Arg-195, and Arg-285 (Fig. 4), were converted by site-directed mutagenesis into neutral residues, R86A, R195A, and R285A. The motif (+X+) on the matrix side close to the first helix also forms a triad via conservation in the three repeats. It is adjacent to a characteristic signature of the mitochondrial carriers (PX-XX+). Within this motif, the following mutations were introduced in the first domain K38A, in the second domain R143A and R145A, and in the third domain all members of the arginine triplet R243A, R244A, and R245A. This triplet is a characteristic of all known AACS. Another target residue, Lys-28, is located only in the first helix and found in all AACS.

Transport Activity—The results of these mutations were assayed after reconstitution of mutant IB-AAC (\textit{N. crassa}) following the standard reconstitution procedure described above. A survey of the mutational influence on the “basic” exchange rate D/D is given in Table II. The intrahelical arginine mutants show generally low activity reaching 21% or less of the wt activity. The mutations of the (+X+) motif in the three repeats on the matrix side resulted in widely varying activities. Although K38A and R145A nearly lost their ability to exchange D/D, R143A still retained 34% of wt activity and R245A had even higher activity than wt. Thus, the involvement of the two positive residues of the (+X+) motif varied between the domains. Although in the second domain the downstream residue was more important, in the arginine triplet of the third domain, only the first two residues were essential.

Inhibition—The degree to which the exchange is affected by the specific AAC inhibitors, CAT and BKA is of interest concerning the possible involvement of the residues in inhibitor binding and the orientation of the AAC incorporation into the liposomes. CAT is membrane-impermeable and binds to the cytosolic-oriented state (c-state), whereas bongkrekate is membrane-permeable and binds to the matrix-oriented state (m-state) of the AAC. Therefore, CAT should inhibit transport only of the right side-outside AAC, whereas BKA may affect both types of incorporated AAC. The inhibition by CAT was 55% in the wt IB-AAC but could reach up to 80% in some of the mutants (Table III). In two mutants the inhibition by CAT was decreased to 19% (R143A) and 38% (R245A). BKA caused very strong inhibition in all mutants with the exception of R243A.

![Fig. 2. Influence of protein-phospholipid ratio on the exchange activity. A, the protein-related exchange activity $Y_1$ (μmol/min/mg protein). B, the directly measured $[^{14}\text{C}]$ADP uptake of 20-s exchange. Results from $n=5$ different reconstitution experiments. Solubilization and reconstitution conditions of heat shock-induced wt IB-AAC were different from the standard reconstitution procedure as follows: IB-AAC solubilization with 1.67% sarkosyl without PEG addition; use of standard PC without methanol extraction step; D (Triton X-114), D/PC of 1:1, protein/PC of 1/100 to 1/1000; CL (6–9.9%); D was removed by stepwise addition of Biobeads (220–350 mg) at 20-min intervals.](http://www.jbc.org/)

![Fig. 3. The influence of KCl and potassium gluconate on the four exchange modes of AAC reconstituted from inclusion bodies (IB-AAC) and from mitochondria (mt-AAC). Isolation, reconstitution, and exchange measurements of the wt mt-AAC were performed as described in Ref. 11. For reconstitution of the heat shock-induced wt IB-AAC, the newly developed standard reconstitution procedure was used. Reconstitution of the mt-AAC and IB-AAC into liposomes was performed without or with internal loading of KCl or potassium gluconate. External KCl respective potassium gluconate and 2 μM valinomycin were added prior to the start of the four various exchange measurements. D/D, $[^{14}\text{C}]$ADP external/ADP internal exchange; D/T, $[^{14}\text{C}]$ADP/ATP exchange; T/D, $[^{14}\text{C}]$ATP/ADP exchange; T/T, $[^{14}\text{C}]$ATP/ATP exchange.](http://www.jbc.org/)
FIG. 4. Folding diagram of the AAC from *N. crassa* and localization of mutated residues. Figure shows the three-repeat structure of the AAC with transmembrane helices in shaded blocks. The mutated residues are represented in black boxes. The frames around the residue symbols signify acidic (○), basic (□), and neutral (©) residues.

