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# Cytoplasmic and mitochondrial forms of yeast adenylate kinase 2 are *N*-acetylated

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**Abstract**

Yeast major adenylate kinase (Aky2p), encoded by a single gene, occurs in two subcellular compartments, mitochondria and cytoplasm. Only 6–8% of the protein which has no cleavable presequence is imported into the organelle (Bandlow et al. (1988) Eur. J. Biochem. 178, 451–457). In the wild type two *AKY2*-derived signals (a major and a minor one) were detected by a monospecific antibody after two-dimensional gel electrophoresis and Western blotting. The signals reflected identical electrophoretic mobilities and were absent from an *AKY2*-disrupted strain suggesting that they were due to differently modified forms of Aky2p. Two similar signals were found in a mutant defective in protein *N*-acetylation, however, the p*I* values of both spots were shifted towards alkaline pH by one charge. This indicated that both forms of Aky2p were *N*-acetylated in the wild type and that their charge difference was not caused by incomplete *N*-acetylation. This observation furthermore suggested that, in the wild type, two different modifications exist one of which is *N*-acetylation. The second modification remains unidentified. We analysed the influence of protein *N*-acetylation on mitochondrial import. Both versions of Aky2p occurred in the cytoplasm and in mitochondria. Their proportion was unchanged in the *N*-acetylation mutant showing that neither modification affected the efficiency of import of adenylate kinase into mitochondria. It is discussed that *N*-acetylation occurs during or immediately after translation in the cytoplasm so that import of adenylate kinase may ensue co-translationally.

*Keywords:* ATP:AMP phosphotransferase; Protein *N*-acetylation; N-terminal processing; Two-dimensional gel electrophoresis; Co-translational import; Mitochondrion; (*S. cerevisiae*)

## 1. Introduction

Adenylate kinases (EC 2.7.4.3) are abundant cellular proteins which provide the ADP required for oxidative and substrate chain phosphorylations and fulfill a very important role in the maintenance of the energy charge. Similar to the adenylate kinase in liver cells, the major ATP:AMP phosphotransferase in yeast, Aky2p, is a so-called 'long-form' isozyme [1,2]. It has an extraordinary subcellular distribution. In yeast, more than 90% of the protein is located in the cytoplasm (and in the nucleus), and only the remainder of 6 to 8% resides in the mitochondrial intermembrane space [3]. Aky2p lacks a cleavable presequence and is active in both compartments [3]. In contrast to Aky2p, all other known mitochondrial proteins are imported into mitochondria very efficiently with undetectably small precursor pools in the cytoplasm. Most precursors reach their sub-mitochondrial destination with the help of a cleavable targeting sequence. Sorting of proteins to the mitochondrial intermembrane space can occur on one of two alternative routes. Cytochromes $b_2$ and $c_1$, for instance, have twopartite presequences, the N-terminal part of which is required and sufficient for recognition of outer membrane receptors and the passage across both membranes. The N-terminal part of the topogenic sequence is removed by the matrix processing peptidase. The import of

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these proteins requires a membrane potential over the inner membrane. It is still unclear, whether the second part of the presequence, which is cleaved off by a peptidase located in the intermembrane space, serves as a signal for re-export from the matrix [4] or as a stop-transfer signal preventing traversion of the inner membrane [5]. Other intermembrane space proteins like cytochrome *c haem* lyase [6] and presumably Aky2p take a direct route of import. They do not have a cleavable prepiece and do not require a membrane potential. However, cytochrome *c haem* lyase is imported very efficiently, whereas most of Aky2p is located outside the organelle in the cytoplasm. So far, no internal mitochondrial target sequences have been identified in adenylate kinase.

The following modifications have been reported to occur on Aky2p: the N-terminal two amino acids, Met and Ser, are clipped off, and the N-terminus of the mature protein, Ser #3, is blocked by *N*-acetylation [2]. In addition, an Asn residue has been shown convincingly to form the C-terminus [2], although an Asp is encoded by the gene sequence [7–9]. In the present study we investigated if we could detect these differently modified forms of Aky2p and analysed in particular, if any of the different versions of Aky2p preferentially occurs in one of the different subcellular compartments so that the type of modification has implications on mitochondrial import and topology of Aky2p. For this purpose we used high resolution two-dimensional polyacrylamide gel electrophoresis [10] in combination with Western blot analysis. Since very little is known about the process of import of adenylate kinase into mitochondria, this approach might give some indication in the direction of either a post- or a co-translational translocation mechanism.

