Two parameters improve efficiency of mitochondrial uptake of adenylate kinase: decreased folding velocity and increased propensity of N-terminal α-helix formation

Michaela Angermayr, Gertrud Strobel, Alfred Zollner[1], Dorothee Korber, Wolfhard Bandlow*

Institut für Genetik und Mikrobiologie der Universität München, Maria-Ward-Straße 1a, D-80638 Munich, Germany

Received 26 September 2001; revised 26 October 2001; accepted 29 October 2001

First published online 8 November 2001

Edited by Gunnar von Heijne

Abstract The long isoform of eukaryotic adenylate kinase has a dual subcellular location in the cytoplasm and in the mitochondrial intermembrane space. Protein sequences and modifications are identical in both locations. In yeast, the bulk of the major form of adenylate kinase (Aky2p) is in the cytoplasm and, in the steady state, only 5–8% is sorted to the mitochondrial intermembrane space. Since the reasons for exclusion from mitochondrial import are unclear, we have constructed *aky2* mutants with elevated mitochondrial uptake efficiency of Aky2p in vivo and in vitro. We have analyzed the effect of the mutations on secondary structure prediction in silico and have tested folding velocity and folding stability. One type of mutants displayed decreased proteolytic stability and retarded renaturation kinetics after chaotropic denaturation implying that deterioration of folding leads to prolonged presentation of target information to mitochondrial import receptors, thereby effecting improved uptake. In a second type of mutants, increased import efficiency was correlated with an increased probability of formation of an α-helix with increased amphipathic moment at the N-terminus suggesting that targeting interactions with mitochondrial import receptors had been improved at the level of binding affinity.

Key words: Adenylate kinase; Protein folding; Mitochondrial import; Sub-mitochondrial sorting; *Saccharomyces cerevisiae*

1. Introduction

Adenylate kinases (AKs) are ubiquitous small soluble proteins which provide the ADP required for substrate chain and oxidative phosphorylations and – based on the reversibility of the formation reaction – serve to maintain the energy charge equilibrium in the cell [1,2]. In eukaryotes, three types of isoenzymes occur [3]. The so-called short version (myokinase or AK1 in vertebrates, Ura6p in yeast) is an exclusively cytoplasmic enzyme. AK1 is the major isoform in muscle cells [4]. In most vertebrate tissues like heart, kidney, lung and intestine, the long isoform, eukaryotic AK (AK2), prevails and has a dual localization in cytoplasm (about 30–70%, varying with the organism [5,6]), and the remainder in the mitochondrial intermembrane space. In yeast, the equilibrium of sorting the major form of AK (Aky2p) to mitochondria is even less favorable, because $<10\%$ of the total is found in this compartment [7]. However, this small fraction has an important role in oxidative metabolism, presumably by interacting with the ADP/ATP translocator thereby creating a microcompartment low in ATP and high in ADP that allows ATP/ADP exchange over the inner mitochondrial against a general concentration gradient [8]. In line with this role, *aky2* mutants have a Pet phenotype presumably because they fail to export oxidatively generated ATP efficiently from mitochondria [7]. AK3 (or Aky3p in yeast) is a long-form isoenzyme, too, and localizes to the mitochondrial matrix of all eukaryotic cells.

Mitochondrial targeting and membrane traversion of most nuclear-encoded mitochondrial proteins depends on N-terminal presequences [9–12]. Often, these target sequences are rich in positively charged and hydroxyamino acids and have the potential to form amphipathic α-helices [13–16]. This structure plays a decisive role in their recognition by the Tom receptor complex and in the interaction with the translocation machinery [17,18] and, in the case of constituents sorted to the inner compartment, is usually removed by the matrix processing peptidase upon traversion of the inner mitochondrial membrane. AK2/Aky2p dispenses with a cleavable presequence like a number of other mitochondrial proteins particularly of the outer compartments, the outer membrane or the intermembrane space, among them, e.g. the cytochrome *c* and c_1 heme lyases [19–21], and cytoplasmic and mitochondrial Aky2p is identically processed and modified in both locations [22,23]. Although not cleaved, the N-terminal seven or eight amino acid residues have been found to carry mitochondrial targeting information, as they suffice to direct heterologous passengers (mouse DHFR or yeast Ura6p) to mitochondria [24]. However, mutants in which this sequence has been deleted or profoundly altered by mutation ([24] and this study) are also imported so that additional intrinsic target information is likely to exist.