**TABLE II**

<table>
<thead>
<tr>
<th>Exchange activity</th>
<th>Relative exchange activity (V/Vo)</th>
<th>μmol/min g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1260 ± 172</td>
<td>100</td>
</tr>
<tr>
<td>R86A</td>
<td>170 ± 60</td>
<td>14</td>
</tr>
<tr>
<td>R195A</td>
<td>78 ± 20</td>
<td>6</td>
</tr>
<tr>
<td>R285A</td>
<td>264 ± 79</td>
<td>21</td>
</tr>
<tr>
<td>K28A</td>
<td>276 ± 82</td>
<td>22</td>
</tr>
<tr>
<td>R28A</td>
<td>13 ± 0.4</td>
<td>1.3</td>
</tr>
<tr>
<td>R143A</td>
<td>427 ± 105</td>
<td>34</td>
</tr>
<tr>
<td>R145A</td>
<td>60 ± 7</td>
<td>5</td>
</tr>
<tr>
<td>R243A</td>
<td>286 ± 93</td>
<td>23</td>
</tr>
<tr>
<td>R244A</td>
<td>190 ± 50</td>
<td>15</td>
</tr>
<tr>
<td>R245A</td>
<td>1600 ± 272</td>
<td>127</td>
</tr>
</tbody>
</table>

* Exchange measurements (n = 5) were performed with heat shock-induced wt and mutant IB-AAC from *N. crassa* using the standard reconstitution procedure. For details see "Experimental Procedures."

The combined addition of CAT and BKA resulted in 100% inhibition in all mutants. To exclude the possibility that the mutant R143A protein is incorporated largely inverted, and thus the binding site for CAT is masked, vesicles with the mutant and wt protein were internally loaded with CAT. Also here the inhibition with R143A was lower (24%) than in the wt (57%). In the combined external and internal inhibition by CAT, also the mutant inhibition was lower than in wt, i.e. 76% versus 98%.

**Exchange Modes**—The distribution of the exchange activities into the four different transport modes of AAC (see above) is of importance in analyzing a more specific influence of the mutations. Previous results with mutants of AAC2 from yeast showed that the elimination of positive groups may affect the transport of ADP and ATP differently. As discussed above, K⁺ and valinomycin were added to compensate charge differences generated by the hetero exchanges T/D and D/T. Fig. 5A shows a general decrease of the four exchange activities of the intrahelical mutants and a change of the exchange mode pattern compared with wild type. ATP-linked modes were more affected by the elimination of the positive charges than the purely ADP-linked basic D/D exchange, with the exception of the R195A mutant. It is also to be noted that the mutations changed the relative activity between the two hetero modes; whereas in the wt the D/T exchange was lower than the T/D exchange, the reverse was true for mutants.

The second group of mutations dealt with the (±X⁺) motif found on the matrix side in the three domains. Fig. 5B shows that here as well the three modes involving ATP were more strongly affected by the removal of the positive charge than the D/D mode with the exception of mutant R245A. The K38A mutant produced an inactive AAC in all exchange modes. The

**TABLE III**

<table>
<thead>
<tr>
<th>Inhibition of the ADP/ADP exchange by CAT and BKA in isolated AAC reconstituted liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitors were added to the proteoliposomes 10 min before the start of the reaction with [14C]ADP for 5 min. Three types of addition were made: 10 μM CAT, 10 μM BKA, and 10 μM CAT plus 4 μM BKA combined. Exchange measurements were performed with heat shock-induced wt and mutant IB-AAC from <em>N. crassa</em> using the standard reconstitution procedure and with the mt-AAC from <em>N. crassa</em> as described previously in Ref. 11. For calculation of the mean value and S.D. for the wt mt-AAC (<em>N. crassa</em>), IB-AAC (<em>N. crassa</em>), and for the mutant IB-AAC (<em>N. crassa</em>), n = 4 experiments were performed.</td>
</tr>
<tr>
<td>Inhibitor</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>wt mt-AAC</td>
</tr>
<tr>
<td>wt IB-AAC</td>
</tr>
<tr>
<td>R86A</td>
</tr>
<tr>
<td>R195A</td>
</tr>
<tr>
<td>R285A</td>
</tr>
<tr>
<td>K28A</td>
</tr>
<tr>
<td>R38A*</td>
</tr>
<tr>
<td>R143A</td>
</tr>
<tr>
<td>R145A</td>
</tr>
<tr>
<td>R243A</td>
</tr>
<tr>
<td>R244A</td>
</tr>
<tr>
<td>R245A</td>
</tr>
</tbody>
</table>

* Mutant K38A lost the ability for nucleotide exchange.
relative decrease of the T-linked modes were particularly drastic in R143A; although the D/D exchange was still largely retained, the T-linked modes were suppressed more than 90%. The mutations in the arginine triplet in the last domain diminished the T-linked modes in the first two arginine mutants (R243A, R244A). The surprising increase of D/D transport activity on eliminating the last arginine, R245A, was not translated into the T-linked modes. The activity of the T/D mode was still as high as in wt, whereas the modes with internal ATP (D/T, T/T) were decreased.

