The Molecular Basis for Relative Physiological Functionality of the ADP/ATP Carrier Isoforms in *Saccharomyces cerevisiae*

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Running Head: Comparing yeast ADP/ATP carriers

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ABSTRACT

AAC2 is one of three paralogs encoding mitochondrial ADP/ATP carriers in the yeast Saccharomyces cerevisiae, and because it is required for respiratory growth it has been the most extensively studied. To comparatively examine the relative functionality of Aac1, Aac2, and Aac3 in vivo, the gene encoding each isoform was expressed from the native AAC2-locus in aac1Δ aac3Δ yeast. Compared to Aac2, Aac1 exhibited reduced capacity to support growth of yeast lacking mitochondrial DNA or of yeast lacking the ATP/Mg-Pi carrier, both conditions requiring ATP import into the mitochondrial matrix through the ADP/ATP carrier. Sixteen AAC1/AAC2 chimeric genes were constructed and analyzed to determine the key differences between residues or sections of Aac1 and Aac2. Based upon the growth rate differences of yeast expressing different chimeras, the C1 and M2 loops of the ADP/ATP carriers contain divergent residues that are responsible for the difference(s) between Aac1 and Aac2. One chimeric gene construct supported growth on nonfermentable carbon sources but failed to support growth of yeast lacking mitochondrial DNA. Nine independent intragenic mutations in this chimeric gene were identified that suppressed the growth phenotype of yeast lacking mitochondrial DNA, identifying regions of the carrier important for nucleotide exchange activities.
INTRODUCTION

Mitochondria are the site for numerous important cellular metabolic processes, and the mitochondrial compartment itself is essential for cell viability of nearly all eukaryotic organisms. Generation of ATP also takes place in this organelle via oxidative phosphorylation, but this activity is not always essential for cell survival if ATP can be generated by other means, such as glycolysis. However, the three major components of the oxidative phosphorylation machinery are also important for maintaining the electrochemical gradient across the inner membrane. The generated electrical potential establishes an energized inner membrane ($\Delta \Psi_M$), which is required for essential proteins synthesized in the cytoplasm to be imported into the inner membrane and matrix (Baker and Schatz 1991; Gasser et al. 1982; Schleyer et al. 1982). First, the electron transport chain energizes the membrane by pumping protons into the intermembrane space. Second, the F$_1$F$_0$-ATPase can couple ATP hydrolysis to the pumping of protons into the intermembrane space, energizing the inner membrane. Finally, the ADP/ATP carrier can exchange ADP$^3^-$ generated by the F$_1$F$_0$-ATPase for ATP$^4^-$ (Giraud and Velours 1997). This electrogenic exchange contributes to the energized inner membrane and provides new substrate for the F$_1$F$_0$-ATPase. Because maintenance of an energized inner membrane is essential for mitochondrial compartment biogenesis and maintenance, at least one of the three electrical potential generating processes is required for cell viability.

Mitochondria also contain a small genome (mtDNA) encoding a subset of mitochondrially-localized proteins. The mitochondrial genome is 75-85 kb in size in Saccharomyces cerevisiae (yeast), and encodes the mitochondrial ribosomal protein
Var1, tRNAs, rRNAs, four cytochrome oxidase subunits that are part of the electron transport chain (Cox1, Cox2, Cox3, and Cob), and three subunits of the proton-translocating F$_O$ portion of the F$_1$F$_O$-ATPase (Atp6, Atp8, and Atp9). Yeast is considered a petite-positive organism because it is able to grow without mtDNA ($\rho^-$) or with a mitochondrial genome severely compromised by extensive deletions ($\rho^-$). Because four subunits of the electron transport chain and three subunits of the F$_O$ portion of the F$_1$F$_O$-ATPase are encoded by mtDNA, yeast lacking a mitochondrial genome must maintain $\Delta\Psi_m$ by exchange of ATP$^\text{4-}$ for ADP$^\text{3-}$ through the ADP/ATP carrier (Giraud and Velours 1997). ADP$^\text{3-}$ is provided by the hydrolysis of ATP$^\text{4-}$, catalyzed by the remaining F$_1$ portion of the ATPase (F$_1$-ATPase) (Giraud and Velours 1997).

Essential for viability of cells lacking mtDNA, the ADP/ATP carrier is an integral inner mitochondrial membrane protein of approximately 300 amino acids and is a member of the mitochondrial carrier family (Walker and Runswick 1993). Yeast contain three paralogous genes encoding ADP/ATP carriers: AAC1, AAC2, and AAC3. The major isoform is encoded by AAC2, and Aac2 shares 77% amino acid sequence identity with Aac1 and 89% with Aac3. Transcription of AAC2 is positively regulated by O$_2$ and heme and negatively regulated by glucose (Betina et al. 1995). Aac2 is the only one of the three carriers that is required for growth of $\rho^-$ yeast or for growth on respiratory media. Numerous mutations affecting yeast growth have been isolated in AAC2, including the classic “op1” mutation ($\text{aac2}^{R96H}$ below), which encodes a missense mutation changing arginine 96 to histidine and fails to rescue growth of aac2$\Delta$ yeast on respiratory media, even in multicopy (Kovacova et al. 1968; Lawson et al. 1990).
The two minor ADP/ATP carrier isoforms are encoded by \textit{AAC1} and \textit{AAC3}.

Transcription of \textit{AAC1} from its native promoter occurs at only very low levels in the presence of \(\text{O}_2\) and is repressed during anaerobic growth in a heme-independent manner \cite{Gavurnikova1996}. \textit{AAC1} only supports growth of \textit{aac2}\textsuperscript{R96H} yeast on respiratory media in multicopy when under control of its native promoter \cite{Lawson1988}, and compared to Aac2, Aac1 has approximately \(\sim\)25\% ADP/ATP exchange capability \cite{Gawaz1990}. In a manner that is reciprocal to \textit{AAC1}, oxygen represses the transcription of \textit{AAC3} \cite{Saboja1993}. Yeast require ADP/ATP exchange activity when grown anaerobically \cite{Drgon1991}, and \textit{AAC3} is required in yeast lacking \textit{AAC2} when grown without \(\text{O}_2\) \cite{Kolarov1990}. Thus, Drgon and coworkers speculated that \textit{AAC3} has kinetic properties that favor ATP uptake during anaerobic growth \cite{Drgon1991}. However, the authors also concluded that all three Aac isoforms have relatively similar ADP/ATP exchange activities and similar affinities for the two nucleotides based upon the \(K_D\) for binding of ADP at the intermembrane space side of the carrier protein (Aac1 = 8.8 \(\mu\text{M}\), Aac2 = 5.4 \(\mu\text{M}\), and Aac3 = 3.4 \(\mu\text{M}\)) \cite{Drgon1992}. It remains unclear whether the activities of the three isoforms of the ADP/ATP carrier in yeast have evolved to accommodate specific metabolic conditions.

As with yeast, there are three paralogous genes encoding different isoforms of human ADP/ATP carrier proteins: \textit{hANT1}, \textit{hANT2}, and \textit{hANT3} (referred to as \textit{Adenine Nucleotide Translocators} in human cells). The human isoforms are expressed at different levels in various tissues (based upon concentrations of mRNA): \textit{hANT1} is specifically expressed in heart, skeletal muscle, and to a lesser extent, brain tissues, \textit{hANT2} is
expressed during development and is downregulated and minimally expressed in most
differentiated tissues, and h\textit{ANT3} is ubiquitously expressed in all tissues at levels
proportional to the level of oxidative phosphorylation (\textit{STEPHEN} \textit{et al.} 1992). Based upon
expression analysis in human cancer cell lines, \textit{Chevrollier} \textit{et al.} observed that \textit{hANT2}
expression increases in some tumor cells, and the authors proposed that \textit{hAnt2} has kinetic
properties favoring uptake of \textit{ATP} into the mitochondria in exchange for \textit{ADP},
important in highly glycolytic cells (\textit{i.e.} developing or transformed cells) (\textit{CHEVROLLIER}
\textit{et al.} 2005). This model is consistent with the observation that \textit{hANT2} mRNA levels are
increased in human cells lacking mtDNA (\textit{ρ}), a situation in which the cells can only
survive by generating ATP via glycolysis (\textit{LUNARDI} and \textit{ATTARDI} 1991).

