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Yeast Mitochondrial ADP/ATP Carriers Are Monomeric in Detergents as Demonstrated by Differential Affinity Purification

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Most mitochondrial carriers carry out equimolar exchange of substrates and they are believed widely to exist as homo-dimers. Here we show by differential tagging that the yeast mitochondrial ADP/ATP carrier AAC2 is a monomer in mild detergents. Carriers with and without six-histidine or hemagglutinin tags were co-expressed in defined molar ratios in yeast mitochondrial membranes. Their specific transport activity was unaffected by tagging or by co-expression. The co-expressed carriers were extracted from the membranes with mild detergents and purified rapidly by affinity chromatography. All of the untagged carriers were in the flow-through of the affinity column, whereas all of the tagged carriers bound to the column and were eluted subsequently, showing that stable dimers, consisting of associated tagged and untagged carriers, were not present. The specific inhibitors carboxyatractyloside and bongkrekic acid and the substrates ADP, ATP and ADP plus ATP were added during the experiments to determine whether lack of association might have been caused by carriers being prevented from cycling through the various states in the transport cycle where dimers might form. All of the protein was accounted for, but stable dimers were not detected in any of these conditions, showing that yeast ADP/ATP carriers are monomeric in detergents in agreement with their hydrodynamic properties and with their structure. Since strong interactions between monomers were not observed in any part of the transport cycle, it is highly unlikely that the carriers function cooperatively. Therefore, transport mechanisms need to be considered in which the carrier is operational as a monomer.

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Introduction

The mitochondrial ADP/ATP carriers are thought to exist as stable homo-dimers in detergents based on size-exclusion chromatography experiments,[1] analytical ultracentrifugation,[1] small-angle neutron scattering,[2] and native gel electrophoresis.[3] However, the structural fold of the yeast mitochondrial ADP/ATP carrier in the membrane is a six α-helical bundle with 3-fold pseudo-symmetry, rather than being an intercalated bundle of 12 α-helices.[4] Crystallographic dimers have been observed in the crystal forms $P2$ and $P22_12_1$ in the membrane[4] and in the crystal form $C222_1$ in 3-laurylamido-N,N'-dimethylpropylaminoxide,[5,6] but they could have formed during crystallization. Both monomers in the crystallographic dimers are in complex with either carboxyatractyloside (CATR) or atractyloside (ATR), and thus they are in the same inhibited state.[4–6] The interface between monomers is struc-

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Abbreviations used: CATR, carboxyatractyloside; ATR, atractyloside; BKA, bongkrekic acid; HA, hemagglutinin tag; His₆, six-histidine tag; AAC2, ADP/ATP carrier 2 from *Saccharomyces cerevisiae*.

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turally different in the two crystallographic dimers; the interactions are mediated by loop regions in the $P22_12_1$ crystal form[4] and by cardiolipins in the $C222_1$ crystal form.[5,6] Thus, the observed interactions are inconsistent and they do not explain the stability of the putative dimers in detergents.[1–3] In contrast to earlier studies, the yeast mitochondrial ADP/ATP carriers have been shown recently to be monomeric in a range of detergents by size-exclusion chromatography and analytical ultracentrifugation,[7] and their dimensions in the detergent micelles agreed very well with those of the bovine ADP/ATP carrier, for which a structure is known.[8]

Here, the putative self-association of the yeast mitochondrial ADP/ATP carriers has been studied by differential tagging. Tagged and untagged versions have been co-expressed in mitochondrial membranes, extracted with mild detergents, and purified rapidly by affinity chromatography. If random dimerisation of monomers occurred then tagged and untagged carriers would co-purify in molar ratios that are dependent on their ratio in the membrane. This approach has been used previously to determine the oligomeric state of the Na^+/H^+ antiporter NhaA from *Escherichia coli*,[9] the mitochondrial ABC transporter Atm1p[10] and phosphate carrier Pic1p[11] from *Saccharomyces cerevisiae*. In comparison with approaches based on size-exclusion chromatography and analytical ultracentrifugation, this method is fast, taking only a few hours. The experiments can be performed with any detergent, because the quantification of the associated detergent in the protein detergent micelle is not required as it is in hydrodynamic studies.[7] Moreover, because the uninhibited ADP/ATP carriers are unstable in detergents, the hydrodynamic studies were limited largely to the CATR-inhibited state.[7,12,13] Differential tagging allows sampling of conformations before extraction, as association and formation of stable dimers may be dependent on the stage in the transport cycle. We report that the stable dimerisation of the yeast mitochondrial ADP/ATP carrier AAC2 (ADP/ATP carrier 2 from *S. cerevisiae*) in detergents cannot be detected, irrespective of whether substrates and inhibitors are present or not. Thus, the results provide support for the notion that

yeast mitochondrial ADP/ATP carriers are monomeric and not dimeric.

