Redundant Mitochondrial Targeting Signals in Yeast Adenylate Kinase*

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† The abbreviations used are: AK, adenylate kinase; Adk1p/Aky2p, yeast major adenylate kinase; AKY2, gene encoding Adk1p/Aky2p; Ura6p, yeast UMP-kinase; URA6, gene encoding Ura6p; Aky3p, yeast GTP:AMP phosphotransferase of the mitochondrial matrix; AKY3, gene encoding Aky3p; DHFR, mouse dihydrofolate reductase; IMS, mitochondrial intermembrane space; FN, family numbering; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

Running title: Two-partite mitochondrial targeting address
SUMMARY

Yeast adenylate kinase (Aky2p, Adk1p) occurs simultaneously in cytoplasm and mitochondrial intermembrane space. It has no cleavable mitochondrial targeting sequence, and the signal for mitochondrial import and submitochondrial sorting is largely unknown. The extreme amino terminus of Aky2p is able to direct cytoplasmic passengers to mitochondria. However, an Aky2 mutant lacking this sequence is imported with about the same efficiency as wildtype. To identify possible import relevant information in the interior, parts of Aky2p have been exchanged by homologous in vitro recombination for the respective segments of the purely cytoplasmic isozyme, Ura6p. Import studies revealed an internal region of about 40 amino acids which was sufficient to direct the chimera to mitochondria, but not for correct submitochondrial sorting. The respective Ura6p hybrid was arrested in the mitochondrial membrane at a position where it was inaccessible to protease, but released by alkaline extraction suggesting that it had entered an import channel and passed the initial steps of recognition and uptake. Site specific mutations within the presumptive address-specifying segment identified the amphipathic helix 5. A Ura6 mutant protein in which helix 5 had been replaced with the respective sequence from Aky2p was imported, and this address sequence cooperates with the N-terminus in the respective double mutant in a synergistic fashion.
INTRODUCTION

Import of most proteins into mitochondria depends on presequences which play important roles in the recognition by the translocation machineries of the outer (TOM complex) and inner membranes (TIM complex) (1-3). Usually, presequences are 15-40 amino acids long and share some structural similarities: they have positive surplus charges, display a pronounced tendency to form amphipathic \( \alpha \)-helices (4, 5) and are removed upon entry by the matrix processing peptidase (1). In the steady state, cytoplasmic precursor pools usually are very small (6-8). Adenylate kinase (Adk1/Aky2p, named Aky2p hereafter, 9) deviates from this general scheme in important aspects: i) Aky2p occurs at two subcellular locations, cytoplasm and mitochondrial intermembrane space (IMS), and most of Aky2p is excluded from entry into mitochondria. ii) It lacks a cleavable presequence; at both locations, Aky2p is derived from the same open reading frame and is identical with respect to primary structure and posttranslational modifications (10, 11). iii) The N-terminal peptide which carries import information is short (7-8 amino acids), electrically neutral and displays a very low hydrophobic \( \alpha \)-helical moment (12).

Adenylate kinases constitute a family of small, abundant, soluble enzymes which serve essential housekeeping functions in energy metabolism of all organisms (13, 14). They have very similar folding structures (15-18) although they differ in length (19) and subcellular distribution (20-22). They can be grouped into three subtypes. AK1\(^1\) (myokinase) is a so-called short form of about 21 kDa molecular mass which is the major species in muscle cells of vertebrates and occurs exclusively in the cytoplasm (20). Ura6p, a UMP/CMP/AMP kinase, is the yeast equivalent of AK1 (23). The other two isoforms have molecular masses of approximately 25 kDa due to an internal insertion of 28 amino acids. AK3/Aky3p is a GTP:AMP phosphotransferase and resides in the mitochondrial matrix (22-25), whereas Aky2p, the major isoform in yeast is found in both, cytoplasm (about 90% of the total adenylate kinase present in a yeast cell homogenate) and mitochondrial IMS (6-8% of total) (12). The small fraction imported plays a prominent role in oxidative metabolism (23) presumably by facilitating ATP/ADP exchange across the inner mitochondrial membrane (12).
The mechanism of mitochondrial targeting and uptake of Aky2p is largely unknown. Recently, it has been reported that, despite its physical properties which are highly unfavorable for import, the extreme N-terminus of Aky2p has the ability to target cytoplasmic passengers, i.e. murine DHFR or Ura6p from yeast, to mitochondrial locations (26). However, the N-terminal region of Aky2p tolerates large sequence variations without abolishing a basal rate of import arguing that it is not the only topogenic sequence in Aky2p and hinting to the presence of additional signal(s) for mitochondrial import. In order to identify internal import information, we constructed chimeras of Aky2p and the purely cytoplasmic isoform, Ura6p, as well as mutant Aky2 proteins affected in the presumptive target sequence, and determined the steady state distribution of hybrid and mutant Aky2 proteins between cytoplasm and mitochondria.
EXPERIMENTAL PROCEDURES