Unlike the human ADP/ATP carriers, little is known about the roles of the yeast
carriers \textit{in vivo}. Because yeast \textit{Aac2} is the only isoform required for respiratory growth or
for growth of \textit{ρ} yeast, it has been the most extensively studied utilizing classic genetic
analyses (\textit{e.g.} mutant screens and suppressor selections). To date, the relative capabilities
of \textit{Aac1} and \textit{Aac3} to support growth of \textit{aac2Δ} yeast lacking mtDNA has not been
carefully assessed. To address these issues, \textit{AAC1}, \textit{AAC2}, and \textit{AAC3} were expressed from
the native \textit{AAC2}-locus in \textit{aac1Δ aac3Δ} yeast. This approach eliminated any variability of
expression caused by different promoters and 3'UTR regions (\textit{MARCE} \textit{et al.} 2002;
\textit{MARGEOT} \textit{et al.} 2002), the requirement to select for plasmids during yeast growth, and
the need to introduce restriction enzyme sites for future experiments. In an effort to
examine the different roles of \textit{Aac1}, \textit{Aac2} and \textit{Aac3} in yeast \textit{in vivo}, the ability of each
version of the ADP/ATP carrier to support growth of yeast that require ATP import into
the mitochondrial matrix was examined. Finally, the clear growth rate difference between
AAC1 and AAC2 ρ⁻ yeast was exploited to screen 16 AAC1/AAC2 chimeric genes to determine the key differences between residues or sections of Aac1 and Aac2.

MATERIALS AND METHODS

Strains and Media: All S. cerevisiae strains were derived from D273-10B and the genotypes of strains used in this study are summarized in Table 1 (THORSNESS and FOX 1993). All strains are haploid unless otherwise indicated. Standard genetic techniques were used to construct and analyze various yeast strains (SHERMAN et al. 1986). Yeast strains were grown in complete glucose medium (YPD) containing 2% glucose, 2% bacto peptone, 1% yeast extract, 40 mg/l adenine, 40 mg/l tryptophan; complete ethanol-glycerol medium (YPEG) containing 3% glycerol, 3% ethanol, 2% bacto peptone, 1% yeast extract, 40 mg/l adenine, 40 mg/l tryptophan; or synthetic dextrose medium (SD) containing 2% glucose, 6.7 g/l yeast nitrogen base without amino acids (Difco), supplemented with the appropriate nutrients (THORSNESS and FOX 1993). For solid media, agar (USB) was added at 15 g/l. Where indicated, ethidium bromide (EtBr) was added at 25 µg/ml (WEBER et al. 1995), geneticin was added at a concentration of 300 µg/ml, or nourseothricin was added at 100 µg/ml. Yeast were grown under anaerobic conditions in a sealed chamber with an AnaeroGen™ packet (Oxoid, Ltd.).

Creation of null mutations: Null mutations of AAC1 and AAC3 were created by homologous gene replacement using DNA fragments generated by PCR. A plasmid containing a hisG-URA3-hisG cassette (pBS31) was used as template for disruption of AAC1 and AAC3 (ALANI et al. 1987). Oligonucleotides used in the PCR reaction to generate DNA for the disruptions were: 5’-
CAGATTCTCGTATCTGTTATTCTTTTCTATTTTTCCTTTTACAGCAGTA
ACTAAAGGAACAAAAGCTGGG -3’ (forward primer) and 5’-
AGAATATGTAGAATTTTTAGATTTTATCGTTTGTGTTTTCTTTTTTTTT
TCATATAGGGCGAATTGGAGCT -3’ (reverse primer) for AAC1, and 5’-
CTCAATTGAAGACGTTTACCTGAAGTGATATCTGTGCCTTGAGAAAACA
ACTAAAGGAACAAAAGCTGGG -3’ (forward primer) and 5’-
CGAAAGGTGAAGAGAAGTCGAGAAATGACTTTTTTCTTCATCCAA
CTGATCACTATAGGGCGAATTGGAGCT -3’ (reverse primer) for AAC3. PCR-generated DNAs were used to transform the indicated strains (Table 1). Transformants capable of growth on media lacking uracil were putative null alleles of AAC1 and AAC3 and were verified by PCR. Once verified, aac1Δ::hisG-URA3-hisG and aac3Δ::hisG-URA3-hisG yeast were grown on 5-FOA to select for yeast that had undergone homologous recombination between the hisG repeat sequences flanking URA3, resulting in the loss of the URA3 gene (aac1Δ::hisG and aac3Δ::hisG) (Alani et al. 1987).

A null mutation of AAC2 was created by homologous gene replacement using DNA fragments generated by PCR. A plasmid containing the kanMX6 cassette (pFA6a-kanMX6) was used as template for disruption of AAC2 (Longtine et al. 1998). Oligonucleotides used in the PCR reaction to generate DNA for the disruption of AAC2 were: 5’-
CTCTTTTATACCTCAGAATCATAACATAACATAAGCAAATAGCCCGG
ATCCCCGGGTAAATTAA -3’ (forward primer) and 5’-
AAAGGAAAATGTGAGAAAGAATTTAGATTAAGAATCAAGCCAGATAGAC
GAATTCAAGCTCTTTAACAC -3’ (reverse primer) for AAC2. PCR-generated DNAs
were used to transform the indicated strains (Table 1). Transformants resistant to
geneticin (Sigma Chemical Corp.) were putative null alleles of AAC2 and were verified
by PCR.

A null mutation of SAL1 was created by homologous gene replacement using DNA fragments generated by PCR. Genomic DNA prepared from a yeast deletion library (Research Genetics) containing the appropriate null allele (replaced with the KanMx4 cassette) was used as template. Oligonucleotides used in the PCR reaction to generate DNA for the SAL1 disruption were: 5’-GGGTCCTACACGATCGTCAAAT-3’ (forward primer) and 5’-ACGAAAGCTTGGCTGACAGTAC-3’ (reverse primer). PCR-generated DNAs were used to transform TCY33. Transformants resistant to geneticin (Sigma Chemical Corp.) were putative null alleles of SAL1 and were verified by PCR, generating TCY187. TCY 189 was derived from a cross of TCY187 with TCY27.

**Creation of AAC1, AAC2, and AAC3 knockins in AAC2-locus:** In a manner similar to disruption of AAC2 described above, AAC1, AAC2 (control), and AAC3 were introduced into the AAC2 locus of aac1Δ::hisG aac2Δ::kanMX6 aac3Δ::hisG yeast (TCY119) by homologous gene replacement using DNA fragments generated by PCR. In this case, DNA was amplified using Pfu Turbo polymerase (Stratagene) to avoid introducing mutations. Genomic DNA prepared from wild type yeast (PTY44) was used as template. Oligonucleotides that contain sequence homologous to the 50 bp immediately up- and downstream of the AAC2 ORF (underlined) and sequence homologous to the 20 bp (italicized) either starting at the start codon (forward primers) or ending at the stop codon (reverse primers) for each gene were used in the PCR reaction to generate DNA for the knockins were: 5’-
CTCTTTATACCTTCAGAATCATACATTAACATACATATAAGCAAATAGCCATG
TCTCACACAGAAACACA -3’ (forward primer) and 5’-
AAAGGAAAATGTGAGAAAGAATTTTAGATTAAGAATCAAGCCAGATTAGACTC
ACTTGAATTITTGCCAA -3’ (reverse primer) for AAC1, 5’-
CTCTTTTATACCTTCAGAATCATACATTAACATACATATAAGCAAATAGCCATG
TCTTCTAACGCACCAAGT-3’ (forward primer) and 5’-
AAAGGAAAATGTGAGAAAGAATTTTAGATTAAGAATCAAGCCAGATTAGACTT
ATTTGAACCTTCATTACAA -3’ (reverse primer) for AAC2, and 5’-
CTCTTTTATACCTTCAGAATCATACATTAACATACATATAAGCAAATAGCCATG
AGTAGCGACAATCAAGCA -3’ (forward primer) and 5’-
AAAGGAAAATGTGAGAAAGAATTTTAGATTAAGAATCAAGCCAGATTAGACTC
ATTTGAATTITTACCGA -3’ (reverse primer) for AAC3. PCR-generated DNAs were
used to transform TCY119. Transformants capable of growth on YPEG were putative
knockin alleles (aac2Δ::AAC1, aac2Δ::AAC2, and aac3Δ::AAC3) and were verified by
PCR and sequence verified.