In contrast to nearly all other cytoplasmically synthesized mitochondrial precursor proteins, which are rapidly and quantitatively imported so that cytoplasmic precursor pools are marginal [25], AK2/Aky2p is peculiar in so far as the bulk of the protein is excluded from mitochondrial uptake. AK2/Aky2p has been reported to be proteolytically highly stable [23] so that one possible reason for the inefficient uptake by

*Corresponding author. Fax: (49)-89-2180 6160.
E-mail address: w.bandlow@lrz.uni-muenchen.de (W. Bandlow).

[1] Present address: Biomax, Am Klopferspitz, D-82152 Martinsried, Germany.

Abbreviations: AK, adenylate kinase; Aky2p, yeast major adenylate kinase; *AKY2*, gene encoding Aky2p; FN, family numbering

0014-5793/01/$20.00 © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(01)03122-2

mitochondria could be that nascent Aky2p folds rapidly into a rigid conformation that is no longer competent for mitochondrial import, and only the small fraction is taken up that reaches a mitochondrial import receptor before folding into a stable structure. To challenge such a model of kinetic limitation of mitochondrial import of Aky2p, we have constructed mutants which displayed improved uptake into the organelle. *aky2* mutants with increased mitochondrial import efficiency have been found to fall into two classes: one, as expected, displayed significantly decreased folding stability, and the other had an elevated propensity of amphipathic α-helix formation near the N-terminus. It is concluded (i) that rapid folding counteracts mitochondrial import constituting a novel mechanism of sorting of a protein to two subcellular compartments, cytoplasm and mitochondria, and (ii) that the equilibrium of partitioning is governed by the velocity of protein folding and the quality of target sequence/receptor interaction, both of which can be modulated by mutation separately.

2. Materials and methods

2.1. Yeast strains, cell growth conditions and preparation of mitochondria

The *AKY2*-disrupted yeast strains DL1-D16 Δaky2 (*aky2*::*LEU2*) [8] or WCG4 Δaky2 (this study) served as hosts for *AKY2* mutant-harboring plasmids. For analysis of import efficiency in vivo, cells were grown on semisynthetic medium supplemented according to the auxotrophic requirements and 3% lactate (or 2% galactose, AKY-C1, AKY-C2) as carbon source. Spheroplasts were prepared from mid-logarithmic cultures ($1–2\times10^7$ cells/ml), lysed in 0.6 M mannitol and nuclei and debris removed ($3000\times g$; 2×5 min). Mitochondria were collected by centrifugation ($10\,000\times g$; 15 min), incubated with proteinase K (50 μg/ml), thoroughly washed and further purified by Percoll gradient centrifugation (28%; Pharmacia, Freiburg, Germany) [26] and analyzed by SDS–PAGE and Western blotting.

2.2. Construction of AKY2 mutants

AKY2 mutants were obtained by site-directed mutagenesis [27]. In AKY-C1, codon 229 FN (for family numbering see [27]), and in AKY-C2 codon 236 were changed to termination codons leading to truncated products lacking 24 or seven amino acids from the C-terminus, respectively. The mature Aky2 wildtype protein has the N-terminal sequence SSESIRMVLIG [23]. In mutant AKY2-N2, the N-terminus was changed to SRLRILRMVLIG by introducing a frameshift mutation into codon 3 and a compensating mutation into codon 7 of the coding sequence [24]. To construct AKY-N9, 15 amino acids from the N-terminus of Aky2p were replaced with the homologous 23 N-terminal residues from Aky3p by homologous in vitro recombination within the conserved P-loop of the ATP-binding motif. AKY-N10 is similar to AKY-N9 with the exception that the amphipathic moment of the N-terminal α-helix that may potentially form at the membrane/aqueous interface has been increased compared to Aky3p. All *AKY2* mutant constructs were expressed in an *AKY2*-disrupted background from 2μ-based plasmids under the control of the original *AKY2* promoter retaining the authentic context of the ATG translational initiation codon.