## 2. Materials and methods

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

The *Saccharomyces cerevisiae* wild type strain DL1 *MATα, his3-11, 15, leu2-3, 112, ura3-251,372,328* [11] served as the parental strain for the construction of the *aky2* deletion, DL1-D16 [12] and the multi-copy transformants DL1-D16 [*URA6*] [12] and DL1-D16 [*PAK3*] [13], respectively. In addition, wild type W303-1A *MATa, ade2-1, his3-11, 15, leu2-3, 112, ura3-1, trp1-1, can1-100* [14] and the *N*-acetylation deficient mutant strain AMR1 *nat1*, other markers isogenic to W303-1A [15], were used. DL1 and DL1-D16 strains were grown on semisynthetic media containing 2% galactose, W303 and AMR1 *nat1* were grown in complete medium (2 l, 30°C, with vigorous shaking) on 3% lactate. Cell homogenates were prepared by breakage of the cells with glass beads by vortexing 5 × 1 min with intervals of cooling on ice for 1 min and subsequent centrifugation (2 × 5 min, 3000 × *g*, presence of 5 mM dithiothreitol and a cocktail of proteinase inhibitors consisting of 350 μM phenylmethylsulfonyl fluoride, 1 mM EDTA, 50 U/ml Trasylol and 0.6 μg/ml Pepstatin). Mitochondria were prepared from lysed spheroplasts (9500 × *g* pellet), purified by Percoll (Pharmacia, Freiburg; 28%) gradient centrifugation and routinely tested for contaminating proteins from other compartments according to published procedures [3,16]. The 105 000 × *g* (60 min, 4°C) supernatant was used as the cytoplasmic fraction. Aliquots of supernatant and mitochondria were frozen in buffer containing proteinase inhibitors and 5 mM dithiothreitol, stored at −70°C and thawed only once. Protein was determined as described [17].

### 2.2. Raising of anti-Aky2p antibodies in chicken

Antibodies against yeast Aky2p were raised in chicken using oligo-His-tagged adenylate kinase as the antigen. The recombinant protein was expressed under the guidance of the bacterial *tac* fusion promoter from plasmid pQE8 (Diagen, Hilden) and purified from bacterial lysates by one-step $Ni^{2+}$-chelate column chromatography according to the protocol supplied by the manufacturer. Two chicken were immunized [18] with about 200 μg of the homogenous recombinant protein per injection, using complete Freund's adjuvant for the first immunisation. Two boost injections with complete Freund's adjuvant were performed four and eight weeks after the first immunization. The polyclonal antibodies were prepared from the egg yolks by dextran sulfate precipitation [19].

### 2.3. Two-dimensional electrophoretic procedure

Two-dimensional gels were prepared according to [20] using an immobilized pH-gradient (IPG) pH 4–7 for isoelectric focusing [21,22]. Two identical gels were prepared in each case. One of the gels was silver stained [23], the other one blotted onto Fluorotrans membrane (Pall, Dreieich) according to Ref. [24]. For detection of Aky2p in a Western blot, the polyclonal chicken anti-Aky2p antibody was used in a 1:2000 dilution in phosphate-buffered saline.

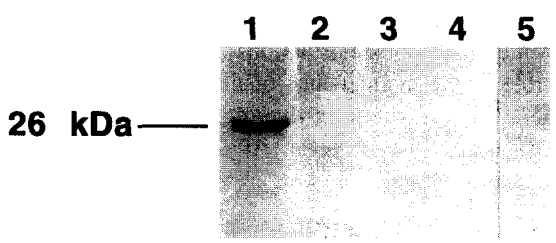


Fig. 1. Western blot analysis of Aky2 protein detected by polyclonal antibodies raised in chicken. 10 μg protein (lanes 1–4) and 60 μg protein (lane 5) from yeast cell homogenates was electrophoretically separated by sodium dodecyl sulfate gel electrophoresis, transferred onto Nylon membrane and immuno-decorated. Lane 1, wild type strain DL1; lanes 2 and 5, *aky2* disruption mutant, DL1-D16; lane 3, transformant strain DL1-D16 [*PAK3*]; lane 4, transformant strain DL1-D16 [*URA6*].