**Measurement of rate of loss of mtDNA (ρ^-/ρ^0 rate):** Approximately 200 yeast cells
from TCY33 (SAL1) and TCY187 (sal1Δ) were spread onto agar plates containing
complete glucose media (YPD) and incubated for 48 hours at 30°C. The number of
colonies was counted and recorded as the total number of cells for each plate. The cells
were transferred (via replica plating) to YPD plates containing wild type ρ^0 yeast of the
opposite mating type and incubated for 24 hours at 30°. The resulting diploid cells were
transferred to fresh YPEG plates, and incubated for 24 hours at 30°. The number of
viable colonies on YPEG were counted and presumed to be a result of an original ρ^- cell
that mated with a $aac1\Delta\ aac3\Delta\ \rho^-$ cell (number of $\rho^+$ cells). The $\rho^+/\rho^0$ rate was calculated by dividing the difference of the total number of cells minus the number of $\rho^+$ cells divided by the total number of cells. Five plates were counted for each yeast strain, and standard deviations were calculated for each.

**FLAG-tagging Aac1 and Aac2:** In a manner similar to introduction of $AAC1$, $AAC2$, and $AAC3$ into the $AAC2$-locus described above, versions of $AAC1$ and $AAC2$ with sequence encoding a single FLAG-tag epitope (DYKDDDDK) (Brizzard et al. 1994) at the C-terminus of the carrier protein were introduced into the $AAC2$ locus of TCY119 by homologous gene replacement using DNA fragments generated by PCR with Pfu Turbo (Stratagene) (Figure 4.1A). Genomic DNA prepared from TCY121 or TCY122 was used as template. Two rounds of PCR were performed. Oligonucleotides used in the first PCR reaction to generate DNA for the knockins were: 5’- CACGATTTTTCCATACGTAACA -3’ (forward primer for both knockins, 400 bp upstream of ATG of $AAC2$-locus) and 5’- TTATTGTCATCGTCATCTTTAATCCTTGAATTTTTTGCCAAAC -3’ (reverse primer) for $AAC1$ or 5’- TTATTGTCATCGTCATCTTTAATCCTTGAACCTTTAATCCAAAC -3’ (reverse primer) for $AAC2$. The amplimers were used as template in a second round of PCR using the forward primer used in the first round and 5’- AAAGGAAATTGTGAGAAAGAATTTAGATTAAGAATCAAGCCAGATTAGACTTTATTGTCATCGTCATCTTTTA-3’ as the reverse primer for both amplimers. PCR-generated DNAs were used to transform TCY119. Transformants capable of growth on YPEG were putative knockin alleles ($aac2\Delta::AAC1\text{-FLAG}$ and $aac2\Delta::AAC2\text{-FLAG}$) and were sequence verified.
**Generation of aac2Δ::aac2^{R96H}-FLAG yeast using the cat5Δ aac2Δ synthetic lethal phenotype:** To create a selection for replacement of aac2Δ with aac2^{R96H}, we first created a null mutation of CAT5 was created by homologous gene replacement using DNA fragments generated by PCR. CAT5 encodes a ubiquinone biosynthesis enzyme (PROFT et al. 1995) and is required for growth of yeast lacking AAC2 (data not shown). A plasmid containing a natMX4 cassette (pAG25) that confers resistance to the antibiotic nourseothricin was used as template for disruption of CAT5 (GOLDSTEIN and MCCUSKER 1999). Oligonucleotides used in the PCR reaction to generate DNA for the disruption of CAT5 were: 5’-

CAATTCAAGGAATAAAGATATCAGTATACGGGAGAGATACATAGAAATTTC
AGGGGCATGATGTGACT -3’ (forward primer) and 5’-

TTCCTTATACCTTTAACCTTGAAACAAAGCTTTTTTTTAAATTACCACAGCT
CGTTTTTCGACACTGGAT -3’ (reverse primer). PCR-generated DNAs were used to transform TCY33. Transformants capable of growth on media containing nourseothricin were putative null alleles of CAT5 and were verified by PCR.

**CAT5** was amplified by PCR and cloned into the BamHI and PstI sites of pCM189 (CEN, URA3, ADH1 promoter) to create pCAT5 (GARI et al. 1997). Genomic DNA from wild type yeast (PTY44) was used for PCR reactions using the following oligonucleotides: 5’- GCGCGGATCCATGTTATCCCGTTCAGT -3’ (forward primer) and 5’- CGCGCTGCGTTTATTATTAGCAGTGGCCC -3’ (reverse primer). The forward primer amplified sequence beginning at the start codon of CAT5 and the reverse primer was located 337 bp downstream of the CAT5 open reading frame. pCAT5 was transformed into TCY118xTCY119, the strain was allowed to sporulate, and tetrads
were dissected onto YPD. \textit{aac2Δ cat5Δ} yeast are inviable (data not shown), but one resulting \textit{aac2Δ cat5Δ} pCAT5 haploid yeast strain was verified (TCY171). This strain was used to introduce \textit{aac2^{R96H}}-\textit{FLAG} into the \textit{AAC2}-locus utilizing the two-step PCR strategy described above for \textit{AAC2-FLAG}. In the case of \textit{aac2^{R96H}}-\textit{FLAG}, genomic DNA prepared from TCY27 was used as template for PCR with the same oligonucleotides used for \textit{AAC2-FLAG}. PCR-generated DNAs were used to transform TCY171, and transformants were selected on 5-FOA to identify \textit{aac2^{R96H}}\textit{cat5Δ} yeast now able to grow without pCAT5 (\textit{URA3}), unlike the \textit{aac2Δ cat5Δ} parent. TCY154 was isolated and sequence verified to contain \textit{aac2Δ::aac2^{R96H}}-\textit{FLAG}.

**Construction of \textit{aac2Δ::AAC1/AAC2-FLAG} chimeric yeast strains:** All chimeric strains were constructed using the PCR strategy described for the introduction of \textit{AAC1} or \textit{AAC2} into the \textit{aac2Δ::kanMX6} locus. Briefly, genomic DNA prepared from TCY151, TCY152, or subsequent chimeric yeast was used as template for PCR with Pfu Turbo polymerase (Stratagene). The specific oligonucleotides used are listed in Table 2. For each transformation, 1 to 3 PCR-generated DNAs were used to transform TCY119. Transformants capable of growth on YPEG were putative chimera or point mutant alleles and were sequence verified. To construct \textit{AAC2(26aa)/1/1/1-FLAG} (TCY175), a similar strategy was employed using the primers AAC2F and AAC2R (Table 2) and the following oligonucleotides: 5’-

\begin{verbatim}
CACTACCTCCAGCCCCAGCTCCAAAGAAGGAATCTAATTTTTTGATTGATTTC
\end{verbatim}

5’-ATCAATCAAAAAGTTAGATT-3’ (forward primer) and 5’-

\begin{verbatim}
CTTATGGGGCGGCGTTTCTGC
\end{verbatim}

ATCAATCAAAAAGTTAGATT-3’ (reverse primer).
Isolation of mitochondria: Mitochondrial isolation was performed essentially as described (DAUM et al. 1982; YAFFE 1991). Yeast were grown in YPD or YPEG, treated with zymolyase (Seikagaku America) to create spheroplasts, and broken with a Dounce homogenizer, and mitochondria collected by differential centrifugation. Mitochondrial yield was determined by spectrophotometric analysis ($A_{280}$).

**AAC2/1/1/2-FLAG Suppressor Selection:** Yeast from individual colonies of AAC2/1/1/2-FLAG yeast grown on complete ethanol-glycerol media (YPEG) were used to inoculate 5 ml liquid complete SD media with ethidium bromide and shaken for 72 hours at 30°. 100 µl of each culture was used to inoculate a second round of SD+EtBr cultures, which were shaken for 72-208 hours at 30°. A sterile inoculating loop was used to transfer cells to plates containing SD+EtBr media, and viable single colonies were streaked to a second SD+EtBr plates to colony purify. Nine intragenic suppressing mutations were identified, sequenced, and backcrossed to TCY8 to generate $\rho^+$ versions.