2.3. In vitro transcription–translation of precursors and mitochondrial import

The genes of wildtype and mutant AKs, as well as of the control protein, F1-β, were ligated to pGEM vectors (Stratagene, Heidelberg, Germany). Capped transcripts were synthesized using SP6 polymerase and the Cap Scribe Kit (Roche, Mannheim, Germany) and translated in micrococcus nuclease-pretreated, methionine-depleted rabbit reticulocyte lysates (Promega, Heidelberg, Germany) in the presence of 50 μCi L-[^{35}S]methionine (1000 Ci/mmol; ICN Biomedicals, Eschwege, Germany) in a final assay volume of 50 μl. Mitochondria were prepared from lysed spheroplasts of strain D273-10B (ATCC 24657), suspended in import mix [28], and the reaction was started by the addition of 3 μl of ^{35}S-labelled urea-denatured (8 M urea) precursor. Valinomycin was used at a final concentration of 2 μM where indicated. After incubation at 25°C for 20 min, the import assays were divided into four aliquots which were diluted with 10 vol 20 mM HEPES, pH 7.4, containing no further additives or proteinase K (generally 50 μg/ml) either in the presence of 250 mM sucrose or without osmotic stabilization or with 1% Triton X-100 (final concentration). Samples were incubated at room temperature (30 min) and digestions terminated by addition of 2 mM phenylmethylsulfonyl fluoride (PMSF, final concentration). Mitochondria were collected by centrifugation (4°C; $20\,000\times g$; 7 min), resuspended in 250 μl 250 mM sucrose, 20 mM HEPES, pH 7.4, 150 mM KCl and recentrifuged. Proteins were separated by SDS–PAGE (14% gels) and dried gels exposed to X-ray film (Betamax, Amersham, Braunschweig, Germany).

2.4. Proteolytic stability of proteins

Cells were disrupted with glass beads, the $4000\times g$ supernatant was incubated with 1% digitonin in 0.6 M mannitol at 0°C for 1 min and 150 μg protein (1 mg/ml) of the $15\,000\times g$ supernatant incubated at room temperature with 25 μg/ml proteinase K for the times indicated. The digestion was terminated with 2 mM PMSF (final concentration) and 20 μg total cellular protein separated by SDS–PAGE and Aky2 wildtype and mutant proteins detected by Western blotting or autoradiography.

2.5. Renaturation of proteins

Aky2 wildtype and mutant proteins were denatured in 8 M urea, 20 mM K/HEPES, pH 7.4, 100 μM dithiothreitol at room temperature for 30 min. For renaturation, aliquots were diluted 200-fold with TEA buffer (70 mM triethanolamine, 13 mM $MgSO_4$, 50 mM KCl, pH 8.0) and incubated at room temperature for the periods indicated, and AK activity was measured by a coupled enzymatic test [7]. Proteolytic stability was assayed after a renaturation period of 30 min.

2.6. Miscellaneous procedures

Published procedures were used for determination of protein concentrations [29], for Western blotting and immunodecoration, and all other molecular procedures [30]. Secondary structure predictions were based on the algorithm of Chou and Fasman [31] using the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI, USA) or by using the algorithm developed by Rost and Sander [32,33] (PHD server, www.embl-heidelberg.de/predictprotein). Sequences were screened for surface exposure and flexibility using the algorithm described by Margalit et al. [34]. Hydrophobic α-helical moments were calculated applying the equation developed by Eisenberg [16].

3. Results

3.1. AKY2 mutants with increased efficiency of uptake into mitochondria

The first set of mutations was intended to interfere with folding stability and, simultaneously, not to affect an N-terminal or any internal targeting signal. Therefore, we deleted part (AKY2-C2) or most (AKY2-C1) of the C-terminal α-helix. Based on X-ray diffraction studies [35], this structural element makes contact with the AMP co-substrate and stabilizes the protein like a clamp. Both truncation mutants – which were enzymatically inactive (not shown) – were imported more efficiently than wildtype into mitochondria in vivo (Fig. 1A). Likewise, three N-terminal mutants with increased import efficiency have been found. All of them are enzymatically active and complement the Pet phenotype of the *AKY2* deletion in yeast as well as the *adk1-1ts* phenotype of *Escherichia coli* strain JH17 [36] (data not shown). Mitochondrial uptake efficiency was examined in vivo in the steady state, and Aky2 wildtype and mutant proteins were detected by Western blotting after SDS–PAGE (Section 2). Antisera against marker proteins (hexokinase for the cytoplasmic fraction and cytochrome c_1 for mitochondria) served to indicate mu-

