Structure-Function Studies of Adenine Nucleotide Transport in Mitochondria

II. BIOCHEMICAL ANALYSIS OF DISTINCT AAC1 AND AAC2 PROTEINS IN YEAST*

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AAC1 and AAC2 genes in yeast each encode functional ADP/ATP carrier (AAC) proteins of the mitochondrial inner membrane. In the present study, mitochondria harboring distinct AAC proteins and the pet9 Arg6 to HIS mutant (Lawson, J., Gawaz, M., Klingenberg, M., and Douglas, M. G. (1990) J. Biol. Chem. 265, 14195-14201) protein have been characterized. In addition, properties of the different AAC proteins have been defined following reconstitution into proteoliposomes. Deletion of AAC2 but not AAC1 causes a major reduction in the mitochondrial cytochrome content and respiration, and this level remains low even when the level of AAC1 protein is increased to 20% that of the AAC2 gene product. In reconstitution studies, the rate of nucleotide transport by isolated AAC1 protein is approximately 40% that of the AAC2 protein. Thus, the lack of mitochondrial-dependent growth support by the AAC1 gene product alone may be due to the combination of low abundance and reduced activity. Surprisingly, analysis of the Arg6 to His mutant protein revealed binding and transport activities similar to the functional AAC1 and AAC2 gene products. These observations are discussed in relation to a molecular analysis of this highly conserved small transporter and its function in conjunction with other proteins in the mitochondrial membrane.

The ADP/ATP carrier protein of mitochondria is an abundant and relatively simple membrane transporter of the mitochondrial inner membrane (Klingenberg, 1985). It is a relatively small protein of approximately 300 amino acids which spans either mitochondrial inner or artificial membranes to promote energy linked exchange of adenine nucleotides. As a simple highly conserved transmembrane transporter it is ideally suited to define the molecular features of nucleotide transport and its control. The AAC1 proteins recently defined in yeast provide the basis for a detailed structure function analysis of different AAC molecules in both native and artificial membranes (Adrian et al., 1986; Lawson and Douglas, 1988; Lawson et al., 1990).

The genetic complementation of mutants blocked in adenine nucleotide function (Lawson and Douglas, 1988; Lawson et al., 1990) and the level of AAC protein present in mitochondrial membranes indicate that the AAC2 gene product (or the yeast pet9 gene product) is essentially the only AAC translation product present in mitochondrial membranes grown under derepressing conditions. The reasons for the presence of a silent AAC1 gene capable of encoding a functional translocator have not been defined at present. To gain some insight into the relative contributions of each protein to adenine nucleotide transport, we have examined the biochemical and biophysical properties of the individual AAC proteins including the Arg6 to His, pet9, protein in mitochondrial membranes as well as reconstituted into artificial bilayers. Sufficient levels of the AAC1 protein are expressed from a yeast multicopy vector for preparation of this translocator isoform. In addition, we have characterized the consequences of chromosomal deletion of both AAC genes on the resulting oxidative capacities and cytochrome content of mitochondria. Their absence from the membrane dramatically reduces the extent to which biogenesis of the respiratory chain will occur. These studies confirm that the individual translocators including the Arg6 to His pet9 exhibit little difference in nucleotide binding and less than a 2-fold difference in their translocation properties. Thus, the failure of the pet9 mutant to grow on a nonfermentable carbon source cannot be explained solely on the basis of a defect in binding and transport of adenine nucleotides.