Immuno-detection of proteins and protein complexes: Protein fractions from whole yeast cell extracts (HORVATH and RIEZMAN 1994) or isolated mitochondria were resolved on SDS-PAGE (SAMBROOK et al. 1989) and electroblotted onto nitrocellulose (BioRad) as previously described (HANEKAMP and THORSNESS 1996). Blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed as described (RYAN et al. 1999; SCHAGGER et al. 1994; SCHAGGER and VON JAGOW 1991). Briefly, isolated mitochondrial pellets (100 µg of protein) were lysed in 35 µl of ice-cold digitonin buffer (1% (w/v) digitonin, 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10% (v/v) glycerol). After a clarifying spin, 5 µl of sample buffer (5% (w/v) Serva Blue (Invitrogen), 100 mM Bis-Tris, pH 7.0, 500 mM ε-aminocaproic acid, 25% glycerol) was added to the supernatants. ~50 µg
protein from each sample was separated on a 6-16% polyacrylamide gradient gel at 4°C. Aac-FLAG fusion proteins were detected using ANTI-FLAG®M2 (Sigma-Aldrich) and mtHsp70 was detected using antisera from Dr. Trevor Lithgow. Signals were detected using the ECL detection method (Amersham).

RESULTS

A PCR strategy to introduce yeast AAC1, AAC2, and AAC3 into the AAC2-locus:
The ORFs of AAC1, AAC2, and AAC3 were amplified by PCR using primers that contained the 50 nucleotides immediately up- and downstream of the AAC2 ORF, and the amplimers were transformed into respiratory-incompetent yeast lacking all three AAC genes. Transformants that contained the “knockin” AAC gene of interest were selected based on their ability to grow on media requiring a functional mitochondrial respiratory system (YPEG). The nucleotide sequence of the final candidates was determined to verify that each was correctly integrated into the AAC2 locus. In this way, we created yeast strains in which AAC1, AAC2, or AAC3 are expressed using the AAC2 upstream and downstream sequences, thus allowing us to compare the relative ability of the three transporters to support yeast growth and respiration under conditions that minimize differences in gene expression. The three knockin genes, denoted aac2Δ::AAC1, aac2Δ::AAC2, and aac2Δ::AAC3, all supported growth of yeast via respiration (Figure 1A). Yeast lacking all three AAC genes (aac2Δ::KanMx6) or containing the missense, loss of function mutation aac2R96H failed to grow on respiration media (data not shown). aac2Δ::KanMx6, aac2R96H, and the three knockin-strains grew equivalently on a fermentable carbon source medium (YPD) in the presence of oxygen (Figure 1B, left
panel). However, while four of these five yeast strains grew at similar rates under anaerobic conditions on YPD, $aac2\Delta::\text{KanMx6}$ failed to grow (Figure 1B, right panel) (Kolarov et al. 1990). We hypothesize that yeast completely lacking Aac protein fail to grow in the absence of oxygen because they are unable to form a membrane potential across the inner mitochondrial membrane. Oxygen is required for electron transport chain activity, thus yeast cultured in the absence of oxygen are unable to generate an electrical potential across the inner mitochondrial membrane using the electron transport chain. Additionally, yeast lacking Aac protein are unable to import ATP into the matrix and thus cannot create the essential mitochondrial membrane potential via proton pumping by $F_1F_0$-ATPase when that ATP is hydrolyzed. Finally, when grown on YPD without mtDNA, only the three knockin yeast were capable of growth (Figure 1C). Growth of $aac2\Delta::\text{AAC1}$ yeast was slower than growth of $aac2\Delta::\text{AAC2}$ yeast, and $aac2\Delta::\text{AAC3}$ yeast showed an intermediate growth rate relative to the other two knockin-strains. We suggest that these differences in growth in the absence of mtDNA reflect differences in ATP/ADP exchange rates of the different Aac proteins.

**Different abilities of Aac1, Aac2, and Aac3 to sustain growth of yeast lacking the Mg-ATP/P$_i$ carrier, Sal1:** The ATP-Mg/P$_i$ carrier, originally characterized in rat liver mitochondria, is an antiporter located in the inner mitochondrial membrane that imports ATP-Mg into the matrix in exchange for inorganic phosphate in an electroneutral fashion (Joyal and Aprille 1992). Yeast mitochondria lacking the cognate ATP-Mg/P$_i$ carrier (encoded by $SAL1$) have a 50% reduction in ATP uptake, indicating that Sal1 is responsible for the influx of ATP into the matrix during glucose-based growth (Cavero et al. 2005). Additionally, $sal1\Delta aac2\Delta$ yeast are inviable (Chen 2004), presumably
because in the absence of both proteins the rate of ATP import is too low to support growth. This inviability is rescued by \textit{AAC2, AAC3}, or \textit{aac2}^{R96H} expressed under control of the \textit{AAC2}-promoter on a low-copy plasmid but not by \textit{AAC1} under the same conditions (\textit{Chen} 2004). Thus while biochemical approaches suggest that Aac1, Aac2, and Aac3 have similar exchange activities, this assay identifies clear differences between the isoforms. Based on these data, Chen proposed that the ADP/ATP carrier protein has two distinct functions, denoted V for viability and R for respiration (\textit{Chen} 2004). Sal1, Aac2, Aac2\textit{R96H}, and Aac3 all have the V function capability, whereas Aac1 does not perform the V function (\textit{Chen} 2004). Chen suggested that the V function results in the accumulation of ATP in the matrix of mitochondria, rather than the exchange of ATP\textit{out} for ADP\textit{in}, which is required during respiratory growth (\textit{Chen} 2004).

Consistent with Chen’s results, our strain background (D273-10B) supported growth on glucose without Aac2 because it contained the full-length version of \textit{SAL1} (\textit{Chen} 2004), and the \textit{sal1}\textit{Δ} mutation was synthetically lethal with \textit{aac2}\textit{Δ}. A null mutation of \textit{SAL1} (\textit{sal1}\textit{Δ}) did not affect growth of D273-10B yeast via respiration (ethanol) or fermentation (glucose). However, on fermentative carbon sources \textit{sal1}\textit{Δ} yeast had an elevated \(\rho^-/\rho^0\) rate (9.5%) compared to wild-type yeast (1%). This difference was statistically significant (\(p<0.0001\); Materials and Methods). Thus, the loss of Sal1 destabilizes mtDNA.

The \textit{AAC} knockin strains were used to assess the relative growth capabilities of \textit{sal1}\textit{Δ} yeast expressing similar levels of Aac1, Aac2, and Aac3 (unpublished data and Figure 6). As Chen observed, Aac2 and Aac3 fully supported growth of \textit{sal1}\textit{Δ} yeast on both fermentative (YPD) and respiratory (YPEG) media (Figure 2). \textit{sal1}\textit{Δ aac2}\textit{Δ}::\textit{AAC1}
yeast exhibited a growth defect when dissected onto fermentative media (YPD), but were clearly viable under these experimental conditions (aac2\(\Delta::AAC1\) versus \(AAC1\) expressed on a plasmid) (data not shown) (Chen 2004). Also, when similar tetrads are dissected onto non-fermentable carbon source media (YPEG) and allowed to incubate at 30°C for 14 days, \(sal1\Delta aac2\Delta::AAC1\) yeast grew at a similar rate as \(SAL1 aac2\Delta::AAC1\) yeast (data not shown). When shifted back to a fermentable carbon source, \(sal1\Delta AAC1\) yeast were viable, but exhibited a mix of colony sizes consistent with an elevated rate of \(\rho^-\) formation (Figure 2). Furthermore, \(sal1\Delta aac2\Delta::AAC1\ \rho^-\) yeast grew extremely slowly on glucose, and growth of \(sal1\Delta aac2\Delta::AAC3\) yeast was greatly reduced compared to \(sal1\Delta aac2\Delta::AAC2\) (Figure 2). Several faster growing colonies of \(sal1\Delta aac2\Delta::AAC3\ \rho^-\) yeast appeared, indicating that this phenotype can be readily suppressed. Together with the growth rate differences shown in Figure 1, these observations allow us to conclude that Aac1 and, to a lesser extent, Aac3, both have a reduced capacity to supply ATP to the mitochondrial matrix in support of mitochondrial membrane potential generation than does Aac2, even when expressed at comparable levels (unpublished data and Figure 6). Therefore, we decided to exploit the easily observable differences in growth rates of \(aac2\Delta::AAC1\) and \(aac2 \Delta::AAC2\) yeast lacking mtDNA in an effort to determine the basis for the difference between the carriers encoded by \(AAC1\) and \(AAC2\).