**DISCUSSION**

**Expression of AAC in E. coli**—The expression of recombinant AAC in *E. coli* in IB and its renaturation combined with reconstitution into liposomes should facilitate the mutagenetic approach to the functional role of amino acids. An advantage of the heterologous expression in *E. coli* is the independence of the expression level on functional AAC, whereas the expression level in yeast of some functionally impaired mutants is virtually zero. Unfortunately, the *E. coli* system does not express yeast AAC2 and mammalian AAC, such as human AAC1, due to unfavorable codon usage. Similarly, Fiermonte *et al.* (15) noted only spurious expression of bovine AAC in *E. coli* but retarded cell growth. They speculated that this is due to incorporation of AAC into the cell membrane. In our hands, however, no incorporation of AAC2 (*S. cerevisiae*) or human AAC1 into *E. coli* cell membranes was observed. Even conversion of the unfavorable codons for arginine in the RRR triplet of the yeast AAC2 failed to increase expression in *E. coli*. Obviously, the unfavorable codons remaining for other arginines in AAC are still a barrier. It is noteworthy that among the mitochondrial carriers from yeast only the AAC has unfavorable codons, whereas those used by the phosphate, citrate, and ketoglutarate carriers are favorable for expression in *E. coli* (15, 38, 39). Fortunately, the cDNA of the AAC (*N. crassa*) has an equivalent codon usage for *E. coli* and therefore can be expressed in sufficient amounts.

**Reconstitution of AAC from Inclusion Bodies**—The renaturation/reconstitution of the AAC from inclusion bodies posed a challenge. The various steps described for reconstitution of other carriers (14, 38, 39) had to be partially modified to obtain AAC with transport activity. It seems that the AAC is more sensitive toward the environmental conditions, because it must undergo much larger conformation changes due to its large substrates. The extensive energy changes involved may require stronger interaction with the lipids, as exemplified by the unusually stringent requirement for cardiolipin (see below). Among three anionic detergents tried, all containing the dodecyl group, renaturation was achieved only with sarkosyl. It appears that sarkosyl can be more easily removed from the protein than SDS or lauryl taurine. Only sarkosyl can readily lose its negative charge by protonation at neutral pH in a micellar environment. Sarkosyl was introduced to solubilize actin expressed in *E. coli* IB to prevent coaggregation with bacterial membrane proteins (40). At mitochondrial carriers it was first applied for the solubilization of the bovine ketoglutarate/malate carrier from *E. coli* IB (15). Nonionic detergents are also required to mediate the incorporation of the AAC into the phospholipids. The renaturation was further improved by PEG 4000 in stoichiometric amounts to the AAC. PEG may prevent aggregation of the AAC during the transfer into the nonionic detergent-phospholipid mixture. During this transfer a gradual exchange of the sarkosyl is important. It seems that a sudden addition of Amberlite sequesters sarkosyl from the AAC too rapidly for the slow renaturation process and thus causes aggregation. For reasons yet unknown, detergents with higher critical micelle concentration such as C8E4 or C10E5 seem to be important for the reconstitution of the AAC. Previously C10E5 has been found to be optimal for the reconstitution of the uncoupling protein from mitochondria (26).

The renaturation/reconstitution of the AAC from IB is sensitive to high ionic strength. In the presence of 150 mM KCl, there is virtually no transport activity whereas reconstitution...
from mitochondria profits from KCl. Potassium gluconate is well tolerated, possibly due to the larger anion. The drastic inhibition of transport activity by KCl may be caused by an inhibitory effect of KCl on renaturation, which is a critical step in the reconstitution procedure of IB-AAC, unlike in the reconstitution of mt-AAC. It can be reasoned that a high Cl\(^-\) concentration effectively shields the numerous positive groups in the IB-AAC and thus prevents formation of the ionic bonds necessary for folding. The smaller Cl\(^-\) should be more effective than gluconate in binding to the large excess of positive charges in AAC.

