Influence of N-terminal sequence variation on the sorting of major adenylate kinase to the mitochondrial intermembrane space in yeast

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Major adenylate kinase (Aky2p) from yeast has no cleavable presequence and occurs in identical form in the mitochondrial intermembrane space (6–8 %) and in the cytoplasm (approx. 90 %). To identify the signal(s) on Aky2p that might be required for mitochondrial import, the N-terminal region was examined. The N-terminus of Aky2p can guide at least two cytoplasmic passengers, dihydrofolate reductase from mouse and UMP kinase (Ura6p) from yeast, to the intermembrane space *in vivo*, showing that the N-terminus harbours import information. In contrast, deletion of the eight N-terminal amino acid residues or the introduction of two compensating frameshifts into this segment does not abolish translocation into the organelle's intermembrane space. Thus internal targeting and sorting information must be present in Aky2p as well. Neither a pronounced amphiphilic α-helical moment nor positive charges in the N-terminal region is a necessary prerequisite for Aky2p to reach the intermembrane space. Even a surplus of negative charges in mutant N-termini does not impede basal import into the correct submitochondrial compartment. The potential to form an amphipathic α-helical structure of five to eight residues close to the N-terminus significantly improves import efficiency, whereas extension of this amphipathic structure, e.g. by replacing it with the homologous segment of Aky3p, a mitochondrial matrix protein from yeast, leads to misdirection of the chimaera to the matrix compartment. This shows that the topogenic N-terminal signal of Aky3p is dominant over the presumptive internal intermembrane space-targeting signal of Aky2p and argues that the sorting of wild-type Aky2p to the intermembrane space is not due to the presence in the protein of a specific sorting sequence for the intermembrane space, but rather is the consequence of being imported but not being sorted to the inner compartment. Some Aky2 mutant proteins are susceptible to proteolysis in the cytoplasm, indicating incorrect folding. They are nevertheless efficiently rescued by uptake into mitochondria, suggesting a negative correlation between folding velocity (or folding stability) and efficiency of import.

INTRODUCTION

Adenylate kinases provide the ADP required for oxidative and substrate chain phosphorylations [1]. In accordance with their essential role in energy metabolism, adenylate kinases are ubiquitous and abundant. In eukaryotes two isoforms exist, a so-called short form, AK1 or myokinase, which occurs exclusively in the cytoplasm, and two subtypes of a long isoform, AK2 and AK3, residing in mitochondria (reviewed in [2]). The yeast equivalent of AK1 is Ura6p, which, in addition to UMP and CMP, efficiently phosphorylates AMP [3]. Aky3p is a GTP:AMP phosphotransferase and resides exclusively in the mitochondrial matrix [4,5]. The subcellular distribution of Aky2p, the major adenylate kinase in yeast, is remarkable because the protein, encoded by a single gene and translated from a single start AUG, is active in two compartments: the cytoplasm (plus the nucleus) and the mitochondrial intermembrane space [6]. The importation of Aky2p into mitochondria is unusual for two reasons: (1) in contrast with most other mitochondrial proteins, it reaches its target compartment without the help of a cleavable presequence, and (2) in contrast with all other known mitochondrial proteins, the cytoplasmic precursor pools of which are very small [7] to undetectable in the steady state [8,9], more than 90 % of total Aky2p stays and is active in the cytoplasm and only a small fraction (6–8 %) is imported [6]. Aky2 proteins from both locations are identical in their amino acid sequences and N-terminal modifications, i.e. the two N-terminal residues have been cleaved off, the N-terminal Ser-3 is N-acetylated and the C-terminal Asp is amidated in both topologies [10].

The present study examines the role of the N-terminal sequence of Aky2p and the properties that might influence its mitochondrial import. Usually, cleavable and non-cleavable N-terminal mitochondrial target sequences carry the complete address for their recognition and uptake by mitochondria, and it has been shown, e.g. for the 70 kDa outer-membrane protein, which like Aky2p is not processed on assembly in its target membrane, that this signal is both necessary and sufficient for import [11]. The propensity to form an amphipathic positively charged N-terminal α-helix at the aqueous phase/membrane interface is a prerequisite for recognition by either of two mitochondrial surface receptors [12–16], and the presence of a surplus of positive charges greatly facilitates transport across at least the inner membrane [12,17–19]. We found the N-terminus of Aky2p sufficient to direct the import of mouse dihydrofolate reductase (DHFR) and of the AK1-equivalent cytoplasmic isoenzyme Ura6p from yeast as non-mitochondrial passengers into the mitochondrial intermembrane space. To analyse the importance of the N-terminal region of Aky2p for mitochondrial import, we have varied the propensity to form an α-helix rather than a β-structure, the magnitude of the hydrophobic moment

Abbreviations used: Aky2p, yeast major adenylate kinase; *AKY2*, gene encoding Aky2p; DHFR, mouse dihydrofolate reductase; FN, family numbering; *URA6*, gene encoding the yeast UMP kinase Ura6p.

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and the number and type of charged amino acid residues by site-directed mutagenesis *in vitro* and have measured the efficiency of uptake into mitochondria *in vivo* (steady-state).

EXPERIMENTAL

Yeast strains, cell growth conditions, preparation and subfractionation of mitochondria

The *AKY2*-disrupted yeast strain DL1-D16 *aky2::LEU2* [4] (*AKY2* being the gene encoding Aky2p) served as the recipient for all mutant constructs in transformations with multi-copy yeast shuttle plasmids. In the cases indicated, strain WCG4 *pre1-1*, *pre2-2,* defective in two subunits of the 20 S proteasome [20], was used. Yeast cells were grown on semisynthetic medium supplemented in accordance with the auxotrophic requirements. As carbon source the medium contained either 3 % (w/v) lactate, 2 % (w/v) galactose, up to 8 % (w/v) glucose or 2 % (v/v) ethanol as indicated. Spheroplasts were prepared from mid-exponential cultures [$(1–2) \times 10^7$ cells/ml] and lysed in 0.6 M mannitol. Mitochondria were prepared from the 10000 ***g*** pellet, purified by Percoll (28 %) gradient centrifugation and subfractionated into intermembrane space, matrix, and inner plus outer membranes as described previously [21].