Yeast Strains, Cell Growth Conditions, Preparation and Subfractionation of Mitochondria - The AKY2-disrupted yeast strains DL1-D16 aky2::LEU2 (23) or WCG4 aky2::LEU2 (this work) served as recipients for all mutant constructs in transformations using multi-copy yeast shuttle plasmids. Yeast cells were grown on semisynthetic medium supplemented according to the auxotrophic requirements and 3% lactate or galactose as carbon source. Spheroplasts were prepared from mid-logarithmic cultures (1-2x10^7 cells/ml) and lysed in 0.6 M mannitol in the presence of a mix of protease inhibitors. After discarding whole cells, debris and nuclei (2x3,000g, 5 min), mitochondria were prepared from the 10,000g pellet, washed, further purified by Percoll (28%) gradient centrifugation and subfractionated into intermembrane space, matrix and inner plus outer membranes as described earlier (27). The 10,000g supernatant was used as the cytoplasmic fraction. Aliquots containing equal amounts of protein (usually 15 µg per slot) were immediately mixed with gel loading buffer and used for SDS-PAGE. Wildtype or hybrid proteins were detected using a mixture of anti-Aky2p and anti-Ura6p antisera and the chloronaphthol/peroxidase system for visualization.

Construction of AKY2 Mutants and Aky2/Ura6 Fusion Proteins. - The genes AKY2 and URA6 were divided into four structurally homologous segments each (Fig. 1A): Segment 1, N-terminus (family numbering (FN) pos. 2 in Ura6p or pos. 12 FN in Aky2p to pos. 32 FN; for FN see ref. 28); segment 2 (pos 33-108 FN), segment 3 (pos. 109-185 FN) and segment 4 (C-terminus, pos. 186 to pos. 242 (Aky2p) or pos. 243 (Ura6p) FN). The three junction sites lie within conserved amino acid sequences (Fig. 1A). Fusions of homologous segments were achieved by SOE PCR (29). All fusion constructs were ligated as XbaI/NcoI fragments to the AKY2 expression cassette of vector pEX (24) using the promoter proximal SpeI restriction site and the NcoI site introduced immediately 3′ of the termination codon. The cassette comprising the promoter, the context of the translational initiation triplet and the terminator from AKY2 together with the respective hybrid gene replacing the AKY2-coding sequence was subsequently ligated to YEp352 (URA3) (30). Fusion constructs were named systematically, according to the segments originating from either AKY2 (A) or URA6 (U) (Fig. 1A).
As a first step to build the fusion genes **UAUU, UUAU** and **AUUA**, the two reciprocal hybrid genes **UUAA** and **AAUU** were constructed by fusing the respective segments of **AKY2** and **URA6** within the central DGFRP consensus motif (pos. 106-110, Fig. 1A). **UAUU** was built from genes **UAAA** and **AAUU** by replacing the second two segments of **UAAA**, encoded on a BgIII/NcoI fragment, by the homologous 556 bp BgIII/NcoI fragment of **AAUU**. The resulting protein UAUUp is a Ura6p derivative containing Aky2p sequences between pos. 33-108 FN. Fusion gene **UUAU** was made by exchanging a 400 bp C-terminal HindIII fragment of pUUAA containing the 3'-noncoding region of **AKY2** and the HindIII site of the pUC19 multiple cloning site with a homologous PCR fragment from pURA6 containing the promoter from **AKY2** (23). The internal HindIII was introduced into **URA6** via PCR 6 amino acids N-terminal of the conserved DDN sequence (pos. 184-186 FN). Fusion gene **AUUA** was constructed by replacing the 509 bp SpeI/NcoI fragment of pAUUU by the corresponding 518 bp SpeI/NcoI fragment of pUUUA. Mutants Aky-imp1, Aky-imp3, Aky-P3 and URA6-h5 as well as Aky-LD3 were constructed by use of site-specific in vitro mutagenesis (28, 31). The sequence of the mutagenic primer imp1 (5'-'CCAAAGGGGTACCTTTATTGTCCAATTTTTC-3') covers the region between pos. 120-125 (FN) of Aky2p (Fig. 1B) and replaces the amino acid sequence QMLKEQ by NK (Fig. 1B), thereby introducing a net deletion of 4 amino acids to approximate the sequence to that of the short isoforms. In mutant Aky-P3, the loop motif C-terminal of helix 5, GTP, was changed into TSN by primer 5'-CCAAATGTTGAAAGAACAAACGTCCAACTTGGAAAAAGCCATC-3'. In mutant Aky-imp3, the sequence from pos. 94-98 was adjusted to the one observed in short isozymes (BOAK1) using mutagenic primer 5'-CCAAGATGAACCCTTTCG-AAGTGTCGACATTGGTGTC-3'. Aky-LD3 was designed as a substitution of the long isozyme-specific large loop sequence (pos. 150-181, FN) by the short isozyme-specific consensus sequence (GDTSG) resulting in a net deletion of 28 amino by use of primer 5'-GGCGTCTGCATCAAATCTGGCTCACTCAGTCTCTCTTCCCG-3' (Fig. 1C). In URA6-h5, helix 5 (sequence PRKMDQAI$FERD$IVESK) was replaced by the AKY2/ AK2-derived sequence PRNIPQA$EKLDQMLEK$RKVESK (helical residues given in italics) (primer 5'-CTTAAATTGACGGATTTCTCAGAGAATATCCCTCAAAGCTGAAAAGCTAGATCAGCTGATTGAGAAACGTAAAGTTGAAAGCAAATTCATCCTGTTCGTC-3'). AURA6-h5 was constructed by using the same primer as for URA6-h5 and construct AUUU as the template. In mutant AKY-N1 the ATG translational initiation codon was changed to ATT so that the second Met triplet...
(pos. 7 of the mature protein) was used for initiation of translation. All constructs, including
URA6 (UUUU), were expressed under the control of the AKY2 promoter including the authentic context of the translational start site (except AKY-N1).

