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In vivo import of yeast adenylate kinase into mitochondria affected by site-directed mutagenesis

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Site-directed mutagenesis and deletions were used to study mitochondrial import of a major yeast adenylate kinase, Aky2p. This enzyme lacks a cleavable presequence and occurs in active and apparently unprocessed form both in mitochondria and cytoplasm. Mutations were applied to regions known to be surface-exposed and to diverge between short and long isoforms. In vertebrates, short adenylate kinase isozymes occur exclusively in the cytoplasm, whereas long versions of the enzyme have mitochondrial locations. Mutations in the extra loop of the yeast (long-form) enzyme did not affect mitochondrial import of the protein, whereas variants altered in the central, N- or C-terminal parts frequently displayed increased or, in the case of a deletion of the 8 N-terminal triplets, decreased import efficiencies. Although the N-terminus is important for targeting adenylate kinase to mitochondria, other parameters like internal sequence determinants and folding velocity of the nascent protein may also play a role.

Saccharomyces cerevisiae; ATP:AMP phosphotransferase; in vitro mutagenesis; mitochondrial import

1. INTRODUCTION

Adenylate kinases (AK; EC 2.7.4.3) are abundant, small, soluble enzymes which catalyse the reversible transfer of the γ-phosphate group from MgATP to AMP. They are mainly required for the biosynthesis of ADP. Due to their essential function, they are ubiquitous and highly conserved in evolution. Two isoforms have been identified which, in vertebrates, occur in mitochondria (long-form enzymes, AK2 and AK3) and in the cytoplasm (short-form, AK1), respectively (for a review see [1]). The differences between the two versions are mainly due to considerable sequence divergence, including length variation, in the central portion of the enzymes. Nevertheless, vertebrate AK1 and yeast AK2 were shown to have very similar folding structures by X-ray crystallographic analyses [2–4]. Consistent herewith, the two isoforms, AK1 and AK2, have very similar kinetic parameters. In yeast, only the long-form isozyme, Aky2p, has been studied extensively [5–7]. In contrast to vertebrates, it has been found to occur simultaneously in active form in cytoplasm (about 90%) and mitochondrial intermembrane space [7–9]. Since the protein lacks a cleavable presequence and occurs in apparently identical form in the two compartments, it was of considerable interest to determine which parts of the protein harbour the mitochondrial import address or which parameters modulate transport efficiency and influence the distribution between the cytoplasmic and the mitochondrial locations.

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2. MATERIALS AND METHODS

2.1. *Yeast strains, growth conditions, cellular subfractionation and enzyme assays*

The *Saccharomyces cerevisiae* wild-type strain, DL1 *MATa his3-11, 15 leu2-3, 112, ura3-251, 372, 328* [10], served as the parental strain for the creation of an aky2 deletion strain, termed DL1-D16 *aky2, sir4::LEU2* (constructed analogously as described for akyDD0 [7]). This mutant was used as the host for transformations with in vitro mutated *AKY2* alleles. Cells were grown on semisynthetic media containing 3% of either glucose, galactose or glycerol. Mitochondria were prepared from lysed spheroplasts, purified by Percoll (Pharmacia, Freiburg; 28%) gradient centrifugation and tested for contaminations by proteins from other compartments according to published procedures [7,11]. The 46,000 × *g* (30 min, 4°C) supernatant of the first homogenate was used as the cytoplasmic fraction. Adenylate kinase, hexokinase and citrate synthase were detected after sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretic transfer onto nylon membranes (Immobilon, Millipore, Eschborn) by immune reaction with polyclonal monospecific sera [12]. Protein was determined as described [13].