EXPERIMENTAL PROCEDURES

Yeast Strains—The following yeast strains were used in this study: wild type W303a MATa ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100; YD73-10Bc; ATCC 24657; AH2200 MATa leu2-3,112 his3-11,15 ura2 trp1-1; JLY73 MATa asc2::HIS3 his 3-11,15 trp1-1 ura3-1 ade2-1 leu2-3,112; JLY1053 Mat a AAC2::his3 AAC1::leu2 his 3-11,15 leu 9-3,112 trp1-1 ura 3-1 can 1-100 ade2-1; JLY-1B MATa leu2-3 leu112 ura3-1 pet9 JLY73 (YEpAAC1) AAC2 strain with AAC1 on the multicopy yeast plasmid pSEY8 (Lawson and Douglas, 1988); JLY1053 (YCPAAC2) AAC2::AAC2 strain with AAC1 on the multicopy yeast plasmid pSEY8 (Lawson and Douglas, 1988); JLY1053 (YCPAAC2) AAC2::AAC2 strain with AAC2 on the single copy yeast plasmid pSEY6 (Emr et al., 1986); JLYW2 Mat a

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aac1::LEU2 his 3-11.15 trpl1 ura 3-1 canl-100 ade 2-1 leu 2-3,112

Culture Media and Growth Conditions—All yeast strains were grown aerobically in a semisynthetic medium (0.5% yeast extract, 0.5% peptone, 0.1% glucose, 0.05% CaCl2, 0.05% NaCl, 0.07% MgSO4, 7H2O, 0.1% KH2PO4, 0.12% (NH4)2SO4, 0.005% FeCl3 with lactate (2%) for wild type, JLY2w, JLY1053(YCPAAC2). JLY73 and JLY70(YCPAAC2) were grown on galactose (Sherman et al., 1979).

Preparation of Mitochondria—Protoplasts were formed by enzymatic digestion of the cell wall (O'Malley et al., 1982, Daum et al., 1982). For this purpose yeast cells were suspended in 0.1 M Tris, 10 mM NaCl, 4% sucrose, 0.1% glycerol, 4% sorbitol, and 0.1% glucose, for 10 min at 0°C. The supernatant was obtained by centrifugation of the resulting supernatant at 9700 X g for 10 min, resuspended at 0.15 g wet weight/ml in 1.2 M sorbitol, 20 mM KH2PO4, pH 7.4, (0.15 g wet weight/ml under addition of zymolyase 20,000 (1 mg/g wet weight). The conversion to protoplasts occurred within 60-90 min at 35°C with gentle shaking. Protoplasts were harvested by centrifugation at 3000 X g for 5 min and washed twice with 1.2 M sorbitol.

For lysis protoplasts were suspended at 4°C in a buffer containing 0.6 M mannitol, 10 mM Tris, pH 7.4, 0.1% bovine serum albumin, 1 mM PMSF at 0.15 g wet weight/ml with a "Dounce" homogenizer (12 strokes) and then incubated for 15 min under moderate stirring. After centrifugation at 9700 X g for 10 min, the supernatant was obtained and used for cell lysis. The mitochondrial fraction was obtained by centrifugation of the resulting supernatant at 9700 X g for 10 min, resuspension in 0.5 M mannitol, 10 mM Tris, pH 7.4, and stirred for 5 min. After centrifugation for 10 min at 1000 X g, the final mitochondrial preparation was obtained by centrifugation of the supernatant at 9700 X g for 10 min. If necessary mitochondria were loaded with carboxyatractylate (CAT) or atractylate (ATR) in the presence of 50 μM ADP and 2 mM MgCl2 before storage at -70°C in liquid nitrogen.

Respiration—Oxygen consumption was determined for cells and mitochondria with the platinum electrode at 25°C (Klingenberg and Winkelmann, 1989). The reaction medium for cells contained 50% potassium phosphate buffer, pH 7.5, 0.6% water, 0.6% nitrogen.

Reconstitution of ATR AAC Complexes—For a better protection of the AAC against degradation, particularly AAC1 protein, the ATR-AAC complex was used for reconstitution. For this purpose AAC in mitochondria was loaded with ATR prior to solubilization. Freshly prepared mitochondria were incubated with ATR at a 200-fold molar excess over the [3H]CAT binding capacity in the presence of 50 μM ADP and 2 mM MgCl2 for 10 min at 0°C. The ATR-AAC complex was isolated and reconstituted according to the procedure for the unliganded protein. The ATR was removed from AAC during the incorporation into vesicles by the combination of the large excess of ATR with subsequent solubilization.