Construction of *AKY2* mutants

Site-specific *in vitro* mutagenesis was performed as described previously [22]. In all mutants except AKY-N1, wild-type sequences 5′-upstream of the coding region, including the AUG translational start triplet, the triplet of the second residue, serine, and the complete *AKY2* promoter were left unchanged. In addition, sequences beyond triplet 10 (corresponding to Val at position 21 in the family numbering, FN [22]) were not altered. AKY-N2 was derived from AKY2 by the insertion of a C base into codon 2 and the deletion of a T base from codon 7. The N-terminal amino acid sequence of the N-terminally truncated version of Aky2p, named AKY-N1, in which the second AUG was used for translation initiation, was MVLI instead of (MS)SSESIRMVLI in the wild-type. The 5′-terminal sequence of *URA6* or *AKY3* was fused to *AKY2* using the conserved sequence of the glycine-rich loop (within triplet 33, Gln, FN) for homologous recombination *in vitro* between *AKY2* and *URA6* or *AKY2* and *AKY3* sequences respectively. For construction of these hybrid genes, the respective sections of *URA6* and *AKY3* were used as primers for PCR and were subsequently fused to the *AKY2* promoter analogously, as described [3]. The gene encoding DHFR was joined to *AKY2* behind triplet 19, Gly, FN.

Mean hydrophobicity and α-helical hydrophobic moments

The hydrophobicity scale of amino acids used was the normalized consensus scale of Eisenberg et al. [23]. The mean hydrophobicity was calculated by using a constant moving window of four amino acid residues. The hydrophobic moments were calculated using the equation described by Eisenberg et al. [23]. Except where stated otherwise, a constant moving window of seven residues, which corresponds to μH_{max} in the wild-type Aky2p primary structure, was used. μH_{max} of wild-type Aky2p was determined by varying the window size from four to fourteen residues and scanning amino acid positions 14 to 27 beginning with Ser-14, FN, which was shown to form the N-terminus of the mature wild-type protein [24]. The N-terminal Ser residue of mutant proteins is likely to be processed and modified by N-acetylation (as in the wild-type protein) in accordance with the rules for cytosolic N-terminal protein processing in yeast [25] and therefore should have a slightly higher hydrophobicity value than assumed for an unmodified Ser residue. The sequences of the mature polypeptides on which the calculations were based, are given in Figure 3(A). Predictions of secondary structure were based on the algorithm of Chou and Fasman [26], with the use of the Wisconsin Sequence Analysis Package (Genetics Computing Group, Madison, WI, U.S.A.).

Miscellaneous procedures

Published procedures were used for determining adenylate kinase activity [6], for raising antisera in chickens [5] and the purification of antisera from eggs [27], for determining protein concentrations [28], for Western blotting and immunodecoration, DNA sequencing, PCR amplification of DNA and all other molecular procedures [29].

RESULTS

Aky2p-directed import of heterologous passengers into mitochondria *in vivo*

To examine whether topogenic information for mitochondrial import of Aky2p is contained in its non-cleavable N-terminal region, the N-terminal residues of Aky2p were fused to two heterologous, non-mitochondrial passengers, i.e. DHFR (from mouse) and Ura6p from yeast. The fusions were made within the highly conserved ATP-binding site of Aky2p as described in the Experimental section, and the *AKY2*-disrupted strain, DL1-D16, was transformed with the fusion construct. The Aky2p/DHFR-chimaeric protein (referred to as ADHFR) contained the 18 N-terminal residues of Aky2p. The hybrid protein is reasonably stable in yeast but approx. 50 % of the cytoplasmic form is slightly shortened (Figure 1A). A significant fraction of the full-length protein is found associated with mitochondria after isolation from a cell homogenate by gradient centrifugation. To verify that this portion is taken up by the organelle and is not superficially attached, isolated mitochondria were incubated with proteinase K. Figure 1(C) shows that the hybrid protein is resistant to this treatment in intact mitochondria and is sensitive in a mitoplast preparation in which the outer membrane has been disrupted. In contrast, the matrix marker citrate synthetase is protected in mitochondria as well as in mitoplasts. These results suggest that the N-terminal 16 (owing to processing of two N-terminal residues, Met-Ser) or fewer residues of the mature Aky2p are sufficient to direct a heterologous passenger into the correct Aky2p-specific submitochondrial compartment, i.e. to the intermembrane space. It should be noted that only the complete fusion protein (upper band in Figure 1A) and not the slightly truncated version (approx. 10 residues missing) is found in mitochondria. Because such a truncation is not observed in wild-type DHFR (Figure 1B, control), the cleavage is likely to occur within the N-terminal Aky2-derived portion of the hybrid. This is taken as a further indication that the extreme N-terminus of Aky2p is necessary and sufficient for import of the fusion protein.

To obtain independent confirmation of this finding we constructed a second chimaeric protein, a fusion between the two similar proteins Aky2p and Ura6p. Ura6p is a non-specific nucleoside monophosphate kinase of the short, exclusively cytoplasmic isoform that phosphorylates UMP, CMP and AMP [4]. The first 24 residues of Ura6p were replaced with the equivalent N-terminal 14 residues of Aky2p by homologous recombination *in vitro* within the ATP-binding motif. The hybrid (named AUUUp) was detectable by immunological means only in minute amounts in mitochondria of wild-type yeast (results not shown).