Results

Co-expression of tagged and untagged AAC2

Both His_6-AAC2 and HA-AAC2 were expressed in mitochondrial membranes as was untagged AAC2 (Figure 1(a) and (b)). Western blots probed with tag-specific antibodies confirmed the presence of the tags in the fusion proteins (Figure 1(c) and (d)). Blots probed with α-AAC2 antibodies showed that the introduction of the tags caused the expected upward shift in molecular mass (Figure 1(b)). Untagged AAC2 was co-expressed with either HA-AAC2 or His_6-AAC2 in yeast mitochondrial membranes as demonstrated by Western blotting with an α-AAC primary antibody (Figure 2(b)). Two distinct bands were detected, corresponding to AAC2 and the tagged AAC2 (Figure 2(a) and (b)). Western blots probed with tag-specific antibodies confirmed the presence of the tags in the fusion proteins (Figure 2(c) and (d)). The different mobilities of the tagged and untagged carriers in electrophoresis allowed their molar ratio to be determined. Densitometry showed that expression levels of His_6-AAC2 and HA-AAC2 were four and two times lower than those of AAC2, respectively.

Transport activity of single and co-expressed tagged and untagged AAC2

The specific ADP-ADP exchange rates for AAC2, His_6-AAC2, HA-AAC2, AAC2 plus His_6-AAC2, and AAC2 plus HA-AAC2 were determined (Figure 3). The results show that the presence of an N-terminal affinity tag does not affect the transport activity of AAC2 significantly (Figure 3). The ADP exchange rate correlated to the total amount of expressed carrier when AAC2 was co-expressed with either His_6-AAC2 or HA-AAC2, indicating that co-expression did not affect carrier function either. The specific exchange rate in the range of 20–26 nmol min^{-1} mg^{-1}
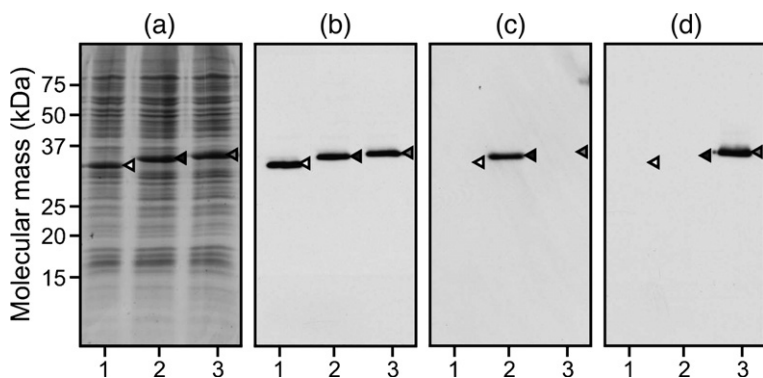
Figure 1. Expression of AAC2, His_6-AAC2 and HA-AAC2 in yeast mitochondria. Coomassie brilliant blue-stained SDS-polyacrylamide gel (a) and Western blots probed against AAC (b), the six-histidine tag (c), and HA epitope tag (d), containing mitochondrial membranes expressing AAC2 (lane 1), His_6-AAC2 (lane 2) and HA-AAC2 (lane 3). White, black and grey arrowheads indicate the approximate molecular mass of the AAC2, His_6-AAC2 and HA-AAC2, respectively. Approximately 12 μg and 1 μg of total membrane protein were loaded per gel lane for Coomassie staining and Western blotting, respectively.
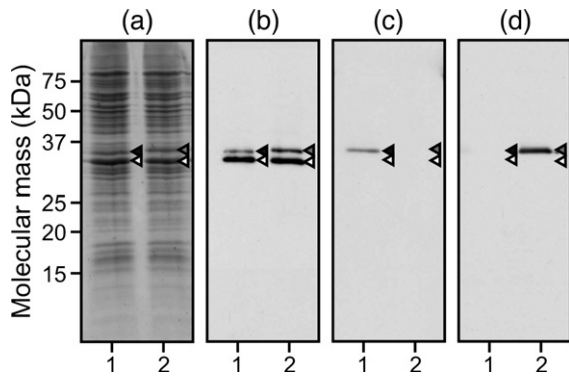
Figure 2. Co-expression of tagged and untagged AAC2. Coomassie brilliant blue-stained SDS-polyacrylamide gel (a) and Western blots probed against AAC (b), the six-histidine tag (c), and HA epitope tag (d), containing mitochondrial membranes co-expressing AAC2 plus His_6-AAC2 (lane 1) and AAC2 plus HA-AAC2 (lane 2). White, black and grey arrowheads indicate the approximate molecular mass of the wild-type, His_6-AAC2 and HA-AAC2, respectively. Approximately 12 μg and 1 μg of total membrane protein were loaded per lane for Coomassie staining and Western blotting, respectively.