Raising Anti-Ura6p Antibodies in Chicken- Antibodies against yeast Ura6p and Aky2p were raised in chicken using purified hexa-His-tagged yeast Ura6p or Aky2p, respectively, as the antigen. Recombinant protein was expressed in E. coli under the bacterial tac fusion promoter from plasmid pQE8 (Qiagen, Hilden) and purified from bacterial lysates by one-step Ni²⁺-NTA column chromatography according to the protocol supplied by the manufacturer. Two chicken were immunized (32) with about 80 µg or 120 µg, respectively, of the homogenous recombinant protein per injection using complete Freund´s adjuvant. Two boost injections using the same amount of protein were performed four and eight weeks after the first immunization. The polyclonal antibodies were prepared from the egg yolks by dextran sulfate precipitation (33).

Miscellaneous Procedures- Published procedures were used to determine adenylate kinase activity (12), for determination of protein concentrations (34), for Western blotting and immunodecoration and all other molecular procedures (35). Helical wheel projections were calculated with the help of the DNA STAR program. Secondary structure predictions were based on the algorithm of Chou and Fasman (36) using the Wisconsin Sequence Analysis Package (Genetics Comp. Group, Madison, Wi., U.S.A.) or by using the secondary structure prediction algorithm offered by the PHD server (www.embl-heidelberg.de/predictprotein; 37). Sequences were screened for surface exposition and flexibility using the algorithm described by Margalit et al. (38) with alternative window sizes of 6 and 10 amino acids. The hydrophobic α-helical moment was calculated applying the equation developed by Eisenberg et al. (39).
RESULTS

The Amino Terminus of Aky2 is not Required for Mitochondrial Targeting- The N-terminus of Aky2p has been shown to contain mitochondrial target information that is sufficient to direct heterologous passengers to the correct sub mitochondrial compartment, the IMS (26). To test whether additional internal import information is present in Aky2p, the N-terminus was either deleted (AKY-N1) or replaced with the N-terminus of the exclusively cytoplasmic isozyme, Ura6p (UAAA). Cell lysates were fractionated into cytoplasm (amounting to about 50% of total homogenate proteins; ref. 12) and purified mitochondria (about 10% of total proteins). Aky2p and marker proteins (hexokinase for the cytoplasm, cytochrome c1 for mitochondrial membranes and Hsp60 for the mitochondrial matrix were used as loading control and to indicate mutual contaminations) were assayed by Western blotting (Fig. 2A). Equal amounts of protein have been applied per slot so that mitochondrial proteins are about fivefold enriched relative to cytoplasm. Only about 10% of total Aky2p (25 kDa) is imported into mitochondria in vivo in the steady state in agreement with previous reports (compare Aky2p mitochondrial (m) and cytoplasmic (c) fractions, positive control), whereas Ura6p (21 kDa) is cytoplasmic (Ura6p, m, negative control). No Ura6p-specific signal is also seen in the AKY2-disrupted recipient strain DL1-D16 (not shown) or in the multicopy AKY2 wildtype transformant (Aky2p, c), since the concentration of Ura6p expressed from the wildtype URA6 gene is too low for detection. Both Aky2 mutant proteins, UAAAp or AKY-N1p, are imported. Subfractionation of AKY-N1 mitochondria shows that AKY-N1p is correctly sorted to the IMS (i in Fig. 2A; IMS proteins amount to about 15% of whole mitochondria). Thus, internal regions of Aky2p are sufficient to direct the hybrid proteins to mitochondria.

Identification of an Internal Signal Sequence- To identify internal targeting information in Aky2p, AKY2 and URA6 were each divided into four homologous segments (see Experimental Procedures, Fig. 1A), and a series of constructs was made in which one or more segments of Ura6p was exchanged for their Aky2p counterparts by homologous in vitro recombination (i.e., AAUU, UUAA, UUAU, UAUU, AUUA and UUUA (the latter fusion protein is not shown in Fig. 2B). The difference in length between short and long isoforms of nucleoside monophosphate
kinases mainly resides in the variable loop contained in segment 3, which is shorter by 28 amino acids in the short versions (i. e. in Ura6p and all constructs carrying segment 3 from Ura6p).