The detection system consisting of the secondary anti-chicken IgG enhancer, horse radish peroxidase-coupled protein A and chloronaphthol as the developer (Bio-Rad, Geretsried) was used according to the prescriptions of the supplier.

## 3. Results

Antibodies directed against Aky2p were raised in chicken and tested for cross-reactivity with other members of the adenylate kinase family in yeast (i.e., Pak3p, the GTP:AMP phosphotransferase of the mitochondrial matrix [12], and Ura6p, the cytoplasmic UMP/CMP/AMP phosphotransferase [12,25]). For this purpose homogenates from wild type strain, DL1, the *AKY2*-disrupted strain, DL1-D16, and the *PAK3* and *URA6* multi-copy transformant strains, DL1-D16 [*PAK3*] and DL1-D16 [*URA6*], respectively, were prepared and transferred onto Nylon membrane after sodium dodecylsulfate polyacrylamide gel electrophoresis. Aky2p was detected after immuno-decoration

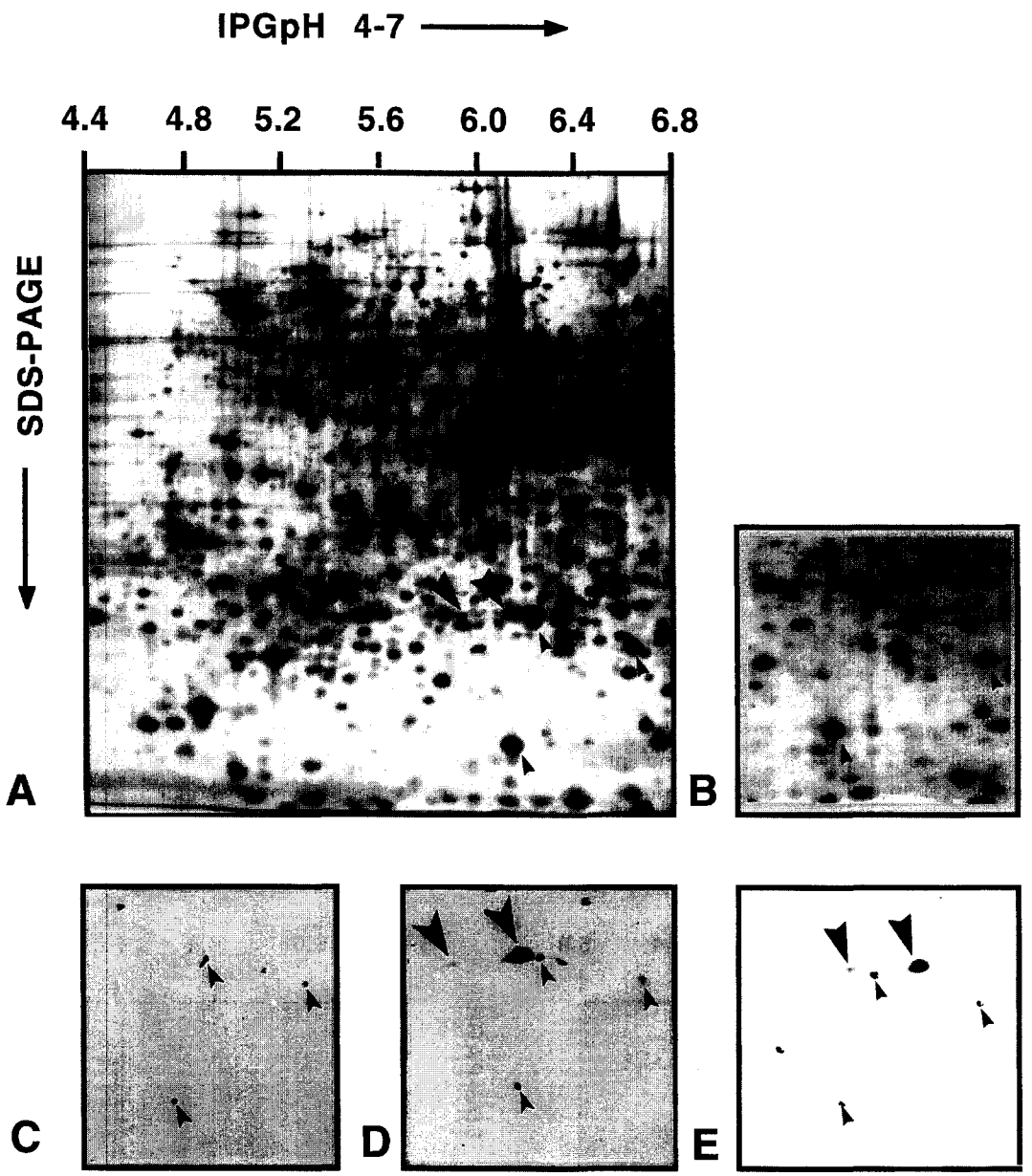


Fig. 2. Two-dimensional electrophoresis and Western blot of yeast cell homogenates decorated with anti-Aky2p antibodies. Two identical samples containing 60 μg of the proteins each were separated by isoelectric focusing in the first dimension and then by SDS electrophoresis in the second dimension. One gel was stained with $AgNO_3$ for identification of the total protein spots (panels A and B). The other gel specimen was electro-blotted, Ponceau S-stained and immuno-decorated with anti-Aky2p antibodies (panels C, D and E). Panels A and D, wild-type W303-1A; panels B and E, *N*-acetylation-deficient mutant strain AMR1; panel C, aky2 disruption mutant DL1-D16. Large arrows point at anti-Aky2p-reactive proteins. Small arrows denote Ponceau S-stained proteins which were found in all three extracts at identical positions and marked with Indian ink in panels C, D and E. They serve as reference spots to demonstrate the p*I* shift of Aky2p. The other spots reflect material detected in this position by Ponceau S staining in only one of the extracts.

using chicken anti-Aky2p as primary antibodies. Western blot analyses of the homogenates from the wild type revealed a single band corresponding to a protein with an apparent molecular mass of about 26 kDa (Fig. 1) which is slightly above the value deduced from the amino acid sequence [2]. The antiserum is specific and does cross-react neither with Ura6p nor Pak3p, even after massive overexpression of either of these latter homologous proteins (lanes 3 and 4). Also homogenates containing 10 μg (lane 2) or 60 μg (lane 5) of homogenate of the *aky2* disruption strain did not reveal any signal, excluding cross-reactivity of the serum with so far undetected closely related adenylate kinase isozymes or other polypeptides from yeast (see Section 4).