2.2. *In vitro mutagenesis*

A 1630 bp *Sspl* fragment harbouring the entire wild-type *AKY2* gene, including about 790 bp of upstream and about 180 bp of downstream sequences [6], was used for in vitro mutagenesis of *AKY2* coding sequences. Two compensatory frameshifts were introduced into the nucleotide sequence (amino acid positions 150 and 181) en-

coding the extra loop of Aky2p by using the procedure and strains described by Kramer and Fritz [14] and Stanssens et al. [15]. Introduced by mutagenesis, plasmid paky123 contained a *Sal*I and a *Sac*II site in the flanks of the nucleotide sequence coding for the extra loop. Sequential restriction with *Sal*I and either *Hin*dIII, *Sac*II or *Bgl*II (*Hin*dIII and *Bgl*II are adjacent sites present in the wild-type sequence [6]), and re-ligation of the blunt ended fragments (made blunt-end with either Klenow polymerase or mung bean nuclease) yielded the in-frame deletion mutants aky2-5, aky2-6 and aky2-7, respectively. Other site-directed mutations were introduced as described by Kunkel et al. [16]. (Mutations are shown in Fig. 1) Introduction of the mutation was verified by hybridization to the mutagenic oligonucleotide, by restriction analysis and nucleotide sequence analysis [17]. Mutated adenylate kinase sequences were re-cloned into the shuttle vector, YEp352 [18], and transformed [19] into the *AKY2*-disrupted strain, DL1-D16. Other molecular procedures were performed as described [20].

3. RESULTS

In vertebrates, only long-form isozymes of adenylate kinase have been observed to reside in mitochondria, whereas short variants occur in the cytoplasm (and in the nucleus). Both types of enzymes have primary structures which exhibit sequence similarities in their N- and C-terminal portions and are folded into very similar tertiary structures [1,3,4]. The sequence alignment shown in Fig. 1 is based on the alignment of the spatial structures of adenylate kinase from *E. coli*, AK3 from cow and AK1 from pig as proposed by Schulz and collaborators [21,22]. In an approach to identify sequences which might target Aky2p to mitochondria, we screened for surface-exposed elements which are present in the long versions but absent from the short ones. Since proteins with mitochondrial destinations interact initially with the lipid bilayer of the membrane by means of a sequence capable of folding into an amphipathic α-helix, we searched particularly for regions in the Aky2p primary structure which fulfilled these requirements. We applied the algorithm developed by Margalit et al. [23] on short and long versions of adenylate kinases using alternative window sizes of 6 and 10 residues. This screen identified three sequence blocks as candidates for carrying mitochondrial import information. They concerned the extreme N- and C-termini and a structure in the central portion displaying limited sequence similarity among the long forms but completely differing between the long and the short versions (Fig. 1). As an additional region, a consecutive block of 38 amino acids was considered (pos. 138–185) which is replaced by 11 amino acids in the short forms. Part of this sequence, the so-called extra loop (pos. 148–180), folds into a β-meander rather than into an amphipathic α-helix [4,22]. Using site-directed mutagenesis, changes were introduced into all four candidate sequence blocks as summarized in Fig. 1.

In mutant akyN1, the first ATG was changed to ATA so that the second ATG (ninth codon) at pos. 20 (according to the family numbering displayed in Fig. 1) was used for translational initiation. Mutant akyN2 contains a compensatory double frameshift in the N-terminal segment. Both mutants were designed to test the relevance of N-terminal sequences for import. akyL1 and akyL8 each harbour a small alteration in the central portion of the protein. akyL1 affects the C-terminal flank of a surface-exposed turn by exchanging Leu^{129} for Arg, whereas in akyL8 the sequence IT (pos. 146 and 147) located in a small surface-exposed helical structure [2,22] is changed to LYVDA. aky2-123 contains two compensating frameshifts near the beginning and the end of the β-meandric loop (amino acid positions 150 and 181, respectively). Mutants aky2-5, aky2-6 and aky2-7 contain deletions eliminating most or all of the loop. aky2-5 and aky2-6 have left 6 and 4 amino acids, respectively, between two conserved arginines (pos. 145 and 182). In aky2-7 the Arg near the C-terminal border of the loop has been deleted together with the neighbouring Ser (Fig. 1). The C-terminus displays an α-helical structure which diverges considerably between short and long versions. akyC1 and akyC2 were constructed to assay the relevance of the C-terminal region. Using site-directed mutagenesis, two stop codons were alternatively introduced into this region eliminating 24 and 7 C-terminal triplets, respectively. With the exception of akyN2p, all mutant proteins are only marginally active (not shown).