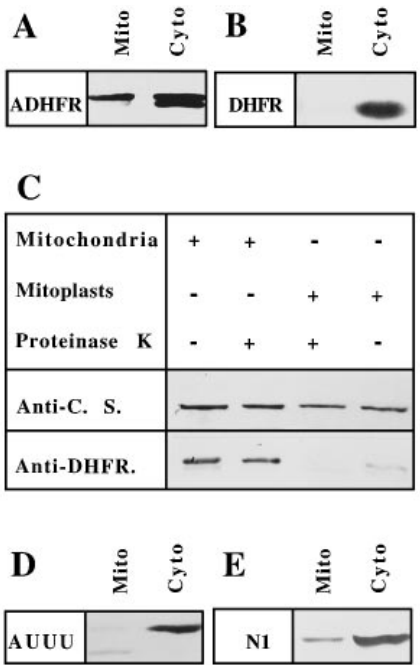

Figure 1 Import of mouse DHFR and yeast Ura6p into mitochondria *in vivo* under the direction of the N-terminal region of Aky2p

The *AKY2*-deleted strain DL1-D16 was transformed with a multi-copy plasmid carrying either the *AKY2*/DHFR hybrid gene (**A**, **C**), the DHFR gene (**B**, control), or the N-terminal truncation construct of *AKY2*, named AKY-N1 (N1, **E**). (**D**) The fusion protein of Aky2p and Ura6p (AUUUp) was expressed in the *AKY2* wild type, WCG4 *pre1*, *pre2* (proteasome-deficient). (**A**, **B**, **D**, **E**) Transformant yeast was converted into spheroplasts, lysed and fractionated into a cytoplasmic fraction (Cyto) and gradient-purified mitochondria (Mito). The proteins (10 μg per lane) were separated by SDS/PAGE and analysed by Western blotting. (**A**) The fusion protein, ADHFR, was detected by a mixture of rabbit anti-DHFR and rabbit anti-Aky2p antibodies and Protein A–horseradish peroxidase conjugate for immunodecoration. For detection of DHFR (**B**), rabbit anti-DHFR, and for AKY-N1p (N1, **E**), chicken anti-Aky2 sera were used as primary antibodies. (**D**) Both the fusion protein of Aky2p and Ura6p (AUUUp) and Aky2p were detected by a mixture of chicken anti-Aky2p and anti-Ura6p antisera and immunodecorated with peroxidase-conjugated goat anti-chicken secondary antibody. The band with the lower mobility is derived from Aky2p, and the faster migrating band from AUUUp. Ura6p does not yield a signal with the amounts of protein used. (**C**) Isolated gradient-purified mitochondria or mitoplasts (swollen in 0.1 M mannitol) were treated (or not) with 100 μg/ml proteinase K for 30 min on ice and then immunoblotted as above. For detection of the fusion protein an anti-DHFR antiserum was used. As a control serum, to verify integrity of the mitochondrial inner membrane, anti-citrate synthetase (anti-C. S.) was used.

We reasoned that the low cellular concentration might be due to low proteolytic stability of the chimaera and, in fact, obtained much higher levels of the chimaera by expressing it in strain WCG4 *pre1*, *pre2*, which was defective in two subunits of the 20 S proteasome [20]. The upper band in Figure 1(D) corresponds to Aky2p because the recipient strain, WCG4, had the wild-type *AKY2* gene. The hybrid protein AUUUp is represented by the lower band. Although AUUUp is expressed from a multi-copy plasmid under the guidance of the relatively strong *AKY2* promoter in exactly the same fashion as ADHFR, still very little protein is seen, and virtually none in the cytoplasm. Because the promoter and the translational start AUG surroundings are identical in all *AKY2* mutant constructs examined, similar cellular concentrations would be expected. Thus the virtual absence of the hybrid protein from the cytoplasm is most probably due to proteolytic degradation. A considerable amount, however, escapes degradation and is rescued by import into mitochondria. It seems that this fraction of AUUUp is even larger than that

Figure 2 α-Helical moment of the N-terminal segment of Aky2p

μH was calculated with moving windows varying from four to fourteen residues. The first residue forming the N-terminus of the mature protein is residue 14 (Ser; see Figure 3A) by the family numbering (FN, [22]), and the last is residue 27 FN (Gly) positioned in the ATP-binding motif. For calculation, the formula of Eisenberg et al. [23] was used; only μH_{max} is given for each particular window size.

imported of wild-type Aky2p (Figure 1D, Mito, upper band). A signal of wild-type Ura6p is absent from Figure 1(D) because the concentration of wild-type Ura6p is too low to yield a signal in a Western blot; in addition, AUUUp is smaller than either Aky2p or Ura6p. The above results show that the N-terminus of Aky2p is sufficient to direct two different cytoplasmically located passengers, DHFR as well as Ura6p, into mitochondria, suggesting that it carries mitochondrial import information. Import efficiencies vary, however, probably owing to intrinsic properties of the respective passenger proteins [30].

Presence of additional internal import information

To examine whether the N-terminal segment of Aky2p harbours the complete targeting signal, an N-terminally truncated version of the protein was expressed by using a construct in which the original translational start triplet had been mutated by *in vitro* mutagenesis. In this construct, AKY-N1, protein synthesis was initiated at the second AUG at position 20 FN (i.e. with the first eight residues of the protein deleted; FN is taken from [22]). Figure 1(E) shows that this shortened protein was still imported. It must be concluded that, in addition to the N-terminus, sequences downstream of the Met residue at position 20 FN are likely to contribute to, or to substitute for, the mitochondrial import address.

Primary structure and hydrophobic moment of the N-terminal section of Aky2p

The presumptive import-relevant target sequence at the N-terminus of Aky2p is likely to be extraordinarily short (see also below) (Figure 2). It is rich in the hydroxyamino acid serine, which is a frequent constituent of mitochondrial target sequences. Apart from the N-terminal Ser residue (i.e. Ser-3 of the coding sequence), which is N-acetylated in the mature protein, the N-proximal peptide harbours two more Ser residues. However, a positive net charge is lacking (one Arg residue present in the N-terminal sequence is compensated for by a Glu residue; Figure 3A) so that the N-terminal segment of Aky2p cannot be addressed as a typical mitochondrial presequence, in line with the observed poor import efficiency.