Adenylate kinase lacks a cleavable mitochondrial presequence, and nothing is known about internal targeting signal(s) and the import mechanism of this protein. First we examined, whether isoforms of Aky2p exist and whether they exhibit differential uptake into the organelle. Indeed, two-dimensional gel electrophoresis (Fig. 2A) and Western blot analyses (Fig. 2D) of a homogenate of the wild type revealed two immuno-reactive spots of Aky2p. The major and the minor spots displayed identical electrophoretic mobilities and had isoelectric points (p*I*) of 6.2 and 5.9, respectively. The calculated isoelectric point of the unmodified protein deduced from the nucleotide sequence (program Isoelectric, UWGCG package) is at pH 6.37. Since in homogenates of the strain in which the *AKY2* gene had been deleted no cross-reacting material is detectable in Western blot analysis (Fig. 2C), both signals must be derived from the single *AKY2* gene.

To examine, how the two spots are related to Aky2p and if the two forms of Aky2p differ by acetylation of the N-terminal amino acid, we compared the two-dimensional protein pattern from the wild type with that obtained with the homogenate of strain AMR1 *nat1*. This mutant strain is deficient in *N*-acetyltransferase 1, the major *N*-acetyltransferase, responsible for N-terminal protein acetylation [15]. The Western blot analysis of a *nat1* extract also revealed two spots of Aky2p again with intensities comparable to the wild type (Fig. 2E). As shown by the silver-stained two-dimensional gels (compare Fig. Fig. 2A with Fig. 2B) as well as by using hexokinase as an intrinsic marker in Western blot analysis the p*I* of which was not affected by the *nat1* mutation (not shown), both *AKY2*-derived spots were shifted to a more alkaline pH in comparison to wild type Aky2p (p*I* values were 6.4 and 6.1, respectively). This clearly demonstrated that both isoforms were acetylated in the wild type and not acetylated in the mutant. Thus, the difference between the two isoforms of Aky2p in the wild type is not caused by incomplete *N*-acetylation.