corresponds approximately to two adenine nucleotides being exchanged per AAC2 per minute. These nucleotide exchange rates are likely to be a large under-representation of values found in mitochondria. First, the experiments were performed at 20 °C rather than the growth temperature to assure that the initial uptake rate was linear. Second, the external substrate concentration was 1.35 μM, which is much lower than local concentration in mitochondria and therefore the overall rate will be much lower. Third, in mitochondria the exchange is driven by the concentration gradients of the substrates and the membrane potential,[14] but here ADP homo-exchange was used.

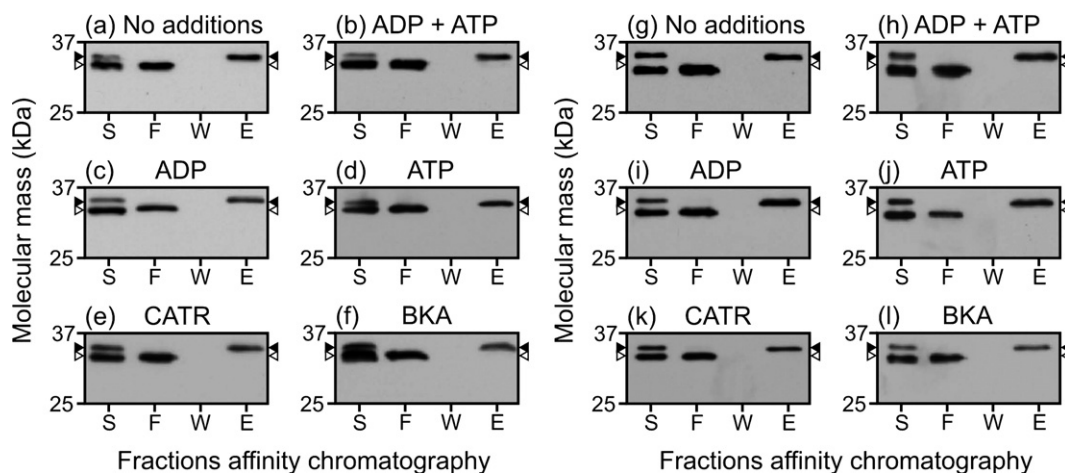### Affinity purification of co-expressed AAC2 and His_6-AAC2

Mitochondrial membranes containing co-expressed AAC2 plus His_6-AAC2 were solubilized in 1% dodecylmaltoside. The molar ratio of the co-expressed tagged and untagged carriers in the solubilized material was identical to that in the membrane, and the efficiency of extraction was >95% (data not shown). The binding of the solubilized carriers to the nickel chelate column was performed in batch to ensure association equilibrium between the tagged and untagged carriers. However, association was not observed as all tagged carriers were bound to the matrix, whereas all untagged carriers were found in the flow-through from the column (Figure 4(a), fraction F). When the His_6-AAC2 fusion proteins were eluted with imidazole, the eluate contained exclusively tagged carriers (Figure 4(a), fraction E). No untagged carriers could be detected even in over-exposed autoradiographs (data not shown). Collec-

tively, these results clearly indicate that tagged and untagged carriers did not form stable dimers in dodecylmaltoside.