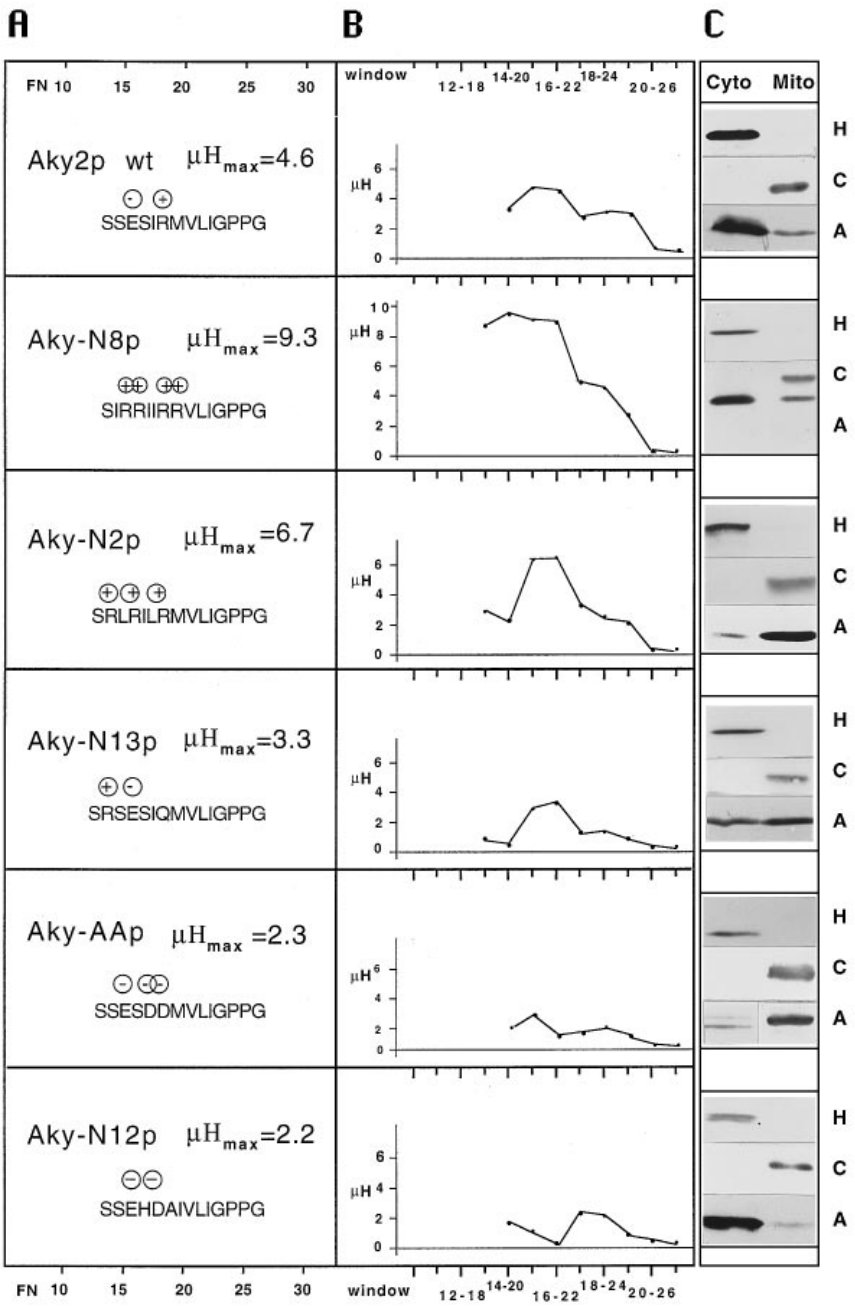

Figure 3 N-terminal sequences of *aky2* mutants, amphiphilic moments and mitochondrial import of Aky2p

(**A**) N-terminal sequences, charge distribution and μH_{max} values of *AKY2* wild-type and selected *aky2* mutants. (**B**) Profile of α-helical amphiphilic moments, calculated for the N-terminal regions of *AKY2* wild-type and mutants by using the normalized scale of hydrophobicity values published by Eisenberg et al. [23] and a moving window of seven residues. (**C**) The mitochondrial *in vivo* import of Aky2 wild-type and mutant proteins assayed by Western blotting in cellular subfractions. Cytoplasmic fraction (Cyto) and gradient-purified mitochondria (Mito) were prepared as described in the Experimental section. DL1-D16 [AKY2] wild-type multi-copy transformant and DL1-D16 *aky2::LEU2* transformed with multi-copy plasmids carrying N-terminal mutations of Aky2p were immune-decorated with anti-hexokinase (cytoplasmic marker; H), anti-cytochrome c_1(mitochondrial marker, C) and anti-Aky2p (A). Aky2 proteins were detected by a chicken anti-Aky2p antiserum and decorated with a horseradish peroxidase conjugate of a goat anti-chicken secondary antibody. Results were derived from different electrophoretic runs with different systems of immunodetection.

To analyse the influence of the N-terminal portion of Aky2p, four parameters affecting mitochondrial import were analysed by sequence variation: (1) number and type of charges [17]; (2) length of the presumed import-relevant sequence [13,19]; (3) the potential to form an α-helical structure [12,13]; and (4) the magnitude of the amphiphilic moment [23].

As a first approach the α-helical hydrophobic moment of the N-terminal segment was calculated for the wild-type protein. Because the magnitude of the calculated moment depends on the distribution of polar and non-polar amino acid residues along the presumed helical axis and on the size of the moving window used in the calculation, the window was varied from four to fourteen residues and μH was determined for each window size. This procedure allowed us to pinpoint those residues that

contribute to the moment. In this way, the window yielding the highest amphiphilic moment (μH_{max}) was determined as being seven (corresponding to about two helical turns). Further calculations were performed with this window size. μH increased when the window was expanded from four to seven or eight residues and then declined with wider windows (Figure 2). From these results it can be deduced that only the residues at positions 2–9 of the mature protein (corresponding to positions 16–23, FN; for N-terminal sequences of Aky2p see Figure 3A) contribute positively to the hydrophobic moment, whereas the inclusion of residues beyond this position leads to its decrease. It seems significant that these downstream sequences are almost invariant in mitochondrial and cytoplasmic isoforms of nucleoside monophosphate kinases from various tissues and organisms, and comprise part of the ATP-binding motif. This motif, situated beyond position 23 (FN), is rich in the helix-breaking residues Gly and Pro and is accordingly unlikely to contribute to the mitochondrial import signal. This view is also in line with the result shown in Figure 1(A) that the N-terminally shortened ADHFR fusion protein fails to be imported into mitochondria. In contrast, because the N-terminally truncated *AKY2* mutant protein Aky-N1p is imported, it must be concluded that in the *AKY2* wild type, sequences further C-terminal to the ATP site bear additional import information.