In the experiments shown in Figs. 2 and 3 the amounts of wild-type and mutant Aky2p are displayed which are taken up by mitochondria in vivo or left in the cytoplasm. It can be seen that all variant forms are imported into mitochondria although with variable efficiencies.

Fig. 2 reflects results obtained with variants affecting the extra loop structure, the most obvious difference between short and long isozymes. A protein product with an electrophoretic mobility similar to the wild type Aky2p (24 kDa) or slightly smaller in the deletion mutants is imported into mitochondria in all cases. The import efficiency is comparable in the wild-type and in the mutants. The unusual differences in electrophoretic mobilities of the deletion mutant proteins may be due to the loss of one and two positive charges in aky2-6p and aky2-7p, respectively, in comparison to aky2-5p. As a control, the *AKY2*-disrupted strain, DL1-D16, fails to display any cross-reacting material either in the cytoplasm or in mitochondria (not shown here, see [7]) confirming that the serum is specific and does not react with any other isozyme. We conclude that the extra loop present in Aky2p does not carry information essential for mitochondrial targeting of this polypeptide.

The results obtained with the other mutant proteins are displayed in Fig. 3. akyN1 carries a deletion of the 8 N-terminal amino acids. It can be seen that the amounts of akyN1p is diminished simultaneously in the cytoplasm and mitochondria relative to the wild-type (Fig. 3, compare lanes 3 and 4 to lanes 1 and 2). The diminished total steady-state concentration of this