It was possible that stable association of monomers had occurred only when the carriers were at particular stages of the transport cycle that differed from those attained above. To test this hypothesis, mitochondrial membranes were pre-incubated with CATR,[15,16] which locks the carrier in a state with the binding site open to the cytoplasm or with BKA,[17,18] which locks the carrier in a state with the binding site oriented to the mitochondrial matrix. The specific initial uptake rate of AAC2 in the absence of inhibitors is ca 20 nmol min^{-1} mg^{-1} of AAC2 (Figure 3). The addition of CATR (4 nmol mg^{-1} of protein)[12,13] inhibited uptake completely, whereas BKA inhibited the uptake of ADP to 80%, whether 4 or 12 nmol mg^{-1} of protein was used. Co-expressed AAC2 and His_6-AAC2 inhibited by CATR or BKA were solubilised and purified in dodecylmaltoside in the presence of the inhibitors. Again, all untagged carriers were found in the flow-through, whereas all tagged carriers were bound to the column (Figure 4(e) and (f)). The column eluates contained only the tagged carriers, showing that the carriers were not associated, neither when they were inhibited in the cytoplasmic state nor in the matrix state. As it was possible that the inhibitors might lock the carrier in some artificial or aborted state that prevented dimerisation, the experiments were also performed in the presence of the substrates ADP, ATP or ADP plus ATP to allow cycling through all intermediate stages in the transport cycle. Again, stable association of tagged and untagged forms of AAC2 was not observed (Figure 4(b), (c) and (d)).
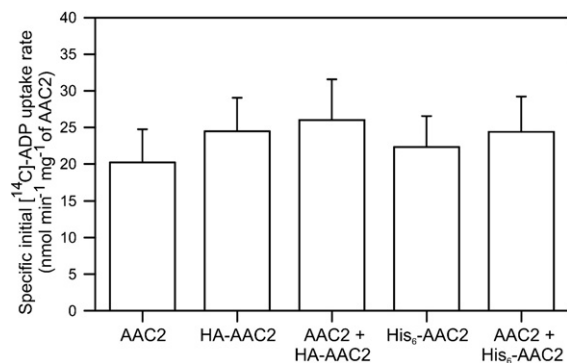
Figure 3. Specific initial uptake rates by untagged and tagged AAC2 in fused mitochondrial membranes. The specific initial rate of uptake of [14C]ADP in the first 15 s (the linear part of the uptake curve) was determined in quintuplicate for AAC2, His_6-AAC2, HA-AAC2, co-expressed AAC2 plus His_6-AAC2, and co-expressed AAC2 plus HA-AAC2. The total amount of AAC2 was determined in triplicate by Western blotting and densitometry by using purified and quantified AAC2 as standards. The values represent the mean and standard deviation. The values were corrected for background binding by using fused mitochondrial membranes of the WB-12 strain that lacks functional ADP/ATP carriers.

Figure 4. Nickel and HA affinity purification of co-expressed AAC2 and tagged AAC2 in dodecylmaltoside. Before solubilisation, mitochondrial membranes containing co-expressed AAC2 plus His$_6$-AAC2 ((a)–(f)) or AAC2 plus HA-AAC2 ((g)–(l)) were incubated in the absence or presence of ADP plus ATP, ADP, ATP, CATR, or BKA. The substrates and inhibitors were also present throughout the solubilisation and purification. Equivalent volumes of the solubilized material (S), flow-through (F), wash (W) and eluate (E) were loaded to determine the molar distribution over the fractions. AAC2 was detected with a primary α-AAC2 antibody. Black and white arrowheads indicate the approximate molecular masses of the tagged and untagged AAC2, respectively. All solubilisation and differential affinity purifications were carried out with the same mitochondrial membrane preparation. The slight dissimilarities in relative intensity and separation are due to differences in loading, separation, blotting and detection.

Affinity purification of co-expressed AAC2 and HA-AAC2

It was also possible that the absence of stable dimers between tagged and untagged AAC2 association could have arisen from the preferred dimerisation of carriers *via* the six-histidine tag, which would have prevented them from forming mixed dimers. Such preferential dimerisation could have been mediated by divalent cations. However, the addition of EDTA to buffers did not alter the outcome of the experiments (data not shown). To rule out any role of the six-histidine tag in preferential dimerisation completely, all the experiments were repeated by HA-affinity purification in dodecylmaltoside of co-expressed AAC2 and HA-AAC2. Once more, no stable associations were detected, irrespective of whether inhibitors or substrates were absent or present (Figure 4(g)–(l)).