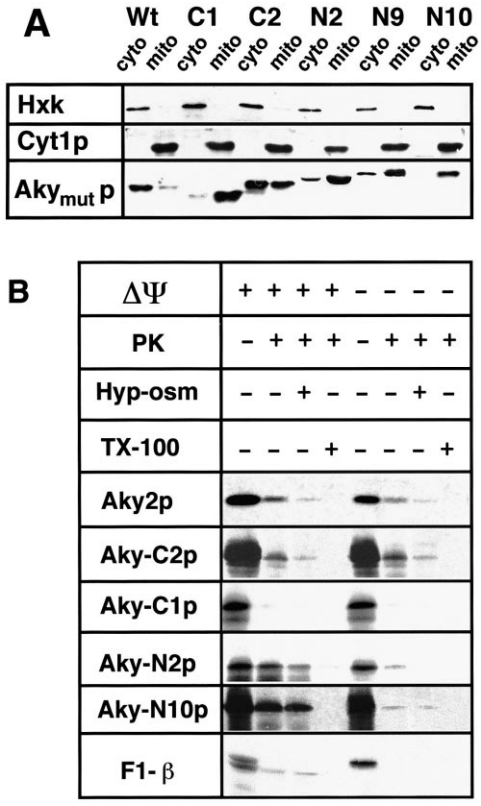

Fig. 1. Improved mitochondrial import of mutant AK in vivo and in vitro. A: Subcellular steady state distribution of Aky2p in wildtype and mutant strains. Proteins were detected by polyclonal antibodies against AK (Aky$_{mut}$p), or marker proteins, cytochrome c_1 (Cyt1p) or hexokinase (Hxk), after SDS–PAGE and Western blotting of cytoplasmic fraction and proteinase K-digested, gradient-purified mitochondria. B: Import of in vitro radiolabelled proteins into isolated mitochondria and dependence on membrane potential ($\Delta\Psi$) (see Section 2). Resistance of the respective protein to proteinase K (PK) in whole mitochondria indicates passage of at least the outer membrane, resistance to the treatment in mitoplasts (Hyp-osm+) but sensitivity in the presence of Triton X-100 (TX-100) documents uptake into the inner compartment/matrix fraction. The β-subunit of F1 ATPase (F1-β) is taken as an indicator of membrane potential-dependent protein uptake into the matrix.

tual contaminations and to standardize protein concentrations (Fig. 1A). The steady state level of each of the mutant proteins in mitochondria was higher than in wildtype. AKY-N10p is almost quantitatively imported and barely detectable in the cytoplasm.

To verify that the proteins are internalized and not merely superficially attached, in vitro synthesized wildtype and mutant proteins were imported into isolated mitochondria. Since in vitro import of bovine AK2 has been reported [6] to depend on membrane potential, we examined a possible dependence for yeast Aky2p as well. $\Delta\Psi$ dependence of matrix incorporation of the β-subunit of F1-ATPase served as a control.

After the import reaction, internalization of in vitro synthesized precursors was tested by incubation of re-isolated mitochondria with added proteinase K. In intact mitochondria and hypo-osmotically swollen mitoplasts (Hyp-osm+), the precursor to F1-β was degraded by the protease, whereas the mature form was resistant to this treatment (Fig. 1B), corroborating energy coupling of the mitochondria and correct sorting and processing of the protein. The mature form was, however, degraded after solubilizing both membranes with Triton X-100. As expected, no F1-β was internalized and processed in the presence of the uncoupler, valinomycin.

In vitro translated Aky2 wildtype protein was taken up to a significant extent only after previous denaturation with 8 M urea (Fig. 1B, details not shown). It is resistant to protease in intact mitochondria, but is accessible to degradation in mitoplasts, verifying localization in the mitochondrial intermembrane space. We could detect no uncoupling of import by the presence of valinomycin. Thus, in contrast to $\Delta\Psi$ dependence of uptake of bovine AK2 [6], yeast Aky2p behaves like other intermembrane space proteins without presequence, like cytochrome *c* and the two cytochrome heme lyases [19,20].

Aky2 mutant proteins behaved heterogeneously in this assay. The C-terminal truncations that had retained the wildtype N-terminus were poorly imported to the intermembrane space, the longer form (AKY-C2) slightly better than the shorter (AKY-C1), and uptake was independent of membrane potential. The C-terminal truncation mutant protein, Aky-C1p, associated with mitochondria quite efficiently after previous denaturation (Fig. 1B). However, only a trace of the material was transported to a position where it was protected from protease showing that it was bound to mitochondria but not imported to a significant extent. This result is in contrast to data obtained in vivo, where both mutant proteins were imported quite efficiently to a location where it was protected from proteolytic attack (Fig. 1A). The reason could be that both proteins were readily recognized and bound by mito-