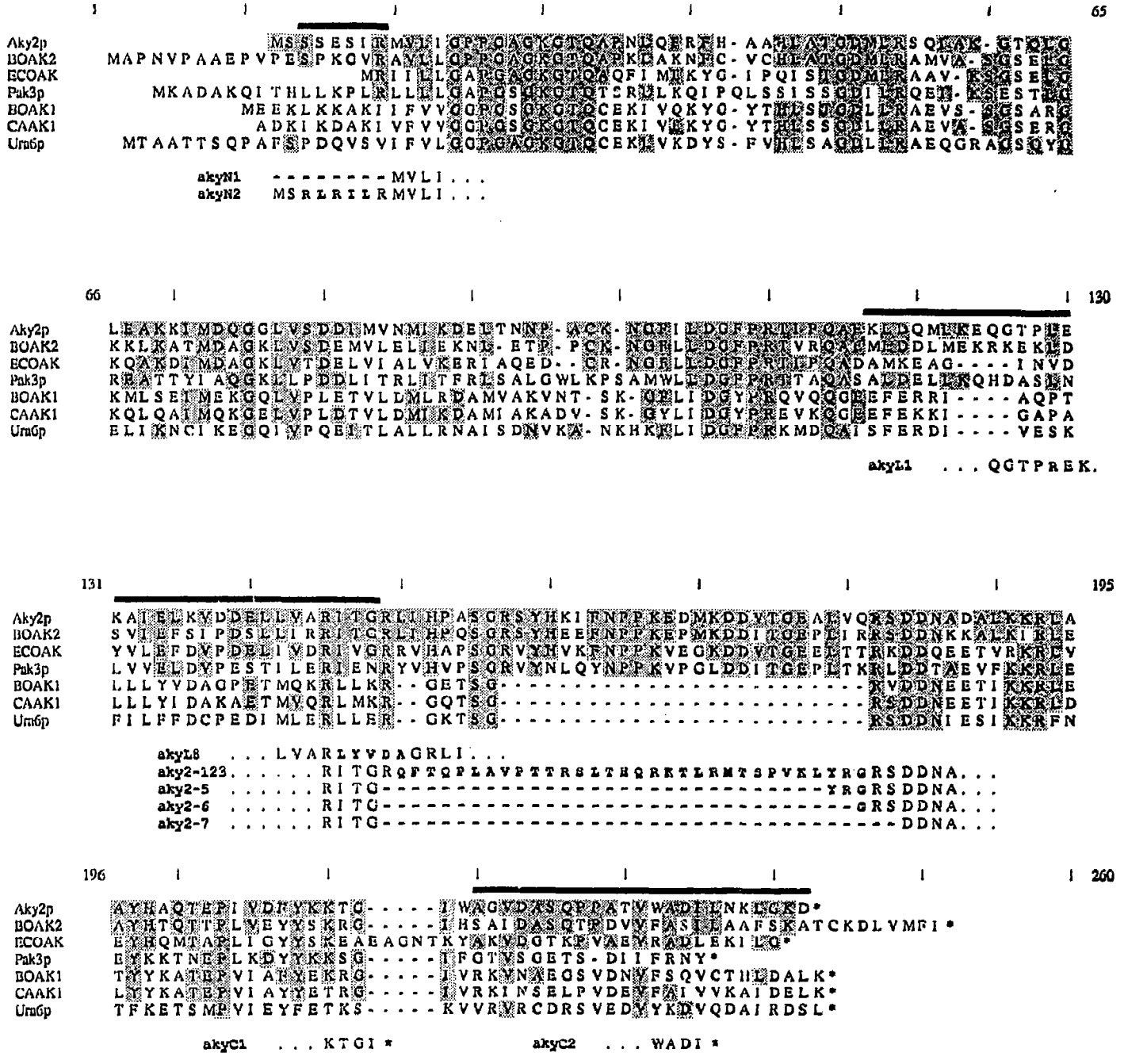

Fig. 1. Alignment of long-form mitochondrial and short-form cytoplasmic nucleoside monophosphate kinases. The alignment was made by the combination of two algorithms, ALIGN [24] and CLUSTAL [25], as described previously [26], and refined according to data on the spatial structure [21,22]. Aky2p, major adenylate kinase isozyme from yeast; Pak3p, long-form yeast mitochondrial isozyme with unknown substrate specificity [27]; BOAK1, bovine AK1; BOAK2, bovine AK2; ECOAK, *E. coli* adenylate kinase; CAAK1, cytosolic carp AK1; Ura6p, ATP:UMP phosphotransferase from yeast. The sequences were taken from previous publications [1,26,27]. Identical amino acids at homologous positions as in Aky2p are shaded. Candidate sequences which may harbour mitochondrial targeting information due to divergence between short and long isozymes are marked by a bar above the sequences. The extra loop is not particularly emphasized. Mutations are indicated below the sequence alignment by smaller sized single letter code; dashes symbolize deleted amino acid residues.

mutant protein could depend on one of the following reasons: (i) translation of the protein is less efficient due to the fact that the context of the akyN1 initiation codon deviates from the consensus in contrast to the wild-type situation; or (ii) the product is less stable. Anyway, it is evident that the N1-polypeptide is still imported into mitochondria although with very low efficiency. The result does not change after proteolytic digestion of the mitochondria (30 min, 25 μg/ml proteinase K, room temperature) or further purification

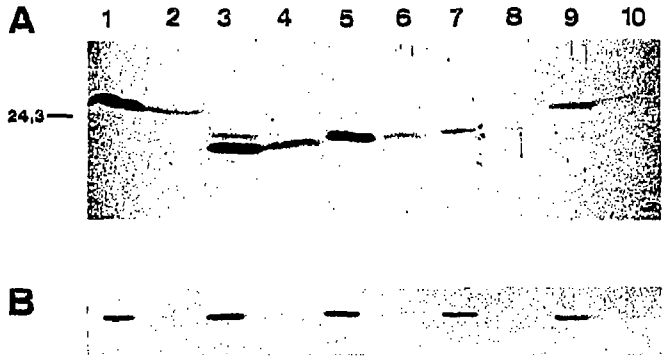

Fig. 2. Import into mitochondria of adenylate kinase mutant proteins altered in the extra loop. Cytoplasmic (odd-numbered lanes) and mitochondrial fractions (even-numbered lanes) were separated electrophoretically, electroblotted onto nitrocellulose and reacted with polyclonal antisera raised against yeast mitochondrial adenylate kinase (A) and against hexokinase as an indicator of cytoplasmic contaminations (B) and decorated with peroxidase-coupled protein A. (A and B were derived from two different gels.) Peroxidase was detected by chloronaphthol oxidation. Gradient-purified mitochondria and cytoplasmic supernatant fractions were prepared from multicopy wild-type transformant McAKY2 (lanes 1 and 2), and mutant strains aky2-7 (lanes 3 and 4), aky2-6 (lanes 5 and 6), aky2-5 (lanes 7 and 8), and aky2-123 (lanes 9 and 10).