All of the experiments described below were performed in a \(SAL1\) background.

**PCR strategy for the construction of Aac1/Aac2-FLAG chimeric proteins:** Based upon the bovine Aac1 crystal structure, the topology of the yeast Aac2 protein is inferred to have 6 transmembrane domains with the N- and C-termini of the carrier facing the intermembrane space side of the inner membrane (Kihira et al. 2004; Pebay-Peyroula
The transmembrane domains are linked by loops, two of which protrude into the intermembrane space (C1 and C2; C = cytosol) and three protrude into the matrix (M1, M2, and M3) (Kihira et al. 2004; Pabay-Peyroula et al. 2003). A closer look at an alignment of Aac1 and Aac2 reveals that three sections of the proteins contain concentrations of divergent residues: the N-terminus, the C1 loop, and the M2 loop (Figure 3). Therefore, the protein was divided into four portions to assess the difference between Aac1 and Aac2 sequence in terms of the individual effect of each divergent section on the carrier’s ability to support ρ⁻ yeast growth (divisions indicated by vertical lines on Figure 3 and jagged lines on Figure 9).

Utilizing the PCR knockin strategy employed to introduce AAC1, AAC2, and AAC3 into the AAC2-locus, the sequence encoding a single FLAG-tag epitope was introduced at the 3’ end of each gene, immediately upstream of the stop codon (see Materials and Methods). Using genomic DNA from AAC1-FLAG and AAC2-FLAG yeast as template, a PCR strategy was used to construct gene fusions encoding chimeric proteins containing 16 different combinations of Aac1 and Aac2 sequence (Figure 4). The four sections of the carrier are referred to as N-term, C1, M2, and C-term (Figures 4A & 9). All 16 chimeric ADP/ATP carrier proteins supported respiratory growth, which was the growth condition used to select the transformants. Figure 4B lists all 16 chimeric strains in terms of genotype, which is denoted as AAC#/#/#/FLAG, where # refers to the source of nucleotide sequence (1 = AAC1 and 2 = AAC2). The integrity of each ADP/ATP carrier gene fusion at the AAC2 locus was verified by DNA sequencing.

It is important to note that the nucleotide sequences of AAC1-FLAG and AAC2-FLAG are more divergent than the amino acid sequences (69% versus 77%). Therefore,
the source of nucleotide sequence for each chimera is marked in Figure 4. Furthermore, several chimeric AACs acquired silent mutations. AAC2/2/2/1-FLAG (TCY156) contains a silent mutation altering codon 140 of AAC1 from TTG to TTA. Both triplets encode leucine and are used at a frequency of approximately 27% by yeast (NAKAMURA et al. 2000). AAC2/1/1/2-FLAG (TCY160), AAC2/1/2/1-FLAG (TCY163), and AAC2/1/2/2-FLAG (TCY164) all contain silent mutations changing codon 103 (serine) of AAC2 from TCG (~9%) to TCT (~23%) (NAKAMURA et al. 2000). The level of identity to AAC1 and AAC2 of each chimera (including the FLAG-tags and silent mutations) and the number of rare codons (less than 20% usage in yeast) are listed in Table 3.

**Growth of chimeric ADP/ATP carrier yeast strains without mtDNA:** Each chimeric strain was backcrossed to aac2Δ yeast to ensure no second site mutations affecting ρ− yeast growth were involved. The backcrossed strains were streaked to various media to assess their relative growth rates (Figure 5). Based upon the size of the colonies resulting from single yeast cells, all 16 chimeric yeast strains grew equivalently on glucose (YPD) when they contained mtDNA (ρ+) (Figure 5A). Fourteen of the 16 chimeric yeast strains grew equivalently on ethanol (YPEG) as well, but AAC2/1/1/1-FLAG and AAC2/1/1/2-FLAG yeast grew slightly more slowly (Figure 5B). The greatest variability of growth rates between the chimeric yeast strains occurred on glucose when they did not contain mtDNA (ρ−) (Figure 5C). Generally, AAC2/2/#/#-FLAG yeast grew most robustly, suggesting that AAC2 sequence encoding the N-term and C1 sections of the carrier ultimately improve its function (Figure 5C). In contrast, AAC2/1/#/#-FLAG yeast grew poorly depending upon the sequence of the M2 and C-term sections of the carrier (Figure 5C). In fact, the two chimeric yeast strains that showed a slight reduction in growth rate
on YPEG failed to grow without mtDNA (Figure 5C). Apparently, AAC2-encoded M2 is necessary for growth of AAC2/1/1/1/1/1/1/1-FLAG ρ- yeast (Figure 5C). All 8 of the yeast that contain AAC1-encoded N-termini grew without mtDNA, but less robustly than AAC2/2/2/2/2/2/2/2-FLAG yeast (Figure 5C). The growth rate of AAC1/1/1/1/1/1/1/1-FLAG yeast generally increased upon the addition of AAC2-encoded sequence in the C1 and M2 sections of the carrier, while the origin of the C-terminus did not greatly affect the growth rate (Figure 5C). Finally, codon usage does not seem to correlate with growth rates of ρ- yeast, exemplified by the growth rate differences between AAC2/1/1/1/1/1/1/1-FLAG yeast (33 rare codons) and AAC1/2/1/1/1/1-FLAG (65 rare codons) (Table 3).

Expression of chimeric ADP/ATP carriers in whole cell extracts: The relative concentration of the chimeric ADP/ATP carriers in whole cell extracts was assessed (Figure 6). Because several of the strains grew poorly without mtDNA, the yeast were grown in rich glucose media (YPD) with their mtDNA (Figure 6A), or on a nonfermentable carbon source (YPEG) (Figure 6B). The blots were probed with a monoclonal antibody directed at the FLAG epitope and with a polyclonal antibody directed at mtHsp70, which was used as an internal standard. The signal for mtHsp70 was relatively uniform and in general the AAC1/1/1/1/1/1/1/1-FLAG yeast appear to have less carrier protein compared to either aac2R96H-FLAG or AAC2/1/1/1/1/1/1/1-FLAG yeast (Figure 6A). Furthermore, AAC2/1/1/1/1/1/1/1-FLAG yeast had equivalent amounts of the carrier protein compared to AAC2/2/2/2/2/2/2/2-FLAG or aac2R96H yeast when grown in rich-glucose media (YPD). Finally, all 16 chimeras appeared to have much greater amounts of ADP/ATP carrier relative to mtHsp70 when grown in media requiring respiration (YPEG), which is not unexpected because AAC2 transcription is repressed by glucose (Betina et al. 1995).
The ability to support ρ⁻ yeast growth does not correlate with the relative amounts of ADP/ATP carrier protein when grown in glucose or ethanol, as evidenced by the fact that \textit{AAC2/1/1/2-FLAG} and \textit{aac2}^{R96H} yeast fail to grow without mtDNA, but in fact have greater amounts of carrier protein compared to \textit{AAC1/2/1/1-FLAG} yeast which does grow without mtDNA.

**The N-terminal 26 amino acids of Aac2 does not improve the Aac1-dependent growth of ρ⁻ yeast:** When the first 11 amino acids of the bovine ADP/ATP carrier protein were replaced with the 26 amino acids of the N-terminus of yeast \textit{AAC2} or the 16 amino acids of the N-terminus of yeast \textit{AAC1}, the chimeric protein could rescue respiratory growth in \textit{aac2Δ} yeast (\textit{HASHIMOTO et al.} 1999). Import of the yeast/bovine chimeric Aac into the yeast inner mitochondrial membrane was increased compared to the bovine AAC (\textit{HATANAKA et al.} 2001a). Similarly, human Ant1 could rescue the respiratory defective phenotype of \textit{aac2Δ} yeast when the first 11 amino acids were replaced with the first 26 amino acids of yeast \textit{AAC2} (\textit{HATANAKA et al.} 2001b). The N-terminal region also impacted the ability of the Aac1/Aac2 chimeric proteins to support growth of ρ⁻ yeast, but in this case it is ρ⁺ yeast growth that was most clearly affected (instead of respiratory growth) (Figure 5 B,C). Two of the chimeric yeast strains containing the N-terminal region encoded by \textit{AAC2} and the C1 region encoded by \textit{AAC1} failed to grow without mtDNA, and each \textit{AAC1/2/1/-FLAG} ρ⁻ yeast strains grew less robustly than its \textit{AAC2/2/-FLAG} counterpart (Figure 5C). In the case of the chimeras, the differences occurred not only between the N-terminal portion that protrudes into the intermembrane space (N-terminal tail), but also at several residues located in the first two transmembrane domains and in the M1 loop.
We hypothesized that the N-terminal 26 amino acids of Aac2 might enhance the ability of an otherwise Aac1 chimeric ADP/ATP carrier to support growth of ρ⁻ yeast. Consequently, a chimera was constructed that contained the first 26 amino acids of Aac2 in place of the first 16 amino acids of Aac1 (TCY 175) (Figure 3). This chimeric ADP/ATP carrier supported growth of ρ⁻ yeast (data not shown) at a rate similar to AAC1/1/1/1-FLAG yeast (Figure 5C). In comparison, AAC2/1/1/1-FLAG yeast, which contain the first 107 amino acids encoded by AAC2, failed to grow without mtDNA (Figure 5C). Based upon these results, we conclude that major determinants of ρ⁻ yeast growth with respect to ATP/ADP carrier activity do not reside in the first 16-26 amino acids of the N-terminal region. Furthermore, the antagonistic elements present in the first 107 amino acids of the AAC2/1/1/1-FLAG yeast that create a translocator that is incapable of supporting growth of ρ⁻ yeast are not located in the first 26 amino acids of the N-terminal section.