Miscellaneous—Protein concentration was determined by the method of Lowry et al. (1951) in the presence of 1% SDS (Schagger and Von Jagow, 1987) and by a modified Biuret method (Lowry and Zitomer, 1984). CNBr cleavage, immunoblot, and preparation of antiserum were performed as published earlier (Knirsch et al., 1989).

For immunoblot and Coomassie blue-stained SDS-polyacrylamide gel electrophoresis, proteins were run on 12.5% Laemmli gels with 5% stacking gel (Laemmli, 1970).

Chemicals—The sources of chemicals were the following: C12E8 was purchased from Fluka, Amberlite XAD-2 beads were from Serva, Sephadex was from Pharmacia LKB Biotechnology Inc., carboxyatractylate, atractylate, and BKA were from Mannheim. [3H]ADP and [3H]ATP were from Amer sham Corp., and Dowex 1-X8 was from Bio-Rad. Zymolyase 20,000 was from Seikagaku Kogyo, Tokyo. Phosphatidylcholine purified from turkey egg yolk was obtained from Sigma, and cholesterol was from Sigma. Hydroxylapatite and [3H]CAT were prepared as described previously.
RESULTS

Yeast Strains—The yeast strains were constructed for growth and characterization of the individual AAC1 and AAC2 gene products (Lawson et al., 1990). The strains used are listed in Table I. The mutants were deleted by deletion of either or both the AAC1 and AAC2 genes. Into the deletion mutants either AAC1 or AAC2 genes were reintroduced on plasmids. By using the centromere plasmid pSEY63, AAC2 was expressed in the ΔAAC1ΔAAC2 host JLY1063 at one to two copies/cell. Using the 2 micron plasmid pSEY8, AAC1 was expressed at approximately 20–50 copies/cell (Table I). This was necessary since AAC1 was not expressed at a detectable level as a unit copy gene (Lawson and Douglas, 1988, Lawson et al., 1990). Only from the 2 micron plasmid YEpAAC1 could the AAC1 product be isolated in amounts sufficient for reconstitution.

Growth Characteristics of the Yeast Cells—Yeast mutants which lack the AAC1 gene (aad1::LEU2) but contain AAC2 either in the chromosome or on a CEN plasmid could all be grown under the same conditions using galactose as a carbon source. AAC2 deletion mutants were unable to grow on glycerol but grew at rates similar to wild type on galactose-containing medium (Table II). Although slower on galactose, the ΔAAC1ΔAAC2 double deletion mutant grew to cell density comparable with the AAC2 mutant alone for biochemical studies.

Respiration and Cytochrome Content of Mitochondria—Mitochondria were isolated from the various yeast strains after growth to log phase on galactose (see “Experimental Procedures”). The cytochrome content noted in isolated mitochondria reflected that measured in whole cells (not shown). The cytochrome aa3 content of ΔAAC2 mitochondria was reduced to about 10% of wild type mitochondria (Table III). The content of cytochromes aa3 and c was lowest in the ΔAAC1ΔAAC2 double deletion mutant but measurable when compared with mitochondria from a rho− strain lacking any cytochrome aa3. These cytochrome contents also reflect the relative respiration rates for each form. The level of cytochrome b was also lowest in the ΔAAC1ΔAAC2 mutant, (40 mmol/g) but increased approximately 3- and 12-fold in the wild type mitochondria (Table III). The level of cytochrome c was also lowest in the ΔAAC1ΔAAC2 mutant, but increased 3- and 12-fold in the wild type mitochondria (Table III). This reduced cytochrome aa3 content results in an elevated cytochrome turnover for the ΔAAC1ΔAAC2 mitochondria (Table IV). We have also observed that the pet9 strain, although fully competent for AAC1 function and blocked for AAC2 function has only one-fifth the cytochrome content of the wild type mitochondria (Table III). Thus, it would appear that assembly of the respiratory chain with its complete complement of cytochromes is limited in some manner by the activity of the AAC1 protein.