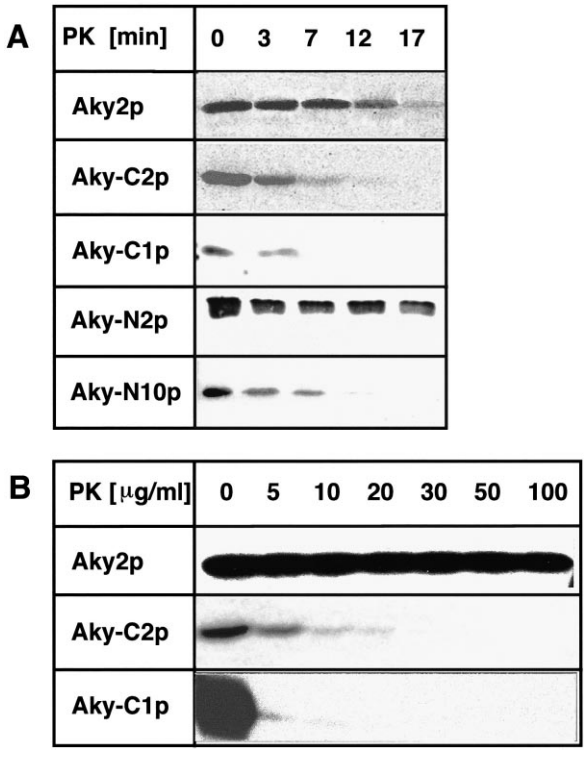

Fig. 2. Resistance to protease of AK wildtype and mutant proteins. A: Incubation of cellular extract (4000×*g* supernatant) at room temperature with 25 μg/ml proteinase K. Samples were taken at the time points indicated and analyzed by SDS–PAGE and Western blotting. B: Incubation of radiolabelled proteins for 30 min at 0°C with the proteinase K concentrations indicated. Proteins were detected by SDS–PAGE and autoradiography.

chondrial surface receptors but folded very reluctantly inside mitochondria (see Section 3.2).

All three N-terminal mutant proteins were transported in part (Aky-N2p) or exclusively to the matrix compartment (Aky-N9p is not shown). Partial resistance to proteinase K digestion, both under isotonic and under hypotonic conditions, but sensitivity in the presence of detergent suggested that some Aky-N2p had been transported across the inner membrane. This suspicion was substantiated by dissipating the membrane potential by valinomycin. In the presence of uncoupler, import efficiency was much lower, and imported Aky-N2p was protease-resistant only under isotonic, but fully sensitive under hypotonic conditions. This demonstrated that it was transported to both compartments, matrix and intermembrane space (IMS), in coupled mitochondria but exclusively to the IMS in the absence of membrane potential. In contrast Aky-N9p (not shown) and, even more efficiently, Aky-N10p, having an Aky3p-derived N-terminus, were transported exclusively to the matrix in a $\Delta\Psi$-dependent fashion, like AK3 [6].

3.2. *In some mutants, increased mitochondrial import is correlated with decreased protein stability and decreased renaturation rates*

The above observation that uptake of Aky-C1p and Aky-C2p is significantly increased over wildtype in vivo (Fig. 1A), whereas the two denatured mutant proteins, on the one hand, readily associate with isolated mitochondria, but on the other, fail to get internalized, can be explained most plausibly by assuming that the proteins are less stable than wildtype and fold only reluctantly. To test this issue and to examine whether folding stability or folding velocity are inversely correlated with import efficiency, resistance of the native proteins to proteolytic degradation (Fig. 2) and renaturation rates after chaotropic denaturation (Fig. 3) were examined.

Proteolytic stability of folded Aky2 wildtype and mutant proteins was measured in total cell extracts, and full length proteins were detected by Western blotting (Section 2). Most pronouncedly, Aky-C1p and, to a slightly lesser extent, Aky-C2p were highly sensitive, whereas wildtype and mutant Aky-N10p were highly resistant (Fig. 3A). Surprisingly, mutant Aky-N2p turned out to be even more resistant to proteolysis than wildtype. At 0°C radiolabelled Aky2p is fully resistant to concentrations of proteinase K as high as 100 μg/ml (Fig. 2B).

The failure of native Aky2p to be imported efficiently into isolated mitochondria and the modest improvement of uptake by previous denaturation by urea is most plausibly explained by a very rapid kinetics of spontaneous folding. This hypothesis was examined by measuring renaturation kinetics after chaotropic denaturation. Aky-N2p, Aky-N9p (not shown) and AKY-N10p (but not AKY-C1 and AKY-C2) were enzymatically active although to a lesser extent than wildtype, so that, as a first approximation, renaturation was followed by recording the return of enzymatic activity in these instances. Aky2 wildtype protein renatures spontaneously as soon as the urea-denatured protein solution is diluted into the renaturation buffer (Fig. 3A). Near to quantitative regain of the original activity argues against aggregation as a possible reason for inefficient uptake into isolated mitochondria. Also Aky-N10p is capable of significant renaturation, although at a much slower rate than wildtype. Strikingly, mutant Aky-N2p renatures even faster than wildtype.