**Import and assembly of the chimeric carrier encoded by AAC2/1/1/2-FLAG:** To examine more closely the roles of the C1 and M2 loops of the ADP/ATP carrier, a suppressor selection was performed with the AAC2/1/1/2-FLAG chimera strain. AAC2/1/1/2-FLAG yeast were chosen because (1) this strain failed to grow without mtDNA, providing a clear phenotype for the selection of suppressors and (2) its ADP/ATP carrier’s C1 and M2 loop regions are encoded by AAC1, and these regions appear to be the likely locations of the key differences between Aac1 and Aac2. Based upon the concentration of Aac2/1/1/2-F in whole cell extracts (Figure 6) and the high level of identity between AAC2/1/1/2-FLAG and AAC2 and the lower number of rare codons used by AAC2/1/1/2-FLAG (Table 3), this chimeric form of the carrier appears to
be expressed at levels that are comparable to Aac2-FLAG. Nonetheless, the import of Aac2/1/1/2-FLAG was examined more in depth.

The relative concentrations of ADP/ATP carrier were assessed in mitochondria isolated from five strains, including AAC2/1/1/2-FLAG yeast (Figure 7A). When normalized to mtHsp70, the concentration of Aac1/1/1/1-FLAG was roughly half of Aac2/2/2/2-FLAG, which is consistent with both the whole cell extract results (Figure 6) and previously reported results (GAWAZ et al. 1990). Aac2R96H-FLAG and Aac2/2/2/2-FLAG from ρ− yeast showed a slight reduction in concentration of carrier relative to Aac2-FLAG (ρ+) (Figure 7). AAC2/1/1/2-FLAG yeast contained more carrier than AAC2/2/2/2-FLAG yeast, indicating that the inability of Aac2/1/1/2-FLAG to support growth of ρ− yeast is likely not caused by reduced import of the protein into mitochondria (Figure 7A). Finally, AAC2/1/1/2-FLAG-encoded carrier from ρ+ yeast also participates in similarly sized oligomers compared to AAC2-FLAG-encoded carrier from yeast containing or lacking mtDNA as assessed by blue native PAGE (Figure 7B).

**Suppressor Selection of AAC2/1/1/2-FLAG:** The inability of the ADP/ATP carrier encoded by AAC2/1/1/2-FLAG to support growth of ρ− yeast is not caused by decreased import of the protein into the mitochondrial inner membrane or its assembly into oligomers. Therefore, a suppressor selection was performed to investigate the reasons this version of the carrier could support respiratory growth but not ρ− yeast growth. Nine independent isolates were recovered from the AAC2/1/1/2-FLAG parent strain, and each had differing abilities to grow in the absence of mtDNA (Figure 8). Each suppressed strain had improved growth in the absence of mtDNA due to mutation of AAC2/1/1/2-FLAG (mutations are listed in Table 4). Seven of the nine strains grew at near wild-type
rates ($AAC2/2/2/2$-FLAG) without mtDNA (Figure 8). The L206V suppressing mutation supported $\rho^-$ yeast growth, but at a slower rate that is comparable to $AAC1/1/1/1$-FLAG $\rho^-$ yeast (Figure 8). The A37T mutation supported the least robust growth of $\rho^-$ yeast, but was clearly capable of growth compared to the non-growing parental $AAC2/1/1/2$-FLAG strain (Figure 8).

The suppressing mutations are localized to two general regions of the ADP/ATP carrier (Figure 9). Four of the mutations occur in the transmembrane domains flanking the C2 loop at or near the interface between the membrane and intermembrane space ($K215M$, $L233F$, $F227C$, and $L206V$). Four of the mutations also occur in the first transmembrane domain ($S21F$, $D26E$, $A37T$, and $A41T$). Finally, the $G300C$ mutation occurs in the sixth transmembrane domain, and the 3-D bovine Aac1 structure suggests that this residue lies near the point(s) of interaction between the first and sixth transmembrane domains (Pebay-Peyroula et al. 2003). $A37T$ and $A41T$ also occur near the point(s) of interaction, suggesting that these residues, along with $G300C$, might alter the strength of interactions between the domains responsible for holding the carrier in a cylinder-like conformation.

**DISCUSSION**

Yeast Aac1 and Aac2 share 77% amino acid sequence identity, and the regions of the carrier with the greatest levels of homology tend to occur in transmembrane domains. Clusters of divergent residues between Aac1 and Aac2 lie at the N-terminus of the carriers, the C1 loop, and the M2 loop. To determine if these areas of divergence cause structural and/or functional differences that would explain the differences between the isoforms in yeast metabolism, we constructed 16 chimeric proteins containing different
combinations of Aac1 and Aac2 with a single C-terminal FLAG-tag epitope (Figure 4). While growth of the chimeric strains did not vary greatly on glucose or ethanol when the yeast contained mtDNA, several of the strains showed dramatically reduced rates of growth on glucose without mtDNA (Figure 5). Based upon the differences in growth rates, it appears that key differences between Aac1 and Aac2 lie in the N-terminus, C1 loop, and M2 loop regions, but do not lie in the C-terminal sequence.

The C1 loop changes conformation between the two functional exchange conformations of the protein, based upon labeling of cysteine mutants with the membrane-impermeable SH-reagent eosin-5-maleimide (EMA) in a cysteine-less form of yeast Aac2 (Kihira et al. 2005). Furthermore, four mutations associated with autosomal dominant Progressive External Ophthalmoplegia (adPEO) disease lie near the C1 loop of human Ant1: A114P, L98P, V289M, and D104G (Fontanesi et al. 2004) (Lodi et al. 2006). Using limited proteolysis and cysteine labeling, Dahout-Gonzalez et al. showed that the M2 loop of Aac2 is accessible to the intermembrane space (IMS) side, and that the accessibility to specific residues depends upon the conformation (Dahout-Gonzalez et al. 2005). The authors suggested that the M2 loop is dynamic and involved in ADP/ATP exchange (Dahout-Gonzalez et al. 2005). Consistent with these observations that C1 and M2 loops are actively involved in nucleotide exchange are our observations that Aac2/1/1/2-FLAG fails to support growth of ρ− yeast and growth is more robust in ρ− yeast containing Aac1/2/2/1-FLAG versus Aac1/1/1/1-FLAG. Biochemical characterization of these chimeras should provide additional insight into the C1 and M2 loops roles in nucleotide exchange.
In an initial attempt to further assess the roles of the C1 and M2 loop sequences in determining relative physiological activity, we utilized a suppressor selection strategy and characterized the effects of nine intragenic mutations in an effort to understand the cause(s) of the inability of Aac2/1/1/2-FLAG to support growth of ρ⁻ yeast. Two of the mutations have been previously isolated under different circumstances. The D26E mutation of Aac2 was initially isolated based upon its suppression of the aac2<sup>R254I</sup>-encoded Aac2 respiratory growth defect, but the mutation was not characterized further (NELSON and DOUGLAS 1993). The F227C mutation, which lies in the C2 loop of Aac2, was generated to perform the same EMA labeling experiment as described previously for the C1 loop (IWASHASHI et al. 2006; KIHIRA et al. 2005). This residue (F227C) is dynamic, and changes state to a more accessible conformation when the carrier is in the c-state compared to the m-state (IWASHASHI et al. 2006). It is also compelling that all of the residues that are changed by point mutations that increase growth of Aac2/1/1/2 ρ⁻ yeast either are exposed in the binding pocket or are immediately adjacent to exposed residues (ROBINSON and KUNJI 2006), creating the real possibility of significant changes in binding interactions between the translocator and ADP/ATP.