All chimeras and Ura6p were expressed from the AKY2 promoter in the context of the translational start triplet of Aky2p (shaded in Fig. 1A) to warrant identical expression. The cytoplasmic concentration of some chimeras, however, was low to undetectable (AAUUAp, UUAUUAp and AUUAp. In these cases, total adenylate kinase present in cytoplasm plus mitochondria is considerably lower than wildtype (e. g. about 10% in AAUU), indicating proteolytic instability in the cytoplasm. Nevertheless, all constructs were taken up by mitochondria suggesting that each of the constructs harbors import information. The uptake efficiencies vary, however (Fig. 2B). In detail, fusion proteins containing either half of Aky2p were both targeted to mitochondria to about the same extent as wildtype (AAUUAp, UUAAP) indicating that both the N- and the C-terminal halves of the Aky2 molecule harbor mitochondrial targeting information which suffices as import address. Among the constructs containing a single segment of Aky2p, only UUAAP was imported efficiently, pointing to segment 3 as the carrier of a major internal mitochondrial target signal. The remaining three constructs, UAUUP, AUUAp and UUUAP (latter not shown), were imported poorly. The extremely low efficiency of import of UUUAP (not shown) suggested that the C-terminal region of Aky2p did not carry sequences relevant to mitochondrial import. This conclusion agrees with the observation that a deletion mutant missing the last 24 amino acid residues of Aky2p showed an import efficiency which was even increased compared to wildtype protein (40).

In an independent approach to identify candidate internal mitochondrial targeting information of Aky2p, we screened the amino acid sequence for surface-exposed regions (38) which differed between members of the 25 kDa mitochondrial subfamily and cytoplasmic 21 kDa isozymes. One obvious divergence is constituted by the so-called "extra loop", a surface-exposed structure forming a β-meander in the long-form enzymes which is absent from the short versions (15). Additionally, the region following the DGFPR consensus motif (AMP-binding motif), comprising helix 5 (FN 111-125), was identified as a candidate. Both motifs are contained in segment 3. They were tested individually for the presence of mitochondrial target information in order to narrow down the region containing the targeting element in question.
In mutant Aky-LD3, the surface-exposed variable loop was exactly replaced by the respective loop of the short isoforms on the basis of primary structure alignments taking into account recent X-ray crystallographic data (15, 16) to achieve the exact excision and replacement of the loop sequence on a structural basis (Fig. 1C; pos. 150-181 FN). Aky-LD3 mutant protein is imported into mitochondria with about the same efficiency as wildtype (Fig. 3A, Aky-LD3 c and m) ruling out the suggestive involvement of the variable loop in mitochondrial targeting and leaving helix 5 as the prime candidate.

In Aky2p (and other long-form AKs), helix 5 is more amphipathic (Fig. 4, left panel), more surface-exposed and four amino acids longer than in the short versions (thirteen helical residues in BOAK2 and Aky2p compared to nine in BOAK1 and Ura6p). To check whether helix 5 carries import-relevant information, several mutations were established at or near the region of heterology (Fig. 1B). Unfortunately, those mutants that affected helix 5 directly (by deleting the whole structure or by changing the number of charges, the propensity of helix formation, or the amphiphilic $\alpha$-helical moment,) were highly unstable so that, even in a proteasome-negative background ($\text{pre}1\text{ pre}2$) or by rapid preparation of cellular proteins, mutant adenylate kinase was barely detectable (not shown). Two types of stable mutants could be obtained: the one affected the helix-flanking regions (Aky-imp1, Aky-imp3 and Aky-P3). The other was, in a complementary approach, a $\text{URA6}$ mutant, URA6-h5, in which helix 5 was replaced with an Aky2/BOAK2-derived helix 5.

Amino acid residues C-terminally adjacent to helix 5 are altered by mutations Aky-imp1 and Aky-P3 (Fig. 1B). Mutant Aky-imp1 deletes 4 $\alpha$-helical amino acids which are absent from short isoforms and thus adjusts the amino acid sequence of Aky2p to the short-form cytoplasmic isozymes in this respect. According to the algorithm of Chou and Fasman (36) the propensity of helix formation extends beyond helix 5 in long-form isozymes by about one helical turn (see refs. 15, 16). Mutation Aky-P3 immediately follows the Aky-imp1 mutational site on the C-terminal side. It contains a change of pos. 126-128 (FN) including Pro128 (Fig. 1B). Aky-imp3 is mutated in the most heterologous part of segment 2 to make it resemble the sequence of the short isozymes in this region. This stretch lies in the vicinity of the imp1 and P3 sites in the folded protein (15), but is contained in segment 2 rather than in segment 3 (Fig. 1B). The amounts of
Aky-imp1p and Aky-P3p found in mitochondria is not significantly altered compared to wildtype (Fig. 3A) suggesting that these two mutations do not affect sequences relevant to import. As expected from results obtained with the UAUU fusion protein, also mutant Aky-imp3 does not show a significant change of cytoplasmic/mitochondrial distribution.

**Helix 5 Contains Import Information**- Helix 5 of Aky2p is amphipathic and comprises 13 amino acids (Fig. 4, left part), whereas the respective structure from Ura6p is significantly less amphipathic and four amino acids shorter (Fig. 4, center). Since AKY2 mutants directly affecting helix 5 were proteolytically unstable in vivo, we exchanged helix 5 in the exclusively cytoplasmic short-form isoprotein Ura6p for the corresponding sequence derived from what was considered the optimization of helix 5 from both, Aky2p and BOAK2 (Experimental Procedures, Fig. 1C and Fig. 4, right panel). This hybrid protein, URA6-h5p, not only is relatively stable in the cytoplasm (although it is evidently degraded to some extent), but is also imported into mitochondria with at least the same efficiency as Aky2p (Fig. 3B) (the band in the cytoplasmic fraction exhibiting slightly higher mobility likely represents a crossreacting protein that can serve as loading control).