The secondary structure prediction, with the algorithm of Chou and Fasman [26] and moving windows of four and six residues, suggests that only the N-terminal 7–9 residues of the mature wild-type Aky2 protein might have a certain (low to moderate) propensity to form an α-helix (results not shown). This capacity might be slightly higher in the presence of lipidic membranes [12].

The length of the presumptive N-terminal amphipathic helix of Aky2p [seven to nine residues according to the Chou and Fasman prediction (results not shown), or a maximum of 10 residues according to Figure 2], as well as the maximal helical moment $\mu H = 4.6$ (Figure 3A) and an $\langle H_{max} \rangle$ value of 1.6 (not shown) are somewhat below the values expected for mitochondrial target sequences (generally, three to five helical turns, $\mu H \geqslant 7.3$ and $\langle H_{max} \rangle \geqslant 4.5$ are regarded as representative of membrane-seeking peptides [12]). However, other examples of mitochondrial proteins exist that have extraordinarily low μH and $\langle H \rangle$ values such as cytochrome *c* and the 70 kDa outer membrane protein [12,31,32].

Variation of charge and amphiphilicity in the N-terminal segment of Aky2p and efficiency of import into mitochondria

For a closer study of the N-terminal determinants that influence import of Aky2p into the organelle, the charges and the α-helical amphiphilic moment of this region were varied by site-directed mutagenesis and the import efficiencies of the Aky2 mutant proteins were tested. Figure 3(A) gives the primary structures of the wild-type protein and a selection of mutants, and lists the maximal μH values. In Figure 3(B) the α-helical amphiphilic moment along the presumptive helical axis of the complete N-terminal region is given (residues 1–14 of the mature proteins, corresponding to positions 13–27, FN). Mutant proteins are ordered in accordance with decreasing μH_{max}.

Figure 3(C) shows that Aky2 wild-type and all mutant proteins are taken up by mitochondria *in vivo*. The import into mitochondria is partial in all cases as in the wild type; however, the efficiency of uptake varies greatly. Wild-type Aky2p is imported into mitochondria not very efficiently, which accords with previous quantitative evaluations [6]. All of the mutant proteins are taken up with at least the same efficiency as the wild type. Surprisingly, some are imported even better than the wild type.

The *AKY2* mutant AKY-N8 accommodates the positive charges of four Arg residues in its N-terminal region. It displays a potentially high μH of more than 8 over a length of four windows (corresponding to 10 residues) and of more than 4 over 12 residues (Figure 3, second panel from the top). The propensity of this sequence for forming an amphipathic helix is extremely low but the efficiency of import is comparable to the wild type. In contrast, among the mutant Aky2 proteins that exhibit relatively low amphiphilicity, mutants AKY-AA and AKY-N12 excel by harbouring three and two negative charges respectively. AKY-N12 contains the amino acid sequence Glu-His-Asp, which is particularly unusual for a conventional mitochondrial presequence. Both mutant proteins are imported into mitochondria, Aky-AAp even more efficiently than the wild type. Also Aky-N13p has a high tendency to get imported, although its N-terminal peptide is electrically neutral and exhibits a rather low amphiphilic moment. This suggests that there is no simple correlation between translocation efficiency and either the magnitude of the α-helical amphiphilic moment or the charge distribution. Positive charges or a high hydrophobic moment are not decisive for basal import of Aky2 proteins into the mitochondrial intermembrane-space compartment.

The mutant construct AKY-N2 displays three positive charges and a μH_{max} of 6.7; it can therefore be addressed as (moderately) membrane-seeking [12,23]. Aky-N2p shows the highest import efficiency of all Aky2 proteins, including the wild type. This mutant protein is taken up so efficiently that the ratio of signal intensities in mitochondria and cytoplasm is approximately inverted compared with the wild type (the sum of the two being about the same as in the wild type). In contrast with the wild type, and in particular with Aky-N8p, Aky-N2p exhibits a significant tendency to form an α-helix comprising eight residues, which is the highest among wild-type and all mutant proteins.

Mutant AKY-N2 has been obtained by introducing two compensatory frameshift mutations into its N-terminal sequence, and the N-terminal peptide shows no similarity of primary structure to the wild type. Nevertheless Aky-N2p is imported significantly better than the wild type. These results, together with those obtained with the truncation mutant AKY-N1, show that a basal level of Aky2p import into mitochondria was maintained by additional internal target information. Increases above the basal level are observed in some mutants, but they are not necessarily correlated with high helical μH and/or the accumulation of positive charges at the N-terminus, because mutants displaying negatively charged N-termini and a low μH also show increased import efficiency. To be directed into the mitochondrial intermembrane space, a peptide of as few as five or six amino acid residues that exhibit a certain tendency to form an amphiphilic α-helix proved to be optimal. This shows that neither the type and number of charges nor the amphiphilicity of the N-terminal segment determines the efficiency of mitochondrial uptake and suggests that still other parameters might have an influence.