Generally, this work demonstrates an alternative approach to studying the ADP/ATP carrier protein. Genes encoding various forms of the carrier can easily be constructed by amplifying different pieces of DNA and the ability of yeast to readily undergo homologous recombination can be used to reconstruct chromosomally-integrated AACs. Knockins of paralogous genes into the same locus, gene fusions, and point mutation forms of AACs (not described here) can all be generated by this method. Furthermore, the synthetic lethality of aac2∆ and cat5∆ (see Materials and Methods) can
be utilized to introduce genes encoding ADP/ATP carriers that are capable of rescuing this phenotype but are not otherwise capable of supporting growth of yeast via respiration (e.g. \textit{aac}_2^{R96H}). Finally, ADP/ATP carriers of interest can be screened based upon their ability to support growth of \(\rho^{-}\) yeast, which has been an underutilized phenotype in genetic analysis.

What aspect of Aac activity is important for growth of yeast lacking mtDNA? Both from previous work and that presented here, it is clear that adequate ATP import coupled with its hydrolysis by the F\(_1\)-ATPase is required. The charge-neutral import of ATP into mitochondria by Sal1, while important for growth depending upon the form of Aac present (Figure 2), is qualitatively different from ATP import by Aac due to the electrogenic nature of ADP/ATP exchange through Aac. Interestingly, versions of the carrier protein that can effectively support respiratory growth (ATP export from mitochondria) are not always well suited for the process of ATP import in support of \(\rho^{-}\) yeast growth (e.g. – Aac1 and Aac2/1/1/2-FLAG). Exchange of ATP and ADP through Aac is a function of relative nucleotide concentrations on either side of the mitochondrial inner membrane and the membrane potential. In principal, an Aac protein could be biased to facilitate exchange of ATP for ADP in one direction versus the other by virtue of significant differences in the \(K_D\) for the nucleotides on opposite sides of the membrane. For instance, Aac1 may facilitate ATP export from mitochondria (respiratory conditions) by having a low \(K_D\) for ATP and a high \(K_D\) for ADP on the matrix side of the inner membrane and the reverse scenario on the cytosolic side. Current biochemical data is incomplete, with only \textit{in vitro} measurements of nucleotide binding affinities of
endogenous Aac proteins for ADP on the matrix side being available, (Drgon et al. 1992), thus not allowing a critical evaluation of such a model.

Alternatively, in vivo conditions or interactions with other gene products may bias the direction of exchange depending on physiological conditions. Intragenic suppressing mutations of Aac2/1/1/2-FLAG that promote robust growth of yeast lacking mtDNA may act by altering binding constants for nucleotides or changing interactions with proteins or lipids. However, assay of the assembly state of Aac proteins (BN-PAGE, Figure 7B) has not revealed any gross differences between several Aac proteins with varying capabilities to support growth of ρ- yeast. While the analysis of the suppressing mutations did not immediately reveal the basis for the Aac2/1/1/2-FLAG inability to support robust growth of yeast lacking mtDNA, these mutations and the other chimeric carrier proteins do provide effective tools for future studies into structural motifs important for the appropriate ATP/ADP transport activity for different physiological conditions.

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LITERATURE CITED


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TCY159  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::hisG aac2-Δ1::AAC1/1/1/2-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY160  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::hisG aac2-Δ1::AAC2/1/1/2-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY161  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::hisG aac2-Δ1::AAC2/1/1/2-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY162  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::AAC2/1/1/2-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY163  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::AAC2/1/1/2-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY164  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::AAC1/1/2/1-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY165  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::AAC1/1/2/1-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY166  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::AAC1/1/2/1-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY167  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::AAC1/1/2/1-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY168  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::AAC1/1/2/1-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY169  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::AAC1/1/2/1-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY170  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::AAC1/1/2/1-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY171  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 pTC4 (CAT5, URA3) aac2-Δ1::KanMX4 cat5-Δ2::NatMX4 aac1-Δ1::hisG aac3-Δ1::hisG
              his3-Δ1::hisG [p⁺, TRP1]
TCY172  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac2-Δ1::aac2^906H-FLAG cat5-Δ2::NatMX4 aac1-Δ1::hisG
              aac3-Δ1::hisG [p⁺, TRP1]
TCY173  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::AAC2(26aa)1/1/1-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY174  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::AAC2(26aa)1/1/1-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY175  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac2-Δ1::AAC1/1/2/1-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY176  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac2-Δ1::AAC1/1/2/1-FLAG aac3-Δ1::hisG [p⁺, TRP1]
TCY203  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 aac1-Δ1:hisG aac2-Δ1::AAC2/1/1/2-FLAG (D26E) aac3-Δ1::hisG [ρ⁺, TRP1]
TCY204  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::hisG aac2-Δ1::AAC2/1/1/2-FLAG (L233F) aac3-Δ1::hisG [ρ⁺, TRP1]
TCY205  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::hisG aac2-Δ1::AAC2/1/1/2-FLAG (G300C) aac3-Δ1::hisG [ρ⁺, TRP1]
TCY206  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG aac1-Δ1::hisG aac2-Δ1::AAC2/1/1/2-FLAG (S21F) aac3-Δ1::hisG [ρ⁺, TRP1]
TCY207  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 aac1-Δ1:hisG aac2-Δ1::AAC2/1/1/2-FLAG (K215M) aac3-Δ1::hisG [ρ⁺, TRP1]
TCY208  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG sal1Δ::KanMX4 aac1-Δ1::hisG aac2-Δ1::AAC3 aac3-Δ1::hisG [ρ⁺, TRP1]
TCY209  MAT a ura3-52 leu2-3, 112 trp1-Δ1 ade2 his3-Δ1::hisG sal1Δ::KanMX4 aac1-Δ1::hisG aac2-Δ1::AAC2 aac3-Δ1::hisG [ρ⁺, TRP1]
TCY211  MAT α ura3-52 leu2-3, 112 trp1-Δ1 lys2 his3-Δ1::hisG sal1Δ::KanMX4 aac1-Δ1::hisG aac2-Δ1::AAC1 aac3-Δ1::hisG [ρ⁺, TRP1]

a Unless indicated, all strains were created in this study.
b Mitochondrial genome is bracketed.
c Strain source: (THORSNESS and FOX 1993)
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<tr>
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The sequence of each primer that annealed ORF DNA of \textit{AAC1} or \textit{AAC2} is italicized. Primer sequence utilized for homologous recombination is underlined.

\textsuperscript{a}
Table 3 Summary of shared identity codon usage of the *AAC1/AAC2* gene fusions.

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<th>Genotype</th>
<th>% ID (<em>AAC1</em>)&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>% ID (<em>AAC2</em>)&lt;sup&gt;b&lt;/sup&gt;</th>
<th># of Rare Codons&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>37</td>
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<sup>a,b</sup> Identity shared between each of the *AAC1*/AAC2* gene fusions and AAC1 or AAC2 was calculated using CLUSTALW (THOMPSON *et al.* 1994) provided by Biology WorkBench 3.2 (SUBRAMANIAM 1998).

<sup>c</sup> Using General Codon Usage Analysis (www.gcua.de), the number of rarely used codons in *S. cerevisiae* was calculated for each chimera (FUHRMANN *et al.* 2004).
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<td>TCY205</td>
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FIGURE LEGENDS

FIGURE 1 Growth of AAC knockins. Three knockin yeast strains aacΔ::AAC1 (TCY121), aacΔ::AAC2 (TCY122), and aacΔ::AAC3 (TCY123), along with aac2^R96H yeast (TCY27) and aac2Δ::KanMx6 yeast (TCY119) were grown overnight in complete glucose media at 30°. 10^4, 10^3, 10^2, and 10^1 cells (left to right) were dropped onto agar plates to assess growth. (A) Complete ethanol-glycerol (YPEG). (B) Complete glucose (YPD ρ^+), grown either in the presence (left panel) or absence (right panel) of oxygen. (C) Yeast strains, grown initially in synthetic glucose media containing ethidium bromide to induce quantitative loss of mtDNA, were dropped onto complete glucose plates as above (YPD ρ^-) (FOX et al. 1991; SLONIMSKI et al. 1968) and then grown in the presence (left panel) or absence (right panel) of oxygen. Yeast strains that failed to grow are not pictured (e.g. – aac2Δ::KanMx6 and aac2^R96H yeast on YPEG in panel A and as ρ^- variants in panel C).