The crystal structure of yeast adenylate kinase (15) shows that helix 5 and the N-terminus are in close proximity. This raises the possibility that the import specificity of Aky2p may be determined by the interaction of helix 5 with the N-terminal peptide of Aky2p. Hairpin interactions between the N-terminus and an internal amphipathic helix have been found to occur and to be essential for import of the Bcs1 protein (41) which, like Aky2p, is taken up into the IMS without a cleavable presequence. In addition to helix 5, also the N-terminus of Aky2p has mitochondrial target information: Although construct AUUU is highly unstable in the cytoplasm (Fig. 3B), it is evident that some material is rescued by uptake into mitochondria. To test the hypothesis that helix 5 and the N-terminus of Aky2p cooperate, the double mutant AUra6-h5 was constructed which contained both, an Aky2-derived helix 5 and the N-terminal segment from Aky2p. The efficiency of import into mitochondria of AUra6-h5p is significantly increased relative to the one observed with either Ura6-h5 or AUUUp, indicating a synergism with respect to uptake efficiency.

**Submitochondrial Distribution of Aky2 Mutant Proteins**- To check the importance for submitochondrial sorting of the sequences identified above as carrying candidate import
information, mitochondrial subfractionations of the mutations-carrying strains were performed. Gradient purified mitochondria of transformant strains AKY2 (wildtype), UAAA, UUAU, Aky-LD3, Aky-imp3, Aky-imp1, URA6-h5, and Aky-P3 were sonicated and fractionated into membranes (p, containing inner + outer membranes) and supernatant (s, matrix+IMS) (Fig. 5).

In mitochondria, Aky2p is exclusively found in the IMS (12, 26). In mutants Aky-LD3p, Aky-imp3p and Aky-P3p, which have the wildtype N-terminus and helix 5, submitochondrial sorting is not visibly affected compared to wildtype Aky2p: all of the immunodetectable material is found in the soluble mitochondrial fraction. Aky-imp1p, in which helix 5 is shortened by four amino acids - although partially active - and those hybrid constructs which lack the N-terminus of Aky2p, i.e. UAAAp, UUAUp and Ura6-h5p - which are directed to mitochondria highly efficiently - are found partly (Aky-imp1p and UAAA) or entirely (UUAU and Ura6-h5) in the membranous fraction.

Membrane-associated Aky2 proteins might have merely bound to surface receptors and then be prevented from entering the general insertion pore (TOM complex) by premature tight folding of the C-terminal domain on the cytoplasmic side or it could have entered a transport channel and have been arrested as an transport intermediate. To discriminate between these two possibilities, a series of additional experiments was performed. Loosely attached proteins would be accessible to protease in intact mitochondria or mitoplasts and readily extractable into the supernatant by carbonate.

Gradient purified mitochondria from the three transformants in which the Aky2 mutant proteins were found membrane associated (UAAA, UUAU, and URA6-h5) and control mitochondria (wildtype and mutant Aky-P3) were extracted with alkali (Fig. 6A) or treated with protease under isotonic or hypotonic conditions or in the presence of detergent (Fig. 6B). As controls for treatment conditions, a number of compartment-specific marker proteins were tested.

Aky2 proteins, released to the soluble supernatant fraction by sonication, were solubilized also by treatment with alkali (Fig. 6A, wildtype Aky2p and mutant Aky-P3p; s, soluble fraction). Also the membrane arrested import intermediate of UUAUp was completely extractable. On the other hand, UAAAp and URA6-h5 were extracted to about 50%. Thus, they rather behave as membrane proteins and are not correctly sorted. (The CCPO marker indicates some slight losses in the
supernatant fractions of UAAAp, UUAUp and Aky-P3p, whereas the Cyt1p marker indicates some loss of the Ura6-h5p pellet fraction leading to modest underrepresentation of the respective adenylate kinase proteins in these fractions).

To verify that the membrane associated Aky2 mutant proteins have entered the import pathway and are arrested as import intermediates, sensitivity to protease of the material imported in vivo in the steady state was examined. Proteins superficially attached to the mitochondrial surface would be accessible and readily degraded by protease, whereas arrested import intermediates usually are resistant. Under isotonic conditions (with the outer mitochondrial membrane intact), all Aky2 proteins are inaccessible to protease (Fig. 6B). Wildtype Aky2p and the fully soluble Aky-P3 mutant protein (the latter not shown) become accessible as soon as the outer membrane is disrupted under slightly hypotonic conditions. Unfortunately, Aky2 wildtype and mutant proteins are unusually resistant to proteases even at RT and concentrations of 50 µg/ml proteinase K so that a faint band of undegraded material is found in some experiments even after lysis with 1% Triton X-100. IMS location arrest as import intermediates of Aky2 and mutant proteins is further corroborated by the sensitivity to this treatment also of the IMS marker proteins, cytochrome b2 or cytochrome c1, which latter is located on the outer surface of the inner mitochondrial membrane with most of the molecule (apart from the C-terminus) exposed to the IMS (the latter not shown). The matrix marker, citrate synthetase, is resistant under these conditions. Taken together, these results suggest that the membrane associated Aky2 mutant proteins, UUAUp and Ura6-h5p, have entered an import pathway and are arrested within the import channel at a stage where they are resistant to extrinsic protease.