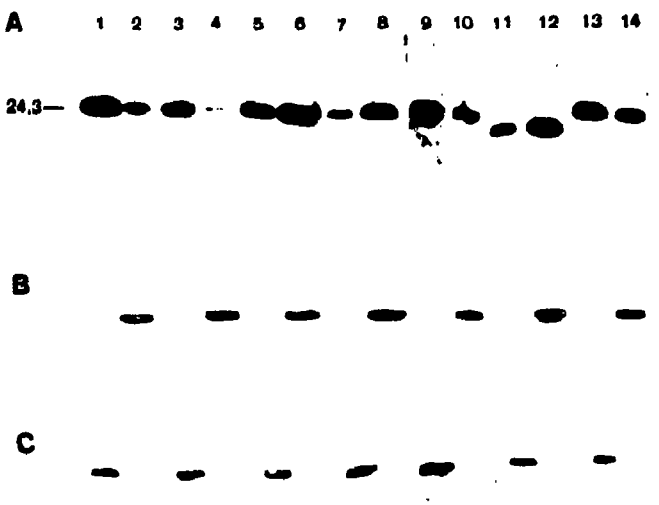

Fig. 3. Import into mitochondria of adenylate kinase mutant proteins altered in N-terminal, C-terminal and central sequences. The primary antibody was decorated with a goat anti-rabbit secondary antiserum coupled to peroxidase and assayed by the luminescence due to luminol oxidation in the presence of hydrogen peroxide. Gradient-purified mitochondria (even-numbered lanes) and cytoplasmic supernatant fractions (odd-numbered lanes) were prepared from multicopy DL1-D16 transformant McAKY2-16 (lanes 1 and 2), and the mutant strains akyN1 (lanes 3 and 4), akyN2 (lanes 5 and 6), akyL1 (lanes 7 and 8), akyL8 (lanes 9 and 10), akyC1 (lanes 11 and 12), and akyC2 (lanes 13 and 14). The same blot was reacted (A) with Aky2p-specific serum, (B) with a serum directed against citrate synthase and (C) with a serum directed against hexokinase.

by another Percoll gradient. These results indicate that the N-terminal sequence of adenylate kinase is partially responsible for import into mitochondria. In addition, other sequences must contain mitochondrial targeting information, since the N-terminally truncated protein still traverses the mitochondrial membrane. akyN2 has an altered amino acid sequence in the interval from residues 3–7 (Fig. 1). This change eliminates a negative charge (known to impede import), introduces two additional positive charges (likely to be favorable for import [28]) and leads to a slightly more hydrophobic sequence. The overall helical hydrophobic moment, however, a measure of amphipathicity [29], is even smaller than in the wild type N-terminal sequence. Lanes 5 and 6 of Fig. 3 show that most of this protein is imported into mitochondria and only little is left in the cytosol, indicating that the relative import efficiency of adenylate kinase can be improved compared to the wild-type by a change in the N-terminal sequence. An enhanced hydrophobic moment appears not to be a prerequisite for this increased efficiency.

Mutants akyL1 and akyL8 are affected in the central part of the protein which is least conserved when long and short isoforms are compared. These sequences diverge strongly also within the group of long-form adenylate kinases. Mutant akyL1 protein is imported into mitochondria slightly better than the wild-type. This may be deduced from a comparison of lanes 8 and 2 in Fig. 3A and B. Fig. 3B shows that the concentrations of mitochondrial protein do not differ much in the two lanes, but still, in Fig. 3A, the signal is significantly stronger in lane 8 (akyL1) than in lane 2 (wild-type). Conversely, a comparison of lanes 1 and 7 in Fig. 3A and C suggests a decrease in the cytoplasmic steady-state concentration of the mutant protein relative to the wild-type, possibly due to diminished proteolytic stability in this compartment. Apparently, the protein is exposed to proteolysis only in the cytoplasm (lane 7) but not in mitochondria so that the relative import efficiency of this protein appears to be increased. However, it should be considered that mitochondrial import shelters the protein from degradation in the cytoplasm. akyL8 seems not to be proteolytically affected, and its import into mitochondria does not significantly differ from the wild-type (lanes 9 and 10).