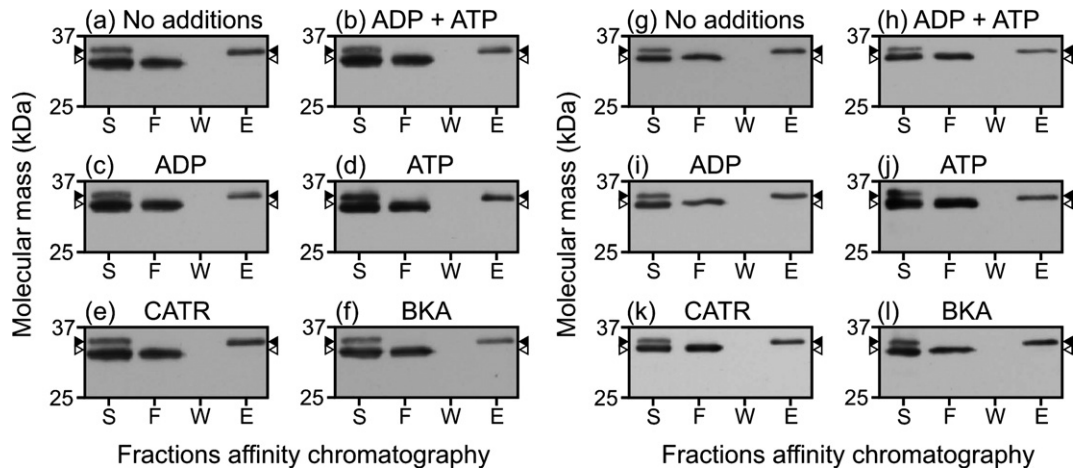## Affinity purification of co-expressed AAC2 and His$_6$-AAC2 in Triton X-100 and digitonin

Although dodecylmaltoside is one of the mildest detergents that preserves the integrity of many membrane protein complexes,[19–22] it was possible that it had not stabilized the putative dimer of AAC2. Therefore, the experiments were repeated with the co-expressed AAC2 and His$_6$-AAC2 in Triton X-100 and digitonin. Triton X-100 has been used previously for the solubilisation and purification of mitochondrial carriers[1,23–25] and has been reported to preserve dimers of mitochondrial carriers in size-exclusion chromatography and analytical ultracentrifugation,[1,24,26] although we could not confirm the presence of dimers in an earlier study.[7] Digitonin is an extremely mild detergent, that has been used to ex-

amine the oligomeric state of the mitochondrial carriers by native gel electrophoresis.[27] However, no stable dimers were observed in 2% Triton X-100 and 4% digitonin in the presence or absence of inhibitors and substrates (Figure 5). The amount of detergents used in the solubilisation was lowered to 1% Triton X-100 and 1% and 2% digitonin, respectively. The amount of protein that was extracted from the membrane was much lower, but again the stable association of protomers was not detected by affinity chromatography (data not shown).

Discussion

We have used differential tagging and affinity purification to investigate the oligomeric state of the yeast mitochondrial ADP/ATP carrier AAC2. This approach is based on the principle that proteins in a stable complex in detergent should co-purify. This method is technically simpler than the determination of oligomeric states by size-exclusion chromatography, analytical ultracentrifugation, neutron scattering or native gel electrophoresis, as the amount of bound detergent and lipid does not have to be determined.[7] It is a quick method that takes into account the possible influence of conformational states on the formation of the oligomeric state.

Tagged and untagged versions of the yeast ADP/ATP carrier were co-expressed in mitochondria and were fully functional as they transported ADP with similar specific initial rates as wild-type AAC2 alone. If the carriers existed and functioned as dimers, dimerisation would have been unaffected by tagging and random association of the carriers should have been observed. If they had dimerized randomly and remained dimeric in detergent solution about 40% of

Figure 5. Nickel affinity purification of AAC2 and His$_6$-AAC2 in digitonin and Triton X-100. The experiments were performed as described in the legend to Figure 4, except that the proteins were purified in digitonin ((a)–(f)) and Triton X-100 ((g)–(l)) in the absence or presence of ADP plus ATP, ADP, ATP, CATR, or BKA rather than in dodecylmaltoside.