The strong increase of the protein-related transport rates with the “dilution” by the phospholipids, i.e. with a higher PC/protein ratio, renders the comparison of reported rates difficult, where the ratios vary between 200 and 10,000 (15, 38, 39). The reason for this observation must be related to larger vesicle size at high PC/protein ratios. Here we used a lower PC/protein ratio, i.e. 200–500. Using a low PC/protein, we could measure the higher absolute rates with improved accuracy. This enabled us to cover a broad range including the low rates with the mutant AAC, although the specific transport rates were lower. The kinetic measurements were facilitated, employing an automated mixing and sampling apparatus.

When estimating the success of renaturation/reconstitution by a comparison with reconstitution of native AAC from mitochondria as shown in Fig. 3, the lower dilution factor of protein to phospholipid (PC/protein = 200 with IB-AAC versus 800 with mt-AAC) must be accounted for, which increases the specific activity more than 2-fold, as shown in Fig. 2. An additional 1.4-fold increase would have been possible by saturating with 12% instead of the suboptimal 8% CL present in the reconstituted IB-AAC. Whereas mt-AAC activity is saturated at this level of CL, IB-AAC activity reaches only two thirds of the maximum activity (Fig. 1). Taking these factors into account, the activity of reconstituted IB-AAC reaches more than 70% of the mt-AAC.

Cardiolipin—The AAC is distinguished by its unusually high content of bound CL. Up to 6 molecules of CL are tightly bound to bovine and yeast AAC, and at least another 2 molecules of CL are bound more loosely (41, 42). The apparent loss of only 3 CL molecules on isolation of the yeast AAC2 causes the transport to become fully dependent on addition of CL (36). Recently we reported the transport activity of reconstituted AAC isolated from mitochondria, obtained from CL-deficient yeast cells (43). The transport activity was virtually only 1% of that measured on CL addition as compared with about 10% of the AAC from mt yeast. The nonlinear dependence of the transport activity of reconstituted IB-AAC (N. crassa) on CL adds a new facet to the interaction of CL with the AAC. Whereas for AAC2 reconstituted from yeast mitochondria the transport activity increases linearly with the content of CL (36), for IB-AAC (N. crassa) a minimum of 4% CL is required, before further CL addition starts to activate transport. It appears that the difference between IB-AAC (N. crassa) and mt-AAC2 (S. cerevisiae) is due to the complete absence of CL in IB-AAC (results not shown). This indicates that a required minimum of CL molecules must be bound to permit activation by further CL additions. This required minimum amount of CL is still present in the isolated AAC2 from yeast mitochondria (36). CL is visualized to bind to positive groups of AAC at the membrane interface. For this purpose AAC is equipped with a large excess of positive charges, in particular lysines (44).

Exchange Mode Pattern—One of the striking features of the single neutralization of these positive residues is the alteration of the exchange mode pattern. In general, the modes involving ATP, particularly the T/T mode, were more strongly decreased than the D/D mode. Additionally, the T/D mode and to a lesser extent the D/T mode were more reduced than the “basic” D/D mode. The explanation for the difference between the mt-AAC and IB-AAC may reside in the renaturation process, which takes place in the presence of high concentrations (20 mM) of ADP or ATP. ADP may facilitate renaturation more than ATP, since the binding of ADP to the AAC is known to induce more flexibility in AAC and a more rapid change between the c- and m-conformations than ATP (7). As a rule, the translocation of ATP is more sensitive to the removal of a positive charge because of its additional negative charge (ATP\(^4^-\) versus ADP\(^3^-\)).

The particular sensitivity of ATP translocation, as observed in all 10 mutations distributed over a wide range in the protein, points to an interdependent charge network in the AAC. Wherever a positive charge is eliminated, its effect is propagated to the translocation channel and sensed more strongly in the ATP\(^4^-\) than ADP\(^3^-\) translocation. Obviously positive charges are involved in the interaction with those highly charged solutes, and the binding of ATP\(^4^-\) requires more positive charges than the binding of ADP\(^3^-\). The fact that positive charge defects, widely spread over the AAC, may inhibit transport suggests that they act within charge network rather than in punctual ion bonds. In this way high mobility is guaranteed and deep energy traps are avoided.

The inhibitors CAT and BKA are believed to bind to sites overlapping with those for ADP and ATP since they displace these substrates. Thus, the removal of a positive charge can be expected to lower the binding affinity also of these ligands. The inhibition by CAT is generally lower than by BKA, due to the partially inverted incorporation of AAC in the vesicles, which does not affect BKA binding (1). Among the present mutants, CAT binding varies much more than BKA binding and is only 19% in R143A. After loading the internal binding site with CAT, the inhibition reaches 98%, as compared with 91% for wt. It further increases to 77% as compared with 88% with external CAT. These results provide evidence for a decreased affinity for CAT rather than a decreased right side location of the R143A-AAC. Additionally, the R245A mutant has a decreased affinity for CAT, although it is less pronounced.