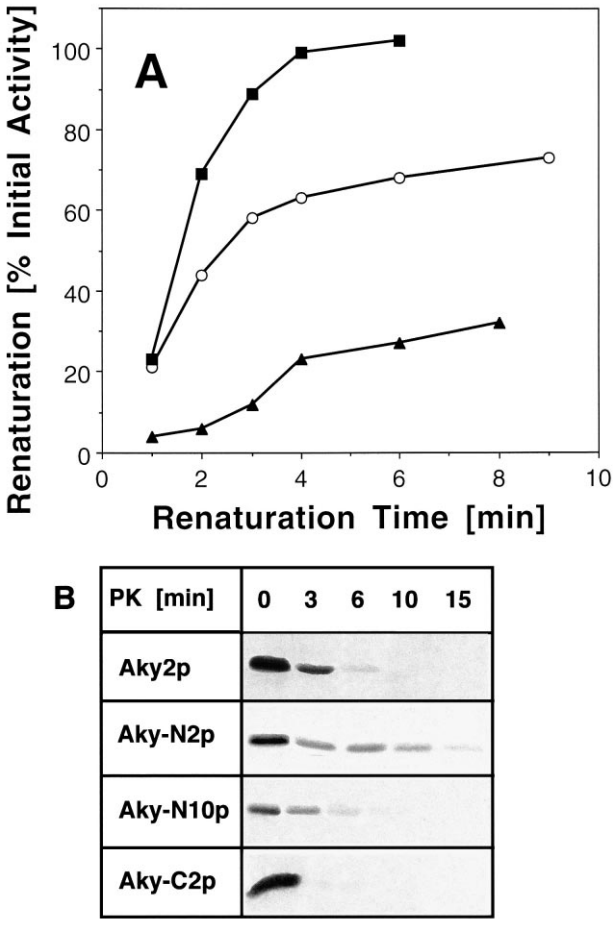

Fig. 3. Renaturation of wildtype and mutant AK. A: cell extract was denatured with 8 M urea for 30 min at room temperature. Aliquots were diluted 200-fold, renatured at room temperature for the periods indicated and enzymatic activities determined as a measure of tertiary structure restoration. ○, wildtype (100% is 40.5 U/mg before denaturation); ■, AKY-N2 (100% is 7.3 U/mg); ▲, AKY-N10 (100% is 19.6 U/mg). B: Cellular extract proteins were renatured for 30 min at room temperature and then incubated with 25 μg/ml proteinase K for the periods indicated and analyzed by SDS–PAGE and Western blotting.

These results were verified and, in addition, folding of mutant Aky-C2p challenged by measuring proteolytic stability of the renatured proteins after 30 min of renaturation (Fig. 3B). Aky-N2p again is highly resistant, whereas the truncation mutant protein, Aky-C2p, is degraded to near completion within 3 min indicating absence of significant folding.

4. Discussion

To find support for the hypothesis that the high velocity of spontaneous protein folding of Aky2p in the cytoplasm leads to (partial) exclusion from mitochondria, we have constructed mutants that exhibited increased mitochondrial uptake. Analysis revealed that two different parameters influence import: folding velocity of the protein and helix probability of the N-terminal peptide. In agreement with the above conclusions, we show that the C-terminal truncation mutants, AKY-C1 and AKY-C2, are readily taken up in vivo and coincidentally are significantly less rigidly folded than wildtype. Additional support for the view that retarded folding improves membrane

traversion comes from mutational analyses of the maltose-binding protein from *E. coli* [37] and of a cytochrome oxidase subunit VII-mouse DHFR fusion protein in yeast [38]. A mutation in the signal peptide of the periplasmic *E. coli* protein leads to its accumulation in the cytoplasm. A destabilizing second site mutation in the interior of the mature part of the protein suppresses the primary defect. In a similar fashion, mutations in the DHFR moiety of the hybrid led simultaneously to increased uptake into isolated yeast mitochondria and increased susceptibility to proteolytic digestion.

After incubation with isolated mitochondria, Aky2 truncation proteins are, however, only marginally imported. The discrepancy between in vivo and in organello import experiments is likely due to the failure to renature spontaneously to a significant extent inside the mitochondrial IMS during the relatively short period of the in vitro incubation, whereas in vivo ample time (and molecular chaperones to assist folding) is available. This suspicion is substantiated by the observation that the truncated proteins fail to renature after previous chaotropic denaturation in vitro and remain extremely sensitive to protease after 30 min under renaturing conditions. The in vitro data show that the Aky-C1 and Aky-C2 mutant proteins superficially adsorb to mitochondria quite efficiently after incubation of the denatured mutant precursor. The interaction survives sedimentation and washing of the mitochondria documenting its stability and suggesting that these proteins have, at least, embarked on the import pathway, but failed to be internalized due to reluctant spontaneous folding inside the outer membrane. Spontaneous folding presumably provides the driving force for irreversible direct uptake of IMS components. These data furthermore suggest that efficiency of import of AKs constitutes a compromise between slow folding to allow efficient receptor recognition outside mitochondria and rapid folding inside to drive internalization and make uptake irreversible.