AAC2 in the eluate would have been untagged. However, no untagged AAC2 was detected in the eluate and all the untagged AAC2 was found in the flow-through, even though the binding had been carried out in batch mode to allow the opportunity for association between the tagged and untagged carriers at equilibrium. Thus, stable dimers consisting of tagged and untagged carriers are not present in the detergents. It was formally possible that the tagged carriers had dimerized preferentially to the exclusion of the untagged carriers, preventing the formation of mixed dimers of untagged and tagged proteins. This explanation is highly implausible, as the same results were obtained with His or HA-tagged carriers and so the observations are independent of the tag. More compellingly, if dimerisation *via* the tags had occurred, then the association of tags would have had to have been stronger than the association of monomers *via* any protein–protein dimerisation interface, as untagged monomers were excluded fully. If this were so, an effect on the specific transport rate would have been anticipated, as the tag would have interfered with efficient dimerisation, but no such effect was observed. Thus, by far the most reasonable explanation is that under these conditions the carriers are monomeric in the detergent-soluble form. This conclusion that the yeast ADP/ATP carrier molecules behave independently of each other during the affinity purification agrees with recent studies of their hydrodynamic properties, which concluded that the carriers are monomeric in a wide range of detergents.[7]

Binding studies have shown that on average two carrier molecules bind one molecule of CATR.[12] Therefore, formally it was possible in our previous study,[7] that the CATR-AAC2 monomer had dissociated from a non-CATR inhibited partner and that the more stable CATR-AAC2 monomer had been purified, whereas the less stable monomer had been lost. Here, we have shown that no stable association of monomers had taken place under conditions

where all transport activity had been inhibited by CATR, and all of the protein has been accounted for. Thus, stable dimers containing at least one CATR-AAC2 monomer do not exist. None of the inhibitors or substrates of the carrier induced the formation of a stable dimer in detergent.

We have shown by three independent methods that the yeast mitochondrial ADP/ATP carriers are monomeric in mild detergents.[7] Cooperativity between protomers has been invoked as an integral part of the transport mechanism,[3,5,28–30] but then substantial structural interactions between protomers are expected to allow for the specific recognition of interacting monomers and for the relay of structural changes. One would expect that such interactions would be partially maintained in the relatively short incubation periods employed here, but no dimers were detected even in the presence of substrates. Structurally, a dimerisation interface consistent with a stable dimer has not been found either.[6,8,9] Taken together these observations may indicate that the yeast mitochondrial ADP/ATP carriers exist and function as monomers rather than dimers.

Materials and Methods

Engineering of an N-terminal six-histidine or hemagglutinin tag to AAC2

Two different tags were engineered at the N terminus of the yeast ADP/ATP carrier AAC2. One was a six-histidine tag, which allows the rapid purification of the fusion protein by nickel-chelate chromatography.[4] The other was the hemagglutinin epitope YPYDVPDYA (HA) from the surface glycoprotein of the influenza virus.[31] Oligonucleotide primers were designed for the introduction of a six-histidine tag (His$_6$-tag) or a hemagglutinin tag (HA-tag), followed by a protease Xa cleavage site at the N terminus of AAC2. The site of introduction was an NcoI site, which contains the start codon of the gene. For the phosphoryla-

tion of primers, 1 µl of the forward and the reverse primer (100 µM stock), 1× ligase buffer (1 µl), and ten units of T4 polynucleotide kinase (New England BioLabs, Ipswich, MA) were mixed in a total volume of 10 µl. The reaction mixture was incubated at 37 °C for 60 min, and the kinase was inactivated by heating at 65 °C for 20 min. Then the sample was cooled to room temperature to anneal the primers. The duplex was diluted to 0.5 µM with water. The expression vector pYES-P*pic2-aac2*[7] (the P*pic2* promoter was previously called *pYER053c*) was digested with NcoI (New England BioLabs) for 2 h at 37 °C, dephosphorylated and mixed with the duplex for ligation. The ligation mixture was electroporated into competent cells of *E. coli*. The ensuing expression vectors, called pYES-P*pic2-His$_6$-aac2* vector and pYES-P*pic2-HA-aac2* vector, were isolated by miniprep (Qiagen, Valencia, CA) and confirmed by PCR, restriction analysis, and sequencing (MRC Geneservice, Cambridge, UK).