It is noteworthy that expression of the AAC1 protein in amounts sufficient to support growth on glycerol (YEpAAC1) only marginally increased the respiratory activity and cytochrome content of the ΔAAC1ΔAAC2 and ΔAAC2 strains. The loss of cytochromes and of respiratory activity is the result of the loss of the AAC2 gene product which is not compensated for by over expression of the AAC1 protein. It would, therefore, appear that the AAC2 protein may serve a structural as well as a nucleotide transport role in the membrane.

Content of AAC in Mitochondria—The carrier protein content in the various yeast mitochondria was determined by measuring the binding of [3H]CAT. The binding of [3H]CAT was determined as a function of the concentration of added CAT in order to evaluate the saturation and the binding constants.

The binding values are summarized in Table V. In strains containing either a chromosomal (ΔAAC1) or centromere plasmid-encoded AAC2 gene product (YEpAAC2) the [3H]CAT binding was the same value as in wild type yeast mitochondria. However, in the mutant harboring only a chromosomal AAC1 or the pet9, the [3H]CAT binding reached 25% of the wild type. When this host harbored the 2 micron YEpAAC1, the binding was increased to about 60% that of wild type.

### Table I

| Yeast strains used in this study |
|-------------------------------|
| Designation | AAC1 | AAC2 | Strain | Reference |
|-------------|------|------|--------|-----------|
| Wild type   | 1    | 1    | W303ay D273-103 | Lawson and Douglas, 1988 |
| ΔAAC1       | Deleted | 1 | JLYW2c | Lawson et al., 1990 |
| ΔAAC2       | 1    | Deleted | JLY73 | Lawson et al., 1990 |
| ΔAAC1ΔAAC2  | Deleted | Deleted | JLY1053 | Lawson et al., 1990 |
| YEpAAC1     | 50+* | Deleted | JLY73 (YEpAAC1) | Lawson et al., 1990 |
| YEpAAC2     | 1    | 2*   | JLY1053 (YEpAAC2) | Lawson et al., 1990 |
| pet9        | 1    | 1    | JLY-10 | Lawson and Douglas, 1988 |

*a In addition to the chromosomal copy of AAC1, the AAC1 gene is also present on the 2 micron plasmid pSEY8 at about 50 copies/cell.

*b Both chromosomal copies of AAC1 and AAC2 are disrupted, and the AAC2 gene is present on a yeast CEN plasmid at 1–2 copies/cell.
In conclusion, the AAC2 deletion results in a marked reduction of AAC content and [3H]CAT binding, whereas the AAC1 deletion does not change the apparent AAC content. These data indicate the presence of a higher level of the AACl product in the AAAC2 strain than was noted using AAC in the wild type. The AACl product in the AAAC2 strain reached a maximum ratio of 5.0 when normalized to the cytochrome aa content (Table I). The molar ratio of [3H]CAT binding to cytochrome aa, was about 2.0 in the wild type and in the AACl mutant. In the AAAC2 host, however, the ratio increased to 3.3 and was about 2.0 in the wild type and in the AACl product in the AAAC2 strain than was noted using AAC.

Table II

| Strain               | Glycerol | Lactate | Galactose |
|----------------------|----------|---------|-----------|
| Wild type            | +        | 2.5     | 1.8       |
| ΔAAC1                | +        | 2.5     | 1.8       |
| ΔAAC1,ΔAAC2          | +        | 2.5     | 3.3       |
| pet9                 | -        | -       | 2.5       |
| YEpAACl              | +        | 2.7     | 2.8       |
| pet9                 | -        | -       | 3.8       |