The results obtained with the C-terminal truncation, akyC1, demonstrate that elimination of the terminal 24 amino acids dramatically decreases stability of the polypeptide in the cytoplasm. Prolonged preparation times or absence of protease inhibitors during the preparation lead to proteolytic degradation exclusively in the cytoplasm. The same tendency, although much less pronounced, can be observed with akyC2 which is shortened by 7 amino acids as compared to the wild-type. This suggests that the C-terminus contains structural information for the stability and proteolytic resistance of the protein. Consistent with this finding it has been observed in the short isozyme AK1 from chicken that any alteration of Leu^{115}, the fourth last amino acid, leads to an unstable protein and an increased K_m value

for AMP [30]. Surprisingly, import of akyC1 mutant protein into mitochondria is improved compared to the wild-type (Fig. 3, lanes 11 and 12). It even appears that import rescues the mutant polypeptide from proteolytic degradation in the cytoplasm. Apparently, the truncation of the C-terminus deletes a determinant impeding mitochondrial import. The sensitivity of the truncated polypeptide to proteolysis by intrinsic proteases suggests that the determinant which hinders import is constituted by a rigid tertiary structure and/or rapid folding kinetics, as indicated by the extreme resistence to protease treatments of the wild-type protein (not shown here). Import of akyC2p is not significantly improved compared to the wild-type (lanes 13 and 14).

4. DISCUSSION

A cleavable presequence is dispensable for polypeptides to reach intramitochondrial destinations. The long-form adenylate kinase from vertebrates, AK2, was found to reside exclusively in the mitochondrial intermembrane space and to lack a cleavable mitochondrial target sequence [31,32]. In yeast, the N-terminus of the Aky2p wild-type protein is shortened post-translationally by two amino acids, and blocked by acetylation [5,6]. The modifications are likely to occur in the cytoplasm, unlinked to mitochondrial import. No evidence for extensive processing of adenylate kinase has been found in this organism either [7]. The N-terminus can be folded into a short amphipathic helix (T.A. Link, personal communication) and may play a role in mitochondrial targeting and import. Since the akyN1 mutant protein, which is truncated at the N-terminus by a total of 8 amino acids, is imported into mitochondria although with diminished efficiency, we conclude that N-terminal sequences play a role in, but do not carry the complete, targeting information. Additional determinant(s) are presumably encoded in the interior of the protein. To a certain extent, the situation resembles the one observed with subunit IV of cytochrome *c* oxidase [33] and *δ*-aminolevulinate synthetase [34]. Although these two precursor polypeptides possess cleavable presequences, it has been shown that they contain additional, internally encoded targeting or intra-mitochondrial sorting information. The data reported here on the variation of the extra loop, the most obvious difference between unimported short and mitochondrially located long versions, demonstrate that this structure lacks important information to direct adenylate kinase into the mitochondrial intermembrane space. Also the surface-exposed helix, which N-terminally borders the loop structure, as well as the C-terminus, do not contain the address for mitochondrial import. Surprisingly, mutant akyL1 and akyC1 proteins display better relative import efficiencies than the wild-type, suggesting that a combination of sequence-inherent determinants and other parameters influence import. Most plausibly the folding velocity and/or the stability of the protein's tertiary structure are the type of determinants which might counteract import and thereby determine the equilibrium between the mitochondrial and cytoplasmic locations of adenylate kinase in yeast cells. It may be taken as evidence in favour of this view that proteins, which are less stable in the cytoplasm, can accumulate in mitochondria. This implies (i) that their uptake is facilitated possibly by retarded folding kinetics of the nascent polypeptide chains, (ii) that, after their synthesis, they do not equilibrate with the cytoplasmic precursor pool and (iii) that their import is irreversible.

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