The correlation between decreased tertiary structure stability of these mutants and increased import efficiency at least in vivo allows the conclusion that in these mutants targeting information is accessible for the mitochondrial import apparatus for a longer time interval than in wildtype. In support of this conclusion, it has been reported that formation of a stable folded protein conformation counteracts import [37–42].

Also mutant Aky-N2p is imported into mitochondria highly efficiently in vitro and in vivo. In the steady state, the equilibrium of cellular partitioning is significantly displaced towards the mitochondrial location when compared to the wildtype. This is surprising, because folding velocity and proteolytic stability of this mutant were even higher than of the wildtype. In contrast to the wildtype, secondary structure prediction for the N-terminal peptide of this mutant protein revealed an elevated tendency to form an α-helix rather than β-sheet (which has been found by X-ray diffraction studies [35]) and a relatively high hydrophobic helical moment (μH about 6.7 in Aky-N2p compared to $\mu H = 4.6$ in wildtype Aky2p [24]). Both parameters positively influence import efficiency [13–15,43] and are likely responsible for increased uptake due to an improvement of the quality of interaction with the import receptors. However, in contrast to the Aky2p wildtype, this import is $\Delta\Psi$-dependent and in part directed to the matrix compartment. The partial $\Delta\Psi$ dependence of import of this mutant protein reflects that a stable amphipathic N-terminal α-helix, indeed, must have formed at least transiently at the aqueous/membrane interface. Although this helix is short (about seven amino acids which is significantly less than in cleavable presequences), it evidently suffices for at least partial import into the inner compartment. Increase in length of the N-terminal α-helix to about 10–11 residues (derived from the N-terminus of Aky3p) in AKY-N9 and AKY-N10 (data not shown) improves import efficiency even further underlining that the primary structure of Aky2p, in principle, is compatible with membrane passage. The Aky2 wildtype protein ends up in the IMS because of lack of interaction with the TIM complex of the inner membrane and not due to an IMS sorting signal.

Thus, efficiency of mitochondrial recognition and uptake can be improved by mutation by two completely different mechanisms: either the propensity of the N-terminal target sequence to form an α-helix at the aqueous/membrane interface may be enhanced, thereby increasing the affinity to the receptor and improving the quality of targeting interactions or, alternatively, the rate of folding may be decreased so that import information which is cryptic in the folded structure (as evident from crystallographic analysis [35]) is exposed over a longer period of time.

Acknowledgements: A.Z. is grateful to Drs. R.A. Stuart and J. Herrmann, Adolf-Butenandt-Institute for Physiological Chemistry, The University of Munich, Germany for the hospitality in their lab and for discussion. Polyclonal antibodies against hexokinase were the kind gift of G. Schatz, The Biocenter, Basel, Switzerland. Yeast wildtype strain WCG4 was obtained from D.H. Wolf, Stuttgart, Germany. The work was supported by the Deutsche Forschungsgemeinschaft within Grant Ba415/24-1.