Submitochondrial localization of Aky2p mutant proteins

Only 6–8 % of the total Aky2 wild-type protein is present in the mitochondrial intermembrane space; the remainder resides in the cytoplasm. The finding that import efficiency is enhanced in several *AKY2* variants is unusual and unexpected. Generally, mutations in the mitochondrial targeting sequence lead to a lowered uptake by the organelle and an increase in the cytoplasmic precursor pool in the steady state. For some mutant

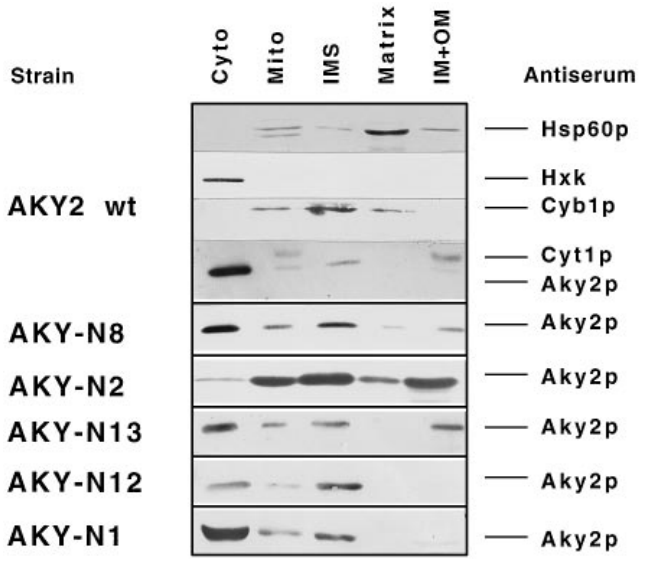

Figure 4 Submitochondrial topology and import efficiency of adenylate kinase-derived proteins of selected Aky2p N-terminal mutants

Cytoplasmic fraction and gradient-purified mitochondria were prepared from yeast spheroplasts. Mitochondria were further subfractionated into intermembrane space (IMS), matrix and inner plus outer membranes (IM + OM) as described in the Experimental section. Upper panel: DL1-D16 [AKY2] wild-type multi-copy transformant, immune-decorated with chicken anti-Aky2p antiserum as described in the legend to Figure 3(C). The following markers were tested as controls for mutual contamination of fractions: hexokinase (Hxk, cytoplasm), cytochrome b_2 (Cyb2p, intermembrane space), cytochrome c_1 (Cyt1p, inner membrane) and Hsp60 (matrix). Other panels: Aky2p N-terminal mutant proteins. Cells and mitochondria were fractionated and immune-decorated as above (only Aky2p is shown). Results were derived from different electrophoretic runs.

Aky2p proteins the increased import efficiency could be due to either an improvement of a presumptive intermembrane space-specific sorting signal or to a prolongation of the time interval during which the protein is import-competent, i.e. before it assumes its rigid folding structure. Alternatively, the specificity of the sorting signal could be altered by the mutation, resulting in transport to another mitochondrial compartment besides the intermembrane space. To discriminate between the last and the first two possibilities, the presence of selected *AKY2* mutant proteins was examined in mitochondrial subfractions (Figure 4). Mutual contaminations by cellular and mitochondrial compartments were tested immunologically by determining specific markers in subfractions of the mitochondria of all *AKY2* mutants. Hexokinase, cytochromes b_2 and c_1 and Hsp60p were assayed as markers for the cytoplasmic fraction, the intermembrane space, the inner membrane and the matrix compartment respectively (results shown only for the wild type).

A selection of Aky2 mutant proteins was examined in this experiment that were stable in the cytoplasm and simultaneously taken up by mitochondria more efficiently than the wild type. Figure 4 shows that the import efficiency of all six mutant proteins is similar to or better than the wild type. In particular, Aky-N2p had a comparably high import efficiency, most of the total mutant protein being imported. Subfractionation showed that most of this protein was, in fact, correctly sorted to the mitochondrial intermembrane space. Only a faint signal, presumably due to slight contamination with intermembrane space protein, could be detected in the matrix fraction. We conclude that the quality of the N-terminal signal targeting the intermembrane space has been improved considerably by the mutation in AKY-N2. In addition, a significant fraction of mitochondrially imported Aky2-derived protein was found associated with the inner plus outer membrane fraction in mutants AKY-N2 and AKY-N8. In this location it is inaccessible to trypsin or proteinase K treatment in whole mitochondria (160 μg/ml trypsin, 30 min, 0 °C) but extractable by sodium carbonate (results not shown).

The results in Figure 4 show that all *AKY2*-derived proteins are sorted to the mitochondrial intermembrane space, although in most mutants the primary structure of the N-terminal region of Aky2p has been altered profoundly. This is most explicit in the case of truncation mutant AKY-N1, which lacks the entire sequence. Nevertheless it is distributed between cytoplasm and mitochondria at a ratio that is about comparable to the wild-type, and all material taken up by mitochondria is correctly sorted to the intermembrane space. This implies that the N-terminal sequence of the wild type, although sufficient to direct DHFR and the AUUUp hybrid to mitochondria, does not constitute the decisive determinant responsible for import into the intermembrane space. Mitochondrial targeting as well as intramitochondrial sorting information must be present in internal sequences as well as at the N-terminus. Structural properties of the N-terminus, however, clearly influence the efficiency of import.

Heterologous targeting sequences

As a further parameter influencing import efficiency, the length of the N-terminal sequence was increased. The nucleotide sequence encoding the 15 N-terminal residues of Aky2p was replaced, by homologous recombination *in vitro*, with that encoding the 23 N-terminal residues of Aky3p. Aky3p is a GTP:AMP phosphotransferase that is similar to Aky2p in both primary [3] and tertiary [33] structures; in contrast with Aky2p, it is located in the mitochondrial matrix [5]. The most prominent difference between Aky2p and Aky3p is the extended N-terminus of Aky3p, which, although also not cleavable, excels in its capacity to form a moderately amphiphilic α-helix over a stretch of 19 residues ($\mu H > 4$ over 15 residues; $\mu H_{max} = 5.47$; four positive and one negative charges) (Figure 5A). The resulting hybrid construct, AKY-N9, was used to examine whether this N-terminal sequence of Aky3p has matrix-targeting information, and which of the two sorting signals in AKY-N9p, the N-terminus of Aky3p or the internal topogenic information of Aky2p, was dominant. Figure 5(B) shows that, even after massive overexpression, most AKY-N9p was found in the mitochondrial matrix. Very little was membrane-associated (in carbonate-extractable form; results not shown) or left in the cytoplasm. In mutant AKY-N10 the length of the amphiphilic helix of the *AKY3*-derived N-terminal sequence was increased ($\mu H_{max} = 5.47$; $\mu H > 4$ over 19 residues; four positive and no negative charges) (Figure 5A). In this case, no hybrid protein was observed in the cytoplasm at all (Figure 5B). This interesting observation suggests that the non-cleavable N-terminal sequence of Aky3p harbours the topological address for sorting into the matrix and that this signal is dominant over the presumed internal intermembrane space-sorting signal in Aky2p.