Next, we analysed, whether the minor spot is attributable to the version preferentially imported into mitochondria and the major spot corresponds to the cytoplasmic form. In addition, we tested if lack of *N*-acetylation in the *nat1* mutant strain excluded Aky2p from mitochondrial import. Fig. 3A shows that the *nat1* mutant displays two spots in the cytoplasmic fraction which are the same as found in the homogenates, and the p*I* values of both isoforms are again shifted by one charge to alkaline pH in the *N*-acetylation-deficient strain relative to the *NAT1* wild type (not shown, but compare Fig. 2D with Fig. 2E). The identical two forms occur also in mitochondria of the *nat1* mutant strain (Fig. 3B) indicating that *N*-acetylation is neither specific for the cytoplasmic compartment, nor is it an indispensable prerequisite for mitochondrial import of Aky2p. It is concluded that this N-terminal modification does not prevent import, since the two isoforms occur at about the same proportion in both compartments. Furthermore, *N*-acetylation is not applied intramitochondrially, i.e. after the protein has been imported, because the *nat1* mutation simultaneously affects the p*I* values of the cytoplasmic and the mitochondrial version. Consequently,

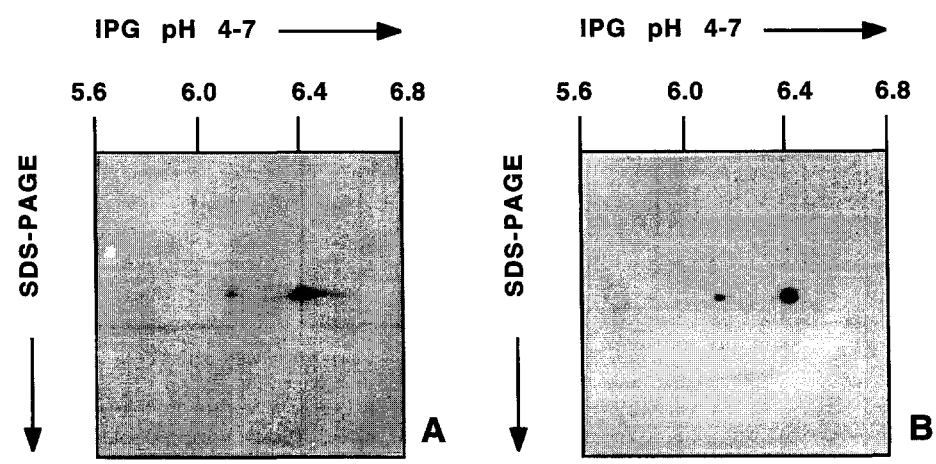


Fig. 3. Two-dimensional Western blot of cytoplasmic (panel A) and mitochondrial proteins (panel B) from strain AMR1. 60 μg of a high speed supernatant and mitochondria purified by Percoll gradient centrifugation [15], respectively, were run in a two-dimensional gel, blotted onto Nylon membrane and proteins identified as described under Fig. 2 and reacted with anti-Aky2 antibodies.

*NAT1* codes for a protein which acetylates both, cytoplasmic and mitochondrial, forms of Aky2p. In addition, the results indicate that also the other modification neither impedes nor promotes import of Aky2p into the organelle.

A closer inspection of the silver-stained gels shown in Fig. 2A,B reveals that the spots, representing Aky2p isoforms (large arrows), are shifted in the *nat1* mutant strain relative to some major spots in the surrounding area which are not affected by the *nat1* mutation (small arrows in Fig. 2A–E). Consequently, the latter proteins are presumably not *N*-acetylated in either strain. In addition to Aky2p, a number of other spots are also shifted by about the same pH difference as each of the two isoforms of Aky2p. This observation allows to estimate, in line with the reports from higher eukaryotes [26], that also in yeast roughly 60 to 80% of all proteins are *N*-acetylated by Nat1p. Also the p*I* values of a number of mitochondrial proteins differ in the two strains (data not shown). This finding demonstrates that, apart from the bulk of mitochondrial proteins which are processed at the N-terminus upon import into the organelle, some proteins exist, the p*I* values of which are affected by the deficiency in *N*-acetylation. These proteins obviously are not processed by the processing peptidase of the mitochondrial matrix (not shown here). Thus, the shift of p*I* of a certain mitochondrial protein in a *N*-acetylation-deficient mutant may be used as a first screen for proteins lacking a cleavable mitochondrial targeting sequence.