**Functional Complementation of Aky2 Mutant Proteins**- To obtain an indication to which extent the above aky2 mutations affect tertiary structure, all mutants were assayed for enzymatic activity in vivo by testing complementation of the Pet+ phenotype of the AKY2-deleted recipient strain DL1-D16 (Fig. 7). Transformants of the AKY2-disrupted mutant DL1-D16 were streaked onto media containing either glucose or glycerol as carbon sources. While growth on glucose-containing medium is largely unimpaired in all transformants (left plate), growth on glycerol could be supported only by three constructs, namely Aky-imp1 (sector 2), Aky-imp3 (sector 4) and Aky-LD3 (sector 1) indicating that these mutant proteins were enzymatically active, and the alteration
did not affect the tertiary structure to a great extent. This is remarkable in particular in the case of the loop deletion construct, Aky-LD3, in which 33 amino acids of Aky2p had been replaced by 5 residues which were similar to those present in this loop in bovine AK1 or yeast Ura6p (Fig. 1C). This deletion mutant protein is functional and retains significant enzymatic activity (approximately 17% wt, not shown) which is sufficient to complement adenylate kinase deficiency in yeast (Fig. 7, sector 1) and *E. coli* (*adk1*-1<sup>ts</sup>, not shown). The Pro128 (FN) mutation in Aky-P3 (sector 5) results in a marginally active protein whereas those strains containing the URA6-h5 (sector 3) mutation or the *UAAA* hybrid gene (sector 6) are completely unable to promote growth of the *AKY2* deleted strain DL1-D16 on non-fermentable carbon sources. This indicates a major defect in folding of these mutant proteins. The double mutant strain AURA6-h5, containing the N-terminus and helix 5 of Aky2p, is enzymatically inactive and does not complement (not shown). Significantly, those mutants that fold into an enzymatically active conformation and complement, are also correctly sorted to the IMS in line with the assumption that folding of Aky2p provides the driving force for membrane traverson.
DISCUSSION

The majority of mitochondrial proteins is nuclear-encoded and posttranslationally imported into mitochondria. The general concept of the initial steps of precursor import into mitochondria (3, 42-44) assumes that the precursor protein associates with cofactors in the cytoplasm which mediate contact to one of two surface receptors, either TOM19/22 or TOM72/30. Preproteins are translocated in a partially unfolded, probably linear conformation (45). Interaction of the prepeptide with the receptor, TOM19/22 in particular, is greatly facilitated by the propensity of the segment to form an amphipathic \( \alpha \)-helix (4, 46) and the presence of positive charges (47, 48). However, a growing number of proteins have been found which differ from this general scheme with respect to the classical address motif. Some of them do not depend on N-terminal mitochondrial targeting sequences such as iso-1-cytochrome \( c \) (49, 50) or contain non-cleavable N-terminal signals, e.g. the 70 kDa outer membrane protein (51) or matrix AK3/Aky3p (24, 52). Other mitochondrial proteins have internal targeting information, such as the ATP/ADP translocator (53), 5-aminolevulinate synthetase (54), the Bcs1 protein (41), or the cytochrome \( c \) and \( c_1 \) heme lyases (55-58). In Bcs1p, the heme lyases and Aky2p, these intrinsic sequences have been confined to short segments (41, 58, this work). However, apart from the presence of amphipathic \( \alpha \)-helices, no obvious sequence similarities are apparent.

Aky2p is different from all other known mitochondrial proteins with respect to import efficiency, since more than 90% of the total is excluded from import and occurs in the cytoplasm. The N-terminus of Aky2p dispenses with any of the common characteristics of mitochondrial presequences, although it definitely carries targeting information. It lacks a positive net charge, displays low amphipathicity and exhibits no recognizable propensity of \( \alpha \)-helix formation. In this view it is not surprising that import of wildtype Aky2p into the IMS is rather inefficient. Nevertheless, the 18 N-terminal residues are sufficient to direct cytoplasmic passengers into mitochondria and, as shown in case of the murine DHFR, also to the correct submitochondrial compartment, the IMS (26).

The present study shows that, in addition to the N-terminal segment, at least one major additional import signal is present in the interior of Aky2p. This is demonstrated in first line by the N-terminal mutants UAAA and AKY-N1 in which the N-terminal 7 amino acids were replaced or
deleted. Since both are imported correctly into mitochondria, redundant targeting information must be present in internal parts of this protein. To identify internal address-containing regions we used hybrid proteins of Aky2p and Ura6p. Ura6p has purely cytoplasmic location. In contrast to wildtype Ura6p, all fusion proteins tested are targeted to mitochondria, although with greatly variable efficiencies. Segment 2 of Aky2p effects a low level of import which is >10% of the rate observed with Aky2 wildtype protein. It cannot be ruled out that minor import-relevant information is located in this segment. This seems plausible especially when construct AAUU is considered, since the simultaneous presence of the N-terminus and segment 2 display a cooperative effect on import efficiency. Moreover, it cannot be rigorously be excluded that cryptic mitochondrial targeting information is present in Ura6 wt that might contribute to the import of the hybrids.