DISCUSSION

Mitochondrial targeting and intramitochondrial sorting of Aky2p

Most mitochondrial proteins are encoded by nuclear genes, translated on cytoplasmic ribosomes as N-terminally elongated precursors and post-translationally imported into mitochondria [34–36]. The information for mitochondrial recognition and

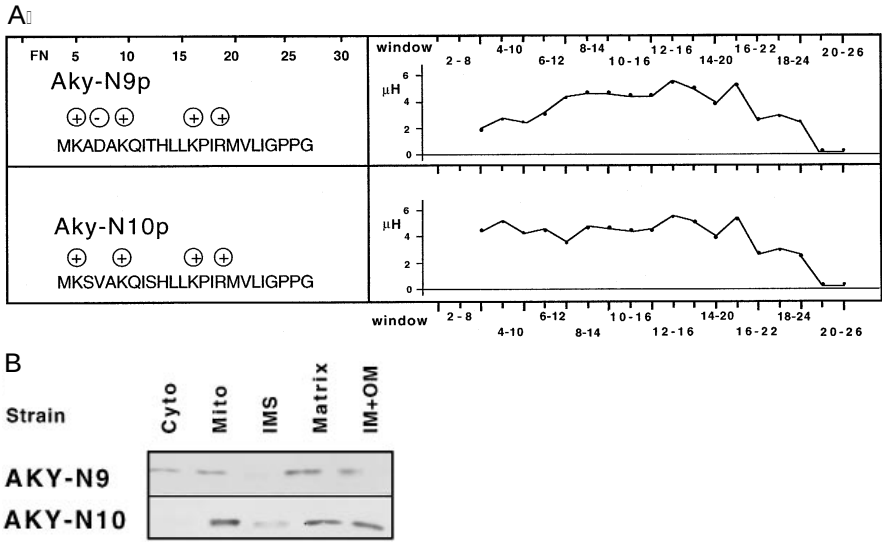

Figure 5 Amphiphilic α-helical moments and mitochondrial subfractionation of Aky2 hybrid proteins carrying *AKY3*-derived N-terminal mitochondrial targeting sequences

(**A**) Sequences and the α-helical amphiphilic moments of hybrid proteins in which Aky2p without the N-terminal 10 residues was fused by homologous recombination *in vitro* to the N-terminus of yeast matrix Aky3p (AKY-N9), and of an *AKY3* N-terminal mutant having a high a amphiphilic moment comprising 19 residues, including the immediate N-terminus (AKY-N10). Amino acid sequences of the mature proteins are given by the one-letter code; positive (+) and negative (−) charges are indicated. (**B**) Cellular and mitochondrial subfractionation and Western blotting of Aky2 hybrid proteins (yeast recipient strain, DL1-D16) carrying mitochondrial targeting sequences of *AKY3* (AKY-N9), and of an *AKY3* N-terminal mutant with an extended length of high α-helical amphiphilic moment (AKY-N10). Subfractionation, designation of subcompartments and immunodecoration procedures were as described in the legend to Figure 4.

import, in most cases, is encoded by the N-terminal segment, which is cleaved as soon as it has traversed the outer and the inner membranes and is exposed to the matrix-processing peptidase. However, a number of mitochondrial proteins lack a cleavable presequence and are nevertheless sorted to any of the submitochondrial compartments. In some of them the mitochondrial address is encoded by the N-terminal segment, although it is not removed on import [e.g. 70 kDa protein of the outer membrane [11,32]; 3-oxoacyl-CoA thiolase of the mitochondrial matrix [37]; GTP:AMP phosphotransferase, Aky3p ([3] and W. Bandlow, R. Schricker and G. Strobel, unpublished work)]. Another subset of proteins obviously has internal targeting sequences (e.g. apocytochrome *c*, the adenine nucleotide translocator or the Bcs1 protein) [38–41]. In any event, mitochondrial targeting sequences are characterized by their capacity to form positively charged amphipathic helices over a stretch of 18 or more amino acid residues [12,17,19].

The N-terminus of Aky2p does not conform these rules, in the following ways. (1) The presumptive target sequence is unusually short. In this respect Aky2p resembles the 70 kDa outer-membrane protein in which 12 residues from the N-terminus are sufficient for targeting and sorting [11]. (2) The first 10 residues have a low tendency to form an α-helix so that in an aqueous environment the predicted secondary-structure probability is significantly higher for the formation of β-structure, and the amphiphilic α-helical moment is low. (3) In the N-terminal segment of the mature Aky2p, surplus positive charges are lacking. The import experiments with N-termini of Aky2p mutated *in vitro* show that neither positive charges nor an amphiphilic moment are decisive for translocation into the organelle. These parameters do not even seem to be essential for a basal level of uptake and sorting to the intermembrane space because Aky-N1 mutant protein, from which most of the sequences forming the presumptive import address of the DHFR and Ura6 fusion proteins have been deleted, is also imported correctly. These findings suggest that the N-terminal region of Aky2p functions as accessory targeting and sorting information that is probably aided by an internal signal.

However, some properties of the N-terminal region of Aky2p, presumably its propensity for forming an amphiphilic helix and the length of this structure, contribute to the import efficiency in a decisive manner. In mutant Aky-N2p the N-terminal segment has been mutated to form a highly amphiphilic, positively charged but rather short α-helix. The improved α-helix might enhance binding to the outer membrane receptor but, being too short, might fail to make contact with the import machinery of the inner mitochondrial membrane that would enable translocation to the matrix. In contrast, an elongated amphiphilic helix that is sufficiently long to allow sorting to the matrix is encountered with the Aky3/Aky2 fusion proteins. In mutant AKY-N9, and in particular AKY-N10, the chimaeric protein ends up in the matrix, showing that in principle the primary structure of Aky2p harbours no constraints opposed to matrix import. Thus mutant AKY-N2 and Aky2 wild-type protein are taken up into the intermembrane space only, because of the shorter helix, suggesting that sorting to the intermembrane space emanates from the absence of a matrix-targeting signal. This argument also conforms to the notion that the import machineries of the inner and outer membranes are self-contained entities and can clearly be uncoupled; the outer membrane interacts with and completely translocates only the subset of proteins that are destined for the mitochondrial outer membrane and the intermembrane space [42]. The driving force for the translocation process of this subset of proteins might be provided by rapid folding in the intermembrane space compartment.