Construction of vectors for co-expression of tagged and untagged AAC2

The *aac2* gene with a promoter region and a transcription terminator were introduced into the SpeI site at the start of the *pic2* promoter region in the pYES-P*pic2-His$_6$-aac2* vector or pYES-P*pic2-HA-aac2* vector. SpeI sites were introduced by PCR with KOD polymerase (Novagen, EMD Biosciences, San Diego, CA) at both ends of the promoter-*aac2*-transcription terminator cassette. Vectors and inserts were digested with SpeI (New England BioLabs). Then, the insert was ligated into the vector and the vector was transformed into *E. coli* cells. The desired orientation of the insert in the vector was identified by colony PCR with Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO) and confirmed by sequencing. The vector containing the tandem expression cassette was transformed into the *S. cerevisiae* strain WB-12.[32]

Growth of yeast strains and the isolation of yeast mitochondrial membranes

Two 50 ml cultures of Synthetic Complete medium minus tryptophan (SC-Trp) supplemented with 3% (v/v) glycerol and 0.05% (w/v) glucose were inoculated with a single colony from a SC-Trp + 3% (v/v) glycerol plate. The cultures were incubated at 30 °C overnight with shaking at 225 rpm. Two 2 l flasks containing 500 ml of YPG medium (10 g/l yeast extract, 20 g/l peptone, 10 g/l D-glucose) were inoculated with the overnight cultures to give an A_{600nm} of ca 0.05.

Cells grown in flasks were harvested by centrifugation at 3000*g* for 5 min, and washed twice with deionised water. The cell pellets were resuspended in an equal volume of breaking buffer (0.65 M sorbitol, 0.1 M Tris-HCl (pH 8.0), 5 mM EDTA, 0.2% (w/v) bovine serum albumin). Immediately before disruption, phenyl-methane-sulfonyl-fluoride (PMSF) was added to a final concentration of 1 mM. Cells were disrupted at 30 kPSI with a 2.2 kW disruptor of the Z series (Constant Systems, UK). Lysed cells were left on ice for 10 min and then unbroken cells were removed by centrifugation at 3000*g* for 10 min. Mitochondria and mitoplasts were harvested by ultracentrifugation at 186,000*g* for 40 min at 4 °C. The pellets were resuspended in buffer (0.65 M sorbitol, 0.02 M Tris-HCl (pH 7.4), 1 mM PMSF) and the centrifugation step was repeated. The mitochondrial pellets were resuspended in buffer lacking PMSF to a final concentration of ca 25 mg ml^{-1}.

Determination of protein concentration

The protein concentrations of yeast mitochondrial membranes and fused membranes were determined by the BCA protein assay (Pierce, Rockford, IL) and with bovine serum albumin as standard. The absorbance was determined at 562 nm with a SpectraMax Plus plate reader (Molecular Devices, Union City, CA).

SDS-PAGE and Western blotting

Proteins were separated by SDS-PAGE in Mighty Small electrophoresis units (Hoefer/G.E. Healthcare, UK) and gels consisting of 15% (w/v) polyacrylamide (Severn Biotech Ltd, UK) at 30 mA for 90 min. Protein bands were visualized by staining with Coomassie brilliant blue (50% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie blue R250) followed by de-staining with 15% (v/v) methanol, 7% (v/v) acetic acid. Proteins were transferred to a polyvinyl-idendifluoride (PVDF) membrane (Immobilon-P) Millipore, Bedford, MA) at 120 mA for 1 h in a Hoefer Semiphor transfer unit (G.E. Healthcare). Before transfer, the membrane was washed with methanol and then in transfer buffer consisting of 0.025 M Tris-HCL, 192 mM glycine, and 10% methanol. Non-specific binding of antibodies to the membrane was prevented by incubating the membrane for ~16 h in blocking buffer, consisting of phosphate-buffered saline (PBS), 0.1% (v/v) Tween 20 and 5% (w/v) skimmed milk powder (Marvel, UK). Proteins were detected with specific antibodies with the following titers; chick α-AAC primary antibody (produced by AgriSera, Sweden) at 1:25,000; α-HA-peroxidase conjugate at 1:5000 (Roche Diagnostics, Germany) or α-His primary antibody (Dianova, Germany) at 1:5000, diluted in PBS with 0.1% Tween. The primary antibody was incubated for 4 h with agitation. Then, the membrane was washed three times with PBS, 0.1% Tween for 10 min. When required, the membrane was incubated for 2 h with rabbit α-chick IgY peroxidase conjugate (Sigma-Aldrich) at a titer of 1:25,000 or goat α-mouse IgG peroxidase conjugate (Calbiochem, EMD Biosciences, San Diego, CA) at a titer of 1:10,000 in PBS, 0.1% Tween. The membrane was washed three times with PBS, 0.1% Tween for 10 min and the labelled protein was detected by chemiluminescence (ECL) (G.E. Healthcare).