References

[1] Noda, L.H. (1973) in: The Enzymes (Boyer, P.D., Ed.), Vol. 8, pp. 279–305, Academic Press, Orlando, FL.
[2] Atkinson, D.E. (1977) in: Cellular Energy Metabolism and Its Regulation, pp. 85–107, Academic Press, New York.
[3] Schulz, G.E. (1987) Cold Spring Harbor Symp. Quant. Biol. LIII, 429–439.
[4] Heil, A., Müller, G., Noda, L., Pinder, D., Schirmer, H., Schirmer, I. and von Zabern, I. (1974) Eur. J. Biochem. 43, 131–144.
[5] Watanabe, K. and Kubo, S. (1982) Eur. J. Biochem. 123, 587–592.
[6] Nobumoto, M., Yamada, M., Song, S., Inouye, S. and Nakazawa, A. (1998) J. Biochem. 123, 128–135.
[7] Bandlow, W., Strobel, G., Zoglowek, C., Oechsner, U. and Magdolen, V. (1988) Eur. J. Biochem. 178, 451–457.
[8] Schricker, R., Magdolen, V., Kaniak, A., Wolf, K. and Bandlow, W. (1992) Gene 122, 111–118.
[9] Schatz, G. and Dobberstein, B. (1996) Science 271, 1519–1526.
[10] Neupert, W. (1997) Annu. Rev. Biochem. 66, 863–917.
[11] Pfanner, N., Craig, E.A. and Hönlinger, A. (1997) Annu. Rev. Cell Dev. Biol. 13, 25–51.
[12] Lithgow, T., Cuezva, J.M. and Silver, P.A. (1997) Trends Biochem. Sci. 22, 110–113.
[13] Von Heijne, G. (1986) EMBO J. 5, 1335–1342.
[14] Roise, D., Horvath, S.J., Tomich, J.M., Richards, J.B. and Schatz, G. (1986) EMBO J. 5, 1327–1334.
[15] Roise, D., Theiler, F., Horvath, S.J., Tomich, J.M., Richards, J.B., Allison, D.S. and Schatz, G. (1988) EMBO J. 7, 649–653.
[16] Eisenberg, D. (1984) Annu. Rev. Biochem. 53, 595–623.
[17] Brix, J., Rüdiger, S., Bukau, B., Schneider-Mergener, J. and Pfanner, N. (1999) J. Biol. Chem. 274, 16522–16530.
[18] Abe, Y., Shodai, T., Muto, T., Mihara, K., Endo, T. and Kohda, D. (2000) Cell 100, 551–560.
[19] Lill, R., Stuart, R.A., Drygas, M.E., Nargang, F.E. and Neupert, W. (1992) EMBO J. 11, 449–456.
[20] Steiner, H., Zollner, A., Haid, A., Neupert, W. and Lill, R. (1995) J. Biol. Chem. 270, 22842–22849.

[21] Diekert, K., Kispal, G., Guiard, B. and Lill, R. (1999) Proc. Natl. Acad. Sci. USA 96, 11752–11757.
[22] Klier, H., Magdolen, V., Schricker, R., Strobel, G., Lottspeich, F. and Bandlow, W. (1996) Biochim. Biophys. Acta 1280, 251–256.
[23] Tomasselli, A.G., Mast, E., Janes, W. and Schiltz, E. (1986) Eur. J. Biochem. 155, 111–119.
[24] Bandlow, W., Strobel, G. and Schricker, R. (1998) Biochem. J. 329, 359–367.
[25] Fujiki, M. and Verner, K. (1993) J. Biol. Chem. 268, 1914–1920.
[26] Müller, G. and Bandlow, W. (1989) Biochemistry 28, 9957–9967.
[27] Magdolen, V., Schricker, R., Strobel, G., Germaier, H. and Bandlow, W. (1992) FEBS Lett. 299, 267–272.
[28] Ungermann, C., Neupert, W. and Cyr, D.M. (1994) Science 266, 1250–1253.
[29] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[30] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[31] Chou, P.Y. and Fasman, G.D. (1978) Adv. Enzymol. 47, 45–148.
[32] Rost, B. and Sander, C. (1993) J. Mol. Biol. 232, 584–599.
[33] Rost, B. and Sander, C. (1994) Proteins 19, 55–72.
[34] Margalit, H., Sponge, I.L., Cornette, I.L., Cease, K.B., Delis, C. and Berzowsky, J.A. (1987) J. Immunol. 138, 2213–2229.
[35] Egner, U., Tomasselli, A.G. and Schulz, G.E. (1987) J. Mol. Biol. 195, 649–658.
[36] Henson, J.M., Blinkowa, A. and Walker, J.R. (1982) Mol. Gen. Genet. 186, 488–492.
[37] Liu, G., Topping, T.B. and Randall, L.L. (1989) Proc. Natl. Acad. Sci. USA 86, 9213–9217.
[38] Vestweber, D. and Schatz, G. (1988) EMBO J. 7, 1147–1151.
[39] Park, S., Liu, G., Topping, T.B., Cover, W.H. and Randall, L.L. (1988) Science 239, 1033–1035.
[40] Wienhues, U., Becker, K., Schleyer, M., Guiard, B., Tropschug, M., Horwich, A.L., Pfanner, N. and Neupert, W. (1991) J. Cell Biol. 115, 1601–1609.
[41] Gärtner, F., Bömer, U., Guiard, B. and Pfanner, N. (1995) EMBO J. 14, 6043–6057.
[42] Eilers, M. and Schatz, G. (1986) Nature 322, 228–232.
[43] Ryan, K.R. and Jensen, R.E. (1995) Cell 83, 517–519.