Distribution of Aky2p between cytoplasm and mitochondria

Generally, targeting sequences are considered specific for each intracellular compartment, so that most proteins are found at

only a single location within the cell. However, an increasing number of exceptions to this rule are being recognized. Usually, topological isoforms are encoded by the same gene in such a way as to allow the expression of differential topogenic information; consequently the two proteins do not have quite the same N-terminal sequence (e.g. the *HTS1* and *MOD5* isoproteins) [43,44]. Fumarase (Fum1p) has been reported to occur in identical forms in mitochondria and cytoplasm; however, Fum1p is a matrix protein and has a cleavable presequence that is processed on all Fum1 molecules by the matrix-processing peptidase. Most of the cleaved Fum1p is subsequently re-exported (or lost from mitochondria by back-slippage of the arrested import intermediate) to the cytoplasm [45].

A number of alterations to the N-terminal sequence of Aky2p have been described in this paper that lead to a more or less pronounced improvement of the uptake efficiency. Because mutations within target sequences usually do not improve the efficiency of translocation across the respective target membrane, it is evident that, in Aky2p, the topogenic properties of the N-terminal sequence do not in themselves determine target recognition and translocation. Because Aky2p harbours internal target information, it seems instead that the sequence of the N-terminus is accessory and primarily influences other import-relevant parameters, most probably the folding velocity (and/or the final three-dimensional folding stability).

Supporting evidence that the velocity or stability of protein folding could determine import efficiency of Aky2p can be derived from the high proteolytic sensitivity in the cytoplasm of some of the Aky2 mutant proteins. Because all mutant constructs have been expressed from the identical promoter, their cellular protein concentrations are expected to be comparable. This is evidently not so; for example, Aky-AAp and Aky-N12p or the hybrid UAAAp are barely detectable, particularly in the cytoplasm. The major reason for unequal cellular *AKY2*-derived protein concentrations seems to be the disposal of mutant proteins that are too reluctantly folded or misfolded in the cytoplasm. Evidence in support of this view is provided by the significant increase of mutant protein concentration in the cytoplasm and, to a smaller extent, also in mitochondria in a *pre1*, *pre2* proteasome mutant background. Destabilizing mutations in the N-terminal segment might lead to delayed folding, and a prolonged exposure of internal targeting sequences could lead to improved uptake (e.g. in Aky-AAp and Aky-N12p or the UAAA fusion protein).

Post-translational compared with co-translational import of Aky2p

The present understanding of post-translational import into mitochondria includes the association of nascent proteins with the presequence-binding protein, Mtf52p, the mitochondrial import-stimulating factor, Msf1p, and molecular chaperones of the Hsp70 class during their synthesis or immediately after release from the ribosomes (reviewed in [46]). Both the presence of a target sequence and the association with a chaperone keep the precursor partly unfolded, i.e. in an import-competent state, until it interacts with the mitochondrial target membrane through its N-terminal amphipathic α-helix. Import of Aky2p dispenses with a presequence that could retard folding. Because partly denatured wild-type Aky2p refolds rapidly in the absence of molecular chaperones (W. Bandlow and A. Zollner, unpublished work), Aky2p folding seems to ensue spontaneously without the help of chaperones. Improvement of import efficiency relative to the wild type of most mutant proteins suggests that the sequence of the N-terminal region nevertheless has an influence. Probably it influences folding and, in the mutants, causes a delay in assuming a stably folded conformation. In fact it has been observed in several cases that the presence of a presequence hinders folding [47,48]. Support for the view that retardation of precursor folding improves import efficiency comes from the finding that a decreased protein folding velocity can suppress a mutation in the leader of the maltose-binding protein of *Escherichia coli*, which caused intracellular accumulation of this protein [49]. In yeast, point mutations in the precursor to a DHFR fusion protein have been found to enhance post-translational import of the chimaera into mitochondria [50]. Thus it appears plausible that the folding velocity is a crucial parameter that determines the extent of import of Aky2p.

As outlined above, folding of Aky2p is spontaneous and very rapid, and the pool of import-competent (partly) unfolded Aky2p molecules is therefore likely to be rather small. This implies that only those molecules of Aky2p are imported into mitochondria that contact the target membrane in a partly unfolded, import-competent state, whereas the rest (more than 90% for Aky2 wild-type protein) remain in the cytoplasm. This suggests further that folding and import are competitive and mutually exclusive. Thus import of Aky2p could largely occur co-translationally. In agreement with this assumption, the results presented here indicate that the pools of cytoplasmic Aky2p and of the fraction imported into mitochondria do not equilibrate to a large extent; otherwise, in those mutants in which Aky2p is unstable in the cytoplasm, little or no Aky2-derived protein would be detected in mitochondria as well (e.g. AUUUp, Aky-AAp, Aky-N12p). However, coupling between translation and membrane translocation cannot be strict, because the proteasome-deficiency of strain WCG4 *pre1*, *pre2* not only leads to an increased half-life of the fraction of the protein retained in the cytoplasm relative to the wild type, but also slightly enhances the amount of the imported polypeptide. This indicates that this fraction is, to a certain degree, accessible to proteolytic degradation by the proteasome in the cytoplasm of the *PRE* wild type. From these observations and considerations it is obvious that, with respect to uptake into mitochondria, Aky2p behaves differently from most other proteins imported into the organelle from the cytoplasm. In any event, limited import of a protein as the consequence of a competition between import and spontaneous folding provides a novel mechanism for intracellular protein sorting to guarantee that a particular protein is active in more than one subcellular compartment.

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