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Table IV

| Strain       | Micromoles/g protein | [3H]CAT | Cytochrome aa3 | Kd |
|--------------|----------------------|---------|---------------|----|
| Wild type    | 0.42                 | 1.9     | 2.1           |    |
| ΔAAC1        | 0.42                 | 2.0     |               |    |
| ΔAAC1,ΔAAC2  | 0                | 2.9     |               |    |
| YCpAAC2      | 0.41                 | 2.9     |               |    |
| ΔAAC2        | 0.15                 | 3.2     |               |    |
| YEpAAC1      | 0.25                 | 5.0     | 3.7           |    |
| pet9         | 0.19                 | 2.0     |               |    |

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| ΔAAC1        | 0.42                 | 2.0     |               |    |
| ΔAAC1, ΔAAC2 | 0                | 2.9     |               |    |
| YCpAAC2      | 0.41                 | 2.9     |               |    |
| ΔAAC2        | 0.15                 | 3.2     |               |    |
| YEpAAC1      | 0.25                 | 5.0     | 3.7           |    |
| pet9         | 0.19                 | 2.0     |               |    |

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FIG. 1. Isolation of AAC proteins. SDS-polyacrylamide gel electrophoresis of detergent-extracted mitochondrial proteins (right) and corresponding hydroxylapatite pass-throughs (left) of wild type, ΔAAC2 ΔAAC1, YEpAAC1, and ΔAAC1 yeast strains. 12.5% Laemmli separating gel was loaded with (50 μg) mitochondrial protein and (15 μg) HTS protein. 1 mg of CAT-loaded mitochondria were solubilized in 4% SDS, 3% glycerol, 10 mM Tris-Cl, pH 6.8, in the presence of 1 mM PMSF. HTS proteins were obtained as described under “Experimental Procedures” using C12E8 for solubilization.

with the fragment lengths predicted from the AAC2 sequence. This agrees with all data published to date that AAC2 is the primary carrier found in aerobically grown wild type yeast (Lawson and Douglas, 1988; Lawson et al., 1990). The level of [3H]CAT binding noted for AAC1 protein in the ΔAAC2 host reflects increased AAC1 protein due in some manner to the loss of the AAC2 gene product. This observation is currently under investigation. In the presence of AAC2 the AAC1 product is not detectable by either antibody or CNBr cleavage pattern.

Reconstitution of AAC1 and AAC2 Gene Products—To further examine the kinetic properties of the AAC1, AAC2, and pet9 gene products, the proteins were isolated and reconstituted into liposomes. For reconstitution studies the carrier proteins were isolated unliganded to the inhibitor CAT (see “Experimental Procedures”). The K_M and V_max values for the AAC2 protein alone and the AAC protein from the wild type strain were the same. We observed that unliganded AAC1 carrier protein is more unstable than AAC2 carrier under these reconstitution conditions. After surveying several detergents and conditions it was noted that the non-ionic detergent C12E8 in the presence of a high concentration of ammonium acetate was useful to solubilize the AAC1 carrier in reasonable yield. These were the conditions used previously for isolating the aspartate-glutamate carrier from bovine heart mitochondria (Kramer and Heberger, 1986) and also rendered a more stable form of the unprotected AAC. Although C12E8 solubilized AAC in lower yield than Triton X-100, it had the advantage that less porin was extracted (see Fig. 1).

In reconstitution studies using the freeze-thaw method, it was necessary to minimize the presence of residual detergent in the proteoliposomes and partial inactivation of the reconstituted protein by sonication. To overcome these difficulties a modification of the method developed for the reconstitution of the uncoupling protein from brown adipose mitochondria was utilized. This led to a reproducibly high exchange activity after reconstitution of both AAC1 and AAC2 proteins (Klingenberg and Winkler, 1986). In this procedure phospholipids dispersed in C12E8 were added to the solubilized carrier protein followed by the addition of Amberlite XAD-2 to gradually remove the detergent (see “Experimental Procedures”).