The results point to one major internal import signal located in segment 3 (pos. 111-183 FN). Insertion of this region into the Ura6p context (UUAU) increases import efficiency about 3-fold compared to wildtype Aky2p. UUAUp is unstable in the cytoplasm, probably resulting from impaired folding and subsequent proteolytic degradation, but associates with mitochondria more efficiently than wildtype. It is tempting to speculate that a reduced velocity of folding results in longer exposure of the internal import signal of mutant proteins and, thus, in increased import efficiency. This view is supported by recent observations implying that the mode of import of the bovine AK2 isozymes may be cotranslational (52) or, in yeast, loosely coupled to translation (own unpublished results).

Segment 3 contains the most pronounced differences between the cytoplasmic short isoform and the long versions which can be imported into mitochondria. About half of this segment is occupied by the so-called extra-loop, specific for long-form isozymes. Excision of the extra loop, however, does not impair import of Aky2p. The same results are obtained with mutants Aky-imp1 and Aky-P3 also ruling out the involvement in import specificity of the long isozyme-specific four-residue insertion (pos. 123-126 FN) extending helix 5.

The region around helix 5 influences submitochondrial sorting as well. After overexpression, several Aky2 mutant proteins are tightly associated with the membrane fraction, inaccessible to protease digestion and poorly extractable at alkaline pH. Similar observations with other hybrid
proteins have been interpreted previously to result from arrest in the import channel due to tight folding of the C-terminal protein domain and close spatial association with the entry site of the protein translocation complex of the outer membrane (59-63). It has also been found recently that folding of Aky2p ensues spontaneously in vitro in the absence of chaperones of the Hsp60 and Hsp70 classes (G. S. and W. B., unpublished observations). If this applies also to folding of Aky2p in the IMS, the pulling force for translocation of Aky2p could be provided by spontaneous intramitochondrial folding and would mean that folding ensues consecutively from the N- to the C-terminus. Arrest of Aky2 mutant proteins could be the consequence of impeded or interrupted consecutive folding. Uncoupled domain folding on both sides of the outer mitochondrial membrane would prevent backsliding to the cytoplasm and access of protease. The data suggest that both, the N-terminus and helix 5, have to be present with the wildtype sequence of Aky2p in order to guarantee folding and, with this prerequisite fulfilled, uptake to the correct destination in the IMS. Since Aky2 wildtype protein folds rapidly in the absence of chaperones, one possible role of this interaction could be to retard premature folding of Aky2p until contact to the mitochondrial import machinery has been established.

Expression of all Aky2 chimeric proteins from the same promoter should lead to about constant concentrations of total AKY2-derived protein. This is true only for some of the mutants (e. g. Aky-imp1, Aky-imp3, Aky-P3, Aky-LD3 and UUAAp). On the other hand, AAUUp and UUAUUp (Fig. 2B) are hardly detectable in the cytoplasmic fraction, and the decrease in the cytoplasm (contributing about 50% to total protein in a cell homogenate) is not compensated for by a corresponding increase of uptake into mitochondria (which comprise only about 10%). This indicates that these proteins are incompletely folded and degraded in the cytoplasm. UUAUUp is, however, imported into mitochondria more efficiently than wildtype leading to the conclusion that retarded folding not only is the cause of proteolytic instability as well as of arrest in the import channel, but also of prolonged presentation of import information to the mitochondrial import receptors.

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serum was provided by A. Zollner, Institute for Genetics and Microbiology, The University of Munich.
REFERENCES

LEGENDS

FIG. 1. Construction scheme of Aky2/Ura6 hybrid proteins. (A) Schematic drawing of homologous in vitro recombination between genes for the long-form isoprotein Aky2p and the short version Ura6p. Conserved sequences at the junctions of segments 1, 2, 3 and 4 were used as sites of recombination, positions of the junctions are indicated (FN). Shaded regions, promoter, translational initiation site (flanked by restriction sites for EcoRI and SpeI) and transcriptional terminator region from AKY2 (flanked by sites for NcoI and BamHI) which were present in all constructs (including UUUU, URA6 wildtype). (B) displays sequences of segment 3 of AKY2, alterations of which led to mutants Aky-imp1, Aky-imp3 and Aky-P3, as well as of Ura6p and URA6-h5, respectively. Dashes indicate deleted amino acid residues relative to the Aky2p wildtype (C) Construct Aky2-LD3: Replacement of the extra loop present in segment 3 of Aky2p by 5 amino acids adapting it to the loop of the short versions. Dashes denote amino acid residues that have no correspondence in the respective other isoenzyme (15, 16). FN, family numbering.