Comparison to Yeast AAC—In all the mutated IB-AAC from N. crassa, with the exception of R143A and R245A, the transport activity is reduced to one fourth or less. The general sensitivity to the elimination of positive charges in these positions was also noted with the AAC2 from yeast mitochondria mt-AAC2 (S. cerevisiae). However, there are several significant differences between the mutational effects on IB-AAC (N. crassa) and mt-AAC2 (S. cerevisiae), which can be attributed to the involvement of the respective residue in import into the

**Table IV**

Comparison of the ADP/ADP exchange activity (%) of different mutants between N. crassa AAC and S. cerevisiae AAC2 in reconstituted proteoliposomes.

<table>
<thead>
<tr>
<th>IB-AAC (N. crassa)</th>
<th>V(_T)</th>
<th>mt-AAC2 (S. cerevisiae)</th>
<th>V(_T)</th>
<th>V(_T) (N. crassa) / V(_T) (S. cerevisiae)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>100</td>
<td>wt</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>R86A</td>
<td>14</td>
<td>R96A</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R195A</td>
<td>6</td>
<td>R204L</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R285A</td>
<td>21</td>
<td>R294A</td>
<td>93</td>
<td>3.72</td>
</tr>
<tr>
<td>K28A</td>
<td>22</td>
<td>K38A</td>
<td>9</td>
<td>0.34</td>
</tr>
<tr>
<td>K38A</td>
<td>1</td>
<td>K48A</td>
<td>2</td>
<td>2.0</td>
</tr>
<tr>
<td>R143A</td>
<td>34</td>
<td>R152A</td>
<td>23</td>
<td>0.43</td>
</tr>
</tbody>
</table>

\(\%\)
mitochondria. In yeast the observed activity decrease is a composite of low AAC content due to an impaired protein import and decreased activity, which are difficult to segregate. Since expression in E. coli is immune to the import problem, a comparison of the results with the corresponding residues obtained with IB-AAC (N. crassa) expressed in E. coli and with mt-AAC2 (S. cerevisiae) in yeast should indicate the roles of the mutated residues in these alternative functions. The ratio of the exchange rates ($V_T$, of $N. crassa$/$V_T$ of $S. cerevisiae$) is useful for differentiation of the two effects (Table IV). According to these criteria, the dominant role of the intrahelical arginines in the first and second domain ($N. crassa$ Arg-86 versus $S. cerevisiae$ Arg-96 and $N. crassa$ Arg-195 versus $S. cerevisiae$ Arg-204), is the import of AAC2 into mitochondria since they have nearly zero activity in yeast but are active in the E. coli system. The transport activity of $N. crassa$-R86A, which is only 14% of wt, also indicates a role in D/D transport. Interestingly, the mutation of the intrahelical arginine ($N. crassa$ R285A versus $S. cerevisiae$ R294A) in the last domain retains activity in the yeast, although activity in E. coli is decreased by 80%. This puzzling result might suggest that the intrahelical arginine in the last domain is more important for refolding from the IB and does not play a role in the AAC import into mitochondria. In yeast AAC2, the three arginines in the triplet were replaced with Ile, which caused a much greater loss of activity than the substitution with Ala in the IB-AAC. In view of the steric difference between Ile and Ala, it is difficult to decide to what extent this is due to impairment of the AAC import.

Acknowledgments—We thank Petra Heckmeier for providing mitochondrial proteins of the inner membrane: Sorting and assembling of PTP, ATPase subunit 9 and the ADP/ATP translocator. Ph.D. thesis, University of Munich, Munich, Germany

REFERENCES

Expression of the Mitochondrial ADP/ATP Carrier in *Escherichia coli*:
RENATURATION, RECONSTITUTION, AND THE EFFECT OF MUTATIONS
ON 10 POSITIVE RESIDUES
Simone Heimpel, Gabriele Basset, Sabine Odoy and Martin Klingenberg

doi: 10.1074/jbc.M010586200 originally published online January 2, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010586200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 7 of which can be accessed free at
http://www.jbc.org/content/276/15/11499.full.html#ref-list-1