The kinetics of transport were obtained by measuring the uptake of [14C]ADP and [14C]ATP at four time intervals at each of four different ADP or ATP concentrations. The initial rates were plotted in v versus v/s graphs for the evaluation of V_max and K_M (Fig. 3). In order to directly compare reconstitution among the different carriers, the molar exchange rates were normalized to the [3H]CAT binding capacity. We noted earlier that [3H]CAT binding was essentially the same for both AAC1 and AAC2 proteins. Therefore the [3H]CAT binding of the HTS protein fraction was determined for each reconstitution experiment (see “Experimental Procedures”).

The results of the exchange measurements obtained with the reconstituted AAC from the various strains are summarized in Table VI. The rates are expressed according to protein content as well as to the [3H]CAT binding capacity. The latter more precisely reflects the carrier content. The K_M and V_max values determined were virtually the same whether AAC protein was isolated from wild type yeast or from strains expressing the plasmid encoded or integrated AAC2 by itself. Sur-
prisingly the pet9 protein exhibited only a 2-fold reduction in \( V_{\text{max}} \) compared with wild type. On the other hand, the AAC1 carrier exhibited a \( V_{\text{max}} \) value which was less than that of the pet9 protein. There was only a small variation in the \( K_M \) values determined for the AAC1, AAC2, and pet9 proteins. Thus, the only major distinction between the activities of the isolated AAC1, AAC2, and pet9 proteins is their turnover number.

For estimating the exchange capacity in mitochondria harboring different carrier proteins, the molecular activity of the reconstituted AAC proteins was multiplied with the number of carrier sites determined in the mitochondria. As shown in Table VI the exchange activity is reduced to approximately 25% in the mitochondria from yeast expressing YEpAAC1 or pet9 for both the ADP and ATP exchange. It is noteworthy that the respiratory capacity of mitochondria is markedly influenced by the type of AAC protein which is present in the membrane. This is expressed as the turnover number normalized to the cytochrome \( a_0 \) content (Table VI). The values where wild type mitochondria are compared with mitochondria containing AAC protein exclusively from YCpAAC2 or YEpAAC1 are similar, supporting the previous observation that the decrease in AAC activity is accompanied by a correspondingly larger decrease in cytochrome content.

**DISCUSSION**

The two AAC proteins encoded by AAC1 and AAC2 in *Saccharomyces cerevisiae* differ much more from each other than those of the mammalian AAC. When the yeast AAC2 and AAC1 are compared, the difference is about 25% (Lawson and Douglas, 1988). With the additional 10 residues at the N terminus in AAC2, the difference is 27%. The rationale that the isoforms may have adapted to tissue specific metabolic requirements is not evident. However, it is clear that both isoforms will support growth of yeast on a nonfermentable carbon source. The possibility that the isoforms may be expressed in yeast under different conditions has not been observed in preliminary studies. At all vegetative stages of the wild yeast only the AAC2 appears to be expressed, whereas the AAC1 gene is essentially silent.3 

### Table VI

| AAC isolated from strains | \( V_{\text{max}} \) | Molecular activity (1/min) | \( K_M \) | Calculated mitochondrial exchange activity | Activity/cytochrome \( a_0 \) (1/min) |
|--------------------------|---------------------|---------------------------|---------|----------------------------------------|-------------------------------|
|                          | \( \mu\text{mol/min/g protein} \) |                          |         |                                        |                               |
| Wild type                | 1150 1150 1100 1040 | 600 850 620 650 450 450 500 | 450 450 | 24 25 23 20 | 400 400 400 400 360 360 360 | 1910 2190 3070 2400 |
| AAC2                     | 1150 1150 1100 1040 | 600 850 620 650 450 450 500 | 450 450 | 24 25 23 20 | 400 400 400 400 360 360 360 | 1910 2190 3070 2400 |
| YEpAAC2                  | 1150 1150 1100 1040 | 600 850 620 650 450 450 500 | 450 450 | 24 25 23 20 | 400 400 400 400 360 360 360 | 1910 2190 3070 2400 |
| YEpAAC1                  | 1150 1150 1100 1040 | 600 850 620 650 450 450 500 | 450 450 | 24 25 23 20 | 400 400 400 400 360 360 360 | 1910 2190 3070 2400 |
| pet9                     | 1150 1150 1100 1040 | 600 850 620 650 450 450 500 | 450 450 | 24 25 23 20 | 400 400 400 400 360 360 360 | 1910 2190 3070 2400 |