FIGURE 2 Effect of sal1Δ on growth of AAC knockins. aac2Δ::AAC1 sal1Δ (TCY211), aac2Δ::AAC2 sal1Δ (TCY209), aac2Δ::AAC3 sal1Δ (TCY208) yeast were streaked to plates containing complete ethanol-glycerol (YPEG) or complete glucose (YPD) with (ρ^+) or without (ρ^-) mtDNA.

FIGURE 3 Alignment of yeast Aac1-FLAG and Aac2-FLAG with chimera boundaries. The nucleotide and corresponding amino acid sequences of yeast AAC1-FLAG (TCY151) and AAC2-FLAG (TCY152) were aligned using CLUSTALW (THOMPSON et al. 1994) provided by Biology WorkBench 3.2 (SUBRAMANIAM 1998). Divergent amino acids are highlighted gray. The sequence was separated into four sections (N-term, C1, M2, and C-term) for construction of chimeric Aac1/Aac2 carriers (denoted by vertical black lines). The sequences to which the various primers listed in Table 2 anneal are italicized, and the sequences added to the amplimers to facilitate homologous recombination are underlined.
FIGURE 4  PCR-strategy for construction of the 16 possible Aac1/Aac2-FLAG chimera proteins. (A) The four sections of the chimeric ADP/ATP carriers were labeled N-term, C1, M2, and C-term. Genomic DNA from aac2Δ::AAC1-FLAG yeast, aac2Δ::AAC2-FLAG yeast (shown here), or the appropriate chimeric yeast was used as template for PCR with different combinations of the primers listed in Table 2 (numbered 1-14). (B) The 16 possible Aac1/Aac2-FLAG chimeras by genotype (AAC#/#/#/F), with # denoting which gene (AAC1 or AAC2) was the source of the nucleotide sequence for each section of the chimera (from left to right: N-term/C1/M2/C-term) and F denoting the FLAG sequence.

FIGURE 5  Growth of 16 Aac1/Aac2-FLAG chimeras. The sixteen chimeric yeast strains (TCY151, TCY152, TCY155-164, TCY167-170) were streaked to the indicated media to assess growth. (A) Complete glucose media (YPD ρ+). (B) Complete ethanol-glycerol (YPEG). (C) Yeast were grown in the presence of ethidium bromide to induce loss of mtDNA and then streaked to complete glucose media (YPD ρ-).

FIGURE 6  Aac chimera expression in whole-cell extracts. Expression of Aac proteins was assessed in AAC2 wild type (TCY33), aac2R96H-FLAG yeast (TCY154) and the sixteen chimeric yeast strains (TCY151, TCY152, TCY155-164, TCY167-170) (all containing mtDNA,). (A) Extracts from cells grown in complete glucose (YPD). (B) Extracts from cells grown in complete ethanol-glycerol (YPEG). After cells were grown in the indicated media, the cells were harvested by centrifugation and boiled in SDS-PAGE buffer, and equivalent amounts of protein (based upon initial cell mass) were resolved by SDS-PAGE. The blot was probed with a monoclonal antibody directed at the FLAG-tag epitope and a polyclonal antibody directed at mtHsp70. The aac2R96H-F yeast did not grow in YPEG media (N.D.). “-F” in lane labels denotes that the FLAG epitope was attached to the indicated AAC chimera.

FIGURE 7 Import and Assembly of the ADP/ATP carrier protein encoded by AAC2/1/1/2-FLAG. (A) Isolated mitochondria from AAC1/1/1/1-F ρ+ (TCY151), AAC2/2/2/2-F ρ+ (TCY152), AAC2/2/2/2-F ρ- (TCY152), aac2R96H-F ρ+ (TCY154), and
AAC2/1/1/2-F ρ⁺ (TCY160) were subjected to SDS-PAGE and probed with antibodies directed against the FLAG-tag epitope and mtHsp70. Using the same blot with a shorter exposure time, the densities of the bands were measured and normalized to mtHsp70 for comparison (Aac2/2/2/2-FLAG/mtHsp70 was set at 100%). (B) Detergent solubilized protein complexes prepared from mitochondria isolated from AAC2 ρ⁺ (PTY44), AAC2/2/2/2-F ρ⁺ (TCY152), AAC2/2/2/2-F ρ⁻ (TCY152), and AAC2/1/1/2-F ρ⁺ (TCY160) were resolved using BN-PAGE and probed with antibodies directed against the FLAG-tag epitope. “-F” in lane labels denotes that the FLAG epitope was attached to the indicated AAC chimera.

FIGURE 8  Growth of 9 intragenic suppressing mutations of the severe slow-growth phenotype of AAC2/1/1/2-FLAG ρ⁻ yeast. AAC2/2/2/2-FLAG (TCY152), AAC1/1/1/1-FLAG (TCY151), AAC2/1/1/2-FLAG (TCY160), and backcrossed versions of each of the AAC2/1/1/2-FLAG yeast containing a single mutation that allowed for growth of ρ⁻ yeast (TCY199-207) were streaked to complete glucose media after growth in the presence of ethidium bromide to induce loss of mtDNA (YPD ρ⁻). Each of these strains expresses a chimeric AAC (as indicated) that also contains the FLAG epitope at the C-terminus.

FIGURE 9  Location of suppressing mutations of the severe slow-growth phenotype of AAC2/1/1/2-FLAG ρ⁻ yeast in the ADP/ATP carrier. The approximate location of each suppressing mutation is marked with a number (listed in Table 4). The six transmembrane domains are labeled TM1-TM6, and the loops linking the transmembrane domains are labeled C1, C2, M1, M2, and M3 (C = cytosol and M = matrix). The N-term and C-term protrude into the intermembrane space side (IMS) of the inner membrane. The jagged lines mark the approximate breakpoints used to construct the AAC chimeras.
Figure 1

A. YPEG $\rho^+$

- $aac2\Delta::AAC1$
- $aac2\Delta::AAC2$
- $aac2\Delta::AAC3$

B. YPD $\rho^+$

- $aac2^{R96H}$
- $aac2\Delta::KanMx6$
- $aac2\Delta::AAC1$
- $aac2\Delta::AAC2$
- $aac2\Delta::AAC3$

C. YPD $\rho^-$

- $aac2\Delta::AAC1$
- $aac2\Delta::AAC2$
- $aac2\Delta::AAC3$
Figure 2

YPD $\rho^+$

$\text{aac}2\Delta::\text{AAC}3\text{ sal}1\Delta$

$\text{aac}2\Delta::\text{AAC}2\text{ sal}1\Delta$

$\text{aac}2\Delta::\text{AAC}1\text{ sal}1\Delta$

$\text{aac}2\Delta::\text{AAC}3\text{ sal}1\Delta$

YPD $\rho^-$

$\text{aac}2\Delta::\text{AAP}1\text{ sal}1\Delta$

$\text{aac}2\Delta::\text{AAC}2\text{ sal}1\Delta$

$\text{aac}2\Delta::\text{AAC}1\text{ sal}1\Delta$

$\text{aac}2\Delta::\text{AAC}3\text{ sal}1\Delta$

YPEG
Figure 4

A.

B.
Figure 5

A. YPD ρ⁺

B. YPEG

C. YPD ρ⁻
Figure 6

A. mtHsp70
   Aac-FLAG

B. mtHsp70
   Aac-FLAG
   N.D.
Figure 7

A.

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\[
\left(\frac{[\text{Aac}]/[\text{mtHsp70}]}{[\text{Aac2}\ p⁺]/[\text{mtHsp70}]}ight) \times 100
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B.

[Image of a gel electrophoresis]
Figure 8
Figure 9

[Diagram of a protein structure with labeled domains and transmembrane regions (TM1 to TM6) and N- and C-termini.]