Preparation of fused mitochondrial membranes and transport assays

Mitochondrial membranes were prepared as described.[33] Liposomes were prepared from *E. coli* total lipid extract and egg yolk phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) mixed in a 3:1 (w/w) ratio in 50 mM potassium phosphate buffer (pH 7.0) (KPi) at a final lipid concentration of 20 mg ml^{-1}. The liposomes (5 mg ml^{-1} in KPi buffer (pH 7.0), 5 mM ADP) and the mitochondrial membranes (1 mg of protein) were mixed. The membranes were fused by freezing in liquid nitrogen and thawing at room temperature seven times. The mixture was extruded nine times through a membrane with 1 µm pores (Whatman, New Jersey, NJ) and membranes were harvested by centrifugation at 300,000*g* for 30 min at 4 °C. The pellet was resuspended in KPi buffer containing 5 mM ADP. The external substrate (ADP) was removed on a Sephadex G-75 gel filtration column (3.5 ml bed volume) equilibrated with KPi. The proteoliposomes were eluted in 1 ml of KPi buffer. Transport was initiated

by diluting 100 μl of fused membranes (100 μg of protein) in 300 μl of KPi buffer containing 1.35 μM [8-^{14}C]ADP (Perkin Elmer, UK). The experiments were performed at 12 °C with constant stirring. At intervals the uptake of radiolabelled substrate was quenched by adding 4 ml of ice-cold KPi, and then the mixture was filtered immediately through cellulose nitrate (0.45 μm pore size). The filters were washed with ice-cold KPi buffer (2 ml), transferred to a scintillation vial, and 2 ml of Ultima Gold AB scintillation liquid (Packard, Netherlands) were added. Radioactivity was measured in a Packard TriCarb 2100 TR–liquid scintillation analyser.

Nickel affinity chromatography

Mitochondrial membranes containing co-expressed AAC2 and His$_6$-AAC2 were diluted to 5 mg ml^{-1} with wash buffer (0.65 M sorbitol, 0.02 M Tris-HCl (pH 7.4)), and incubated in the absence or presence of substrates (5 mM) or inhibitors (4 nmol mg^{-1}) at 4 °C for 20 min on ice. The membrane proteins were extracted in 1% dodecyl-β-D-maltoside, 2% Triton X-100 or 4% digitonin (Anatrace, Maumee, OH) in buffer containing 20 mM imidazole, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), and one tablet of complete protease inhibitor mini minus EDTA (Roche Diagnostics) by mixing at 4 °C for 60 min. Particulate material was removed by ultracentrifugation at 140,000*g* at 4 °C for 35 min. The tagged AAC2 was bound to Ni-NTA superflow (Qiagen) by batch binding at 4 °C for 2 h on a rotating mixer. The matrix (2 ml bed volume) was packed into 10 ml polyprep columns (Biorad, Hercules, CA). The column was washed with five column volumes of buffer containing 0.1% dodecylmaltoside, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 1 μM CATR plus inhibitor or substrate. The protein was eluted with 200 mM imidazole, 0.1% dodecylmaltoside, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 μM CATR plus inhibitor or substrate. The buffers were identical when the purifications were performed in Triton X-100 or digitonin, except for the final concentrations of detergent, which were 0.1% and 0.4%, respectively. To exclude the possibility of tag-mediated dimerisation *via* divalent cations, 1 mM EDTA was added in control experiments.

HA Affinity chromatography

Anti-HA Affinity Matrix (Roche Diagnostics) was pre-equilibrated with ten column volumes of equilibration buffer (0.1% dodecylmaltoside, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA). Mitochondrial membranes containing co-expressed AAC2 and HA-AAC2 (1 mg) were incubated in the absence or presence of substrates (5 mM) or inhibitors (4 nmol mg^{-1}) at 4 °C for 20 min on ice. Proteins were extracted in buffer containing 1% dodecylmaltoside, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), and one tablet of complete protease inhibitor mini minus EDTA (Roche Diagnostics) by mixing at 4 °C for 60 min. Particulate material was removed by ultracentrifugation at 140,000*g* at 4 °C for 35 min. The soluble fraction was bound to anti-HA column (1 mg of protein per ml of bed volume) by batch binding for 30 min. The flow-through fraction was collected and the column was washed with 20 bed volumes of equilibration buffer. The HA-tagged protein was eluted with elution buffer, containing 1 mg ml^{-1} of HA peptide in equilibration buffer, in three 1 ml volumes, each incubated at room temperature for 15 min.

Acknowledgements

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