3 M. Gawaz and M. Klingenberg, unpublished observation.
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proteins. The AACZ protein both in mitochondria and the liposome has only about 40% of the exchange activity of the AAC2 protein. There was, however, no significant difference for the $K_M$ for ADP/ATP between both AAC isoforms as well as pet9.

The AAC1 protein expressed from the YEplAC1 plasmid is present in the mitochondrial membrane to 45% the level of AAC2 protein in the wild type (Lawson et al., 1990). At this level of AAC1 protein in the mitochondria one can calculate that the exchange capacity of the mitochondria is less than 25% that of the wild type. On the other hand AAC2 in single copy yields mitochondria with essentially the same activity as the wild type. We conclude from these studies that the only distinction between AAC1 and AAC2 proteins is from their relative exchange capacities.

Yeast cells still grow at near normal rates on a fermentable carbon source in the absence of both AAC1 and AAC2 gene products. This is surprising in view of the vital importance of nucleotide exchange between the cytoplasmic and mitochondrial compartments. It was anticipated that the loss of all nucleotide transport function would be a lethal event. The maintenance of cell viability could result from some low level of residual adenine nucleotide transport. Respiration and cytochrome content is drastically diminished but still definitively present in mitochondria. The possibility must also be considered that in these cells the very small pool of intramitochondrial nucleotides is segregated from the cytosolic pool. In this case some mitochondrial functions would be sustained by low level endogenous mitochondrial ATP production.

It is now well documented that the loss of ATP-dependent protein folding activities within mitochondria are not viable (reviewed in Hartl and Neupert, 1990). It is anticipated that the maintenance of cell viability likely results from some low level of residual adenine nucleotide transport. Maintenance of cell viability likely results from the possible adoption of low level translocator activity.

It is noteworthy in this regard that AAC1 which appears to be below the limits of detection in membranes containing AAC2 protein is increased to about 10–20% the level of AAC2 protein in membranes which lack an AAC2 gene product. These data suggest an interesting feedback mechanism for sensing the presence of the AAC2 gene product. Alternatively, during rapid growth of yeast there may be a mechanism for segregating pools of adenine nucleotides from the cytoplasm which are sufficient for maintenance of basic vital activities.

Reconstitution studies with the Arg⁶⁸ to His pet9 protein show that nucleotide transport is still present in the artificial membrane. A 2-fold reduction in turnover under conditions in which the $K_M$ for both nucleotides remains essentially unchanged should not reduce the transport of nucleotides below the threshold necessary for mitochondrial-dependent growth. It is possible that the combination of reduced turnover in addition to the 55% reduction in membrane content of the pet9 gene product (Table V) was sufficient to prevent growth on a nonfermentable carbon source. However, a pet9 mutant cannot be restored for growth on glycerol under conditions in which the pet9 allele is also expressed on a multicopy plasmid.

Therefore, lack of complementation by the pet9 gene product from a high copy plasmid cannot be ascribed to a simple reduction in nucleotide transport kinetics. It is possible that transport through the translocator in the mitochondrial membrane is much reduced compared with that observed in the reconstituted membrane.

It is also possible that the mutation at Arg⁶⁸ affects the association of AAC with other proteins in the inner membrane and that the constraints imposed or released by the mutation would not be apparent when the protein is the only protein in the membrane. One approach to determining if the mutation affects the association of AAC with other components of the inner membrane is the potential selection of extragenic suppressors of pet9. One class of extragenic suppressors might be expected to encode compensatory changes in proteins assembled with pet9 AAC in the membrane. These studies are currently in progress.

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