FIG. 2. Subcellular distribution of Ura6/Aky2 chimeric proteins. Strains harboring the Ura6/Aky2p chimeras were fractionated into cytoplasm (c) and purified mitochondria (m). After SDS-PAGE, hybrid proteins (15 µg of protein per lane) were detected by a mixture of anti-Aky2p and anti-Ura6p antisera. Antisera against hexokinase (cytoplasm), cytochrome c1 (mitochondrial membranes) and Hsp60 (mitochondrial matrix) served as indicators of mutual contaminations of the subfractions and loading controls. (A) strain DL1-D16 transformed with wildtype AKY2 on a multi-copy plasmid (Aky2p, control), Ura6p, URA6 multi-copy transformant (expressed from the AKY2 promoter, control), and mutants having an altered N-terminus, hybrid UAAAAp and mutant AKY2-N1p, in which the first AUG was mutated (Experimental Procedures). (B) Aky2p, wildtype control and hybrid proteins AAUUp, UUAAp, UUAUp, UAUUp and AUUAp.

FIG. 3. Subcellular distribution of Aky2 mutant proteins. Cellular subfractionation into cytoplasm (c) and gradient-purified mitochondria (m) of strains overexpressing Aky2p or Aky2 mutant proteins. After SDS-PAGE, proteins were detected by Western blotting and immunodecoration using a mixture of anti-Aky2p and anti-Ura6p antisera and sera directed against compartment-specific marker proteins: Hxk, anti-hexokinase (cytoplasm); Hsp60, anti-
Hsp60 protein (mitochondrial matrix); Cyt1p, anti-cytochrome $c_1$ (inner mitochondrial membrane). (A) Aky2p wt; Aky-imp1p; Aky-imp3p; Aky-P3p; Aky-LD3p; (B) Ura6p expressed from a multi-copy plasmid under the control of the AKY2 promoter (control); AUUUp containing the N-terminus from Aky2p; Ura6-h5p, Ura6p containing helix 5 from Aky2p and AUra6-h5p containing N-terminus plus helix 5 from Aky2p.

FIG. 4. **Helical wheel projection of helix 5.** Helix 5 of Aky2 wildtype protein is shown on the left, of Ura6p in the center (Ura6-helix5 wt) and that of Ura6-h5p on the right panel (Ura6-helix5 Aky). The view is from the C-terminal end of the helix. Hydrophobic residues are black, and polar residues are white.

FIG. 5. **Subfractionation of Aky2-mutant mitochondria.** Gradient-purified mitochondria of AKY2 wildtype and mutants were sonicated and separated into a soluble supernatant fraction (s, containing IMS and matrix) and pellet (p, containing inner plus outer mitochondrial membranes). Soluble proteins were concentrated by precipitation with trichloroacetic acid and both fractions quantitatively applied to the gel. Aky2 proteins and the following markers were detected by Western blotting and immunodecoration: cytochrome $c_1$ (Cyt1p), inner membranes; cytochrome $c$ peroxidase (CCPO), IMS.

FIG. 6. **Alkaline extraction (A) and protease treatment (B) of mitochondria.** (A) gradient-purified mitochondria were extracted with 0.1 M sodium carbonate and fractionated by centrifugation into supernatant (s) containing soluble proteins and peripheral membrane proteins and a pellet fraction (p) containing integral membrane proteins. After SDS-PAGE, Aky2 proteins and compartment-specific markers were detected by Western blotting and immunodecoration as above. (B) gradient-purified mitochondria were either left untreated or incubated at RT with proteinase K (50 µg/ml) in isotonic or hypotonic buffer under conditions where only the outer mitochondrial membrane is disrupted or in the presence of 1% Triton X-100 (indicated at the top of the figure). Aky2 and marker proteins were detected as above, except that in some experiments cytochrome $b_2$ (Cyb1p) and cytochrome $c_1$ (not shown) were assayed alternatively as markers accessible from the IMS under hypotonic conditions. Citrate synthetase served as matrix marker which is inaccessible under hypotonic conditions.
FIG. 7. Test of complementation of the Pet phenotype of the AKY2-disrupted recipient strain by aky2 mutant and AKY2/URA6 chimeric constructs. The following DL1-D16 transformant strains were streaked onto YPD medium (left plate) and YPG medium (right plate): Sector 1, Aky-LD3; sector 2, Aky-imp1; sector 3, URA6-h5; sector 4, Aky-imp3; sector 5, Aky-P3; sector 6, UAAA.
FIG. 1
FIG. 2
**FIG. 3**

### A

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- 25 kDa
- 21 kDa
- Hxk
- Hsp60
- Cyt1p

### B

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- 21 kDa
- Hxk
- Hsp60
- Cyt1p
FIG. 5

25 kDa

Cytp

CCPp

25 KDa

d s d s d s d s d s d s d s d s

P3p

Ura6-hgp

Imp1p

Imp3p

LD3p

Uaup

V4Aap

AVY2p
FIG. 6
Redundant mitochondrial targeting signals in yeast adenylate kinase
Roland Schricker, Michaela Angermayr, Gertrud Strobel, Sigrid Klinke, Dorothee Korber and Wolfhard Bandlow

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