## 4. Discussion

Most mitochondrial proteins are synthesized on free ribosomes in the cytoplasm. They are post-translationally targeted to the organelle by means of a cleavable presequence which is required and sufficient for both, recognition of the mitochondrial surface receptor(s) and translocation. The situation with Aky2p differs from this general scheme in several aspects: (i) Aky2p lacks a cleavable targeting sequence [3], (ii) only a minor fraction of Aky2p is imported into the organelle [3], (iii) the mitochondrial fraction of Aky2p does not equilibrate with a cytoplasmic pool prior to translocation, since it has been observed that some Aky2 mutant proteins are extremely unstable in the cytoplasm, but still are imported into mitochondria very efficiently (G. Strobel, R. Schricker and W. Bandlow, unpublished results), and (iv) the import efficiency can be improved by decreasing the stability of the protein structure or by extending the time required by the protein to assume a folded structure which is likely to terminate the import-competent state [27]. These observations propose a co-translational mechanism of import of Aky2p in contrast to the bulk of mitochondrial proteins. Co-translational import is, indeed, discussed for at least a subset of mitochondrial proteins [28–30]. The observation that mitochondrial Aky2p is modified to the same extent as in the cytoplasmic fraction is consistent with a co-translational import mechanism only, when the modifications are applied to the protein during translation. In fact, amino-terminal processing and *N*-acetylation of proteins are very rapid processes, and evidence has been obtained which implies that the acetyl group is attached to the polypeptide chain co-translationally [31,32]. The observation that in the *NAT1* wild-type non-acetylated Aky2p is not detectable agrees with this view. The finding that the vast majority of wild-type Aky2p is excluded from import into mitochondria suggests that an analogue of a recognition particle and a translational arrest, to bring nascent Aky2p to the mitochondrial membrane, do not exist. A mechanism of uptake of Aky2p which is in agreement with the data presented here could be based on the kinetics of spontaneous protein folding. In this model only those nascent Aky2 molecules are imported by mitochondria which are synthesized on ribosomes located in close proximity to the surface of the organelle and reach their cognate import receptors in an import competent state before tertiary structure formation commences. Such a mechanism implies that import and protein folding of adenylate kinase are competitive and mutually exclusive processes.

In Western blot analyses of two-dimensional gels using a chicken antibody directed against recombinant His-tagged Aky2p two spots were detected. Both spots represent protein products of the single *AKY2* gene, because these antibodies do not cross-react with other proteins in homogenates of a strain in which the *AKY2* gene had been deleted, even when an excess of protein was tested in Western blot analysis. The existence of further AMP kinases in addition to Ura6p [12] and Pak3p [13] is unlikely also for the following reasons: (i) In low stringency hybridization additional signals are absent, *AKY2* is a single copy gene in the genome of *Saccharomyces cerevisiae*; (ii) in polymerase chain reactions on genomic DNA templates from an *AKY2* deletion mutant using primers designed after conserved sequence blocks (ATP-binding site and DGF/YPR sequence) no amplification products were detected in addition to *URA6* and *PAK3*; (iii) functional complementation tests of adenylate kinase deficiency, both in yeast and in *E. coli*, did not reveal complementing genes other than *URA6* [12] and *AKY3* [33].

Another possible explanation of the appearance of the two spots could be partial hydrolysis of the protein during preparation. However, (i) proteinase inhibitors were present throughout all steps of the preparation; (ii) aliquots of the samples were frozen immediately after preparation and thawed only once, and (iii) the coincidence of the electrophoretic mobility and the identical proportion of the two spots in homogenate, mitochondria and cytoplasm of two different strains argues against this possibility.

Thus, the appearance of two versions of Aky2p is more likely due to an incomplete protein modification in addition to *N*-acetylation. This modification alters the p*I* by one charge. Like *N*-acetylation it is also present both on

the cytoplasmic and the mitochondrially imported fraction of Aky2p. The nature of this additional modification has not yet been established. Both, the adenylate kinase protein [2] and the *AKY2* gene [7–9] have been sequenced. The reading frame of the nucleotide sequence of *AKY2* ends with a triplet encoding Asp, whereas it has been shown that the C-terminal amino acid of Aky2p is Asn. It is tempting to speculate that the second modification consists in the amidation of the C-terminal aspartic acid in agreement with the published protein sequence. This would imply that amidation is not quantitative. Indeed, the major isoform of Aky2p shown in Fig. 2A has a more alkaline p*I*. Nothing is known about the kinetics and topology of protein amidation. The fact, that Aky2p is modified at the C-terminus, suggests a post-translational event. Such a mechanism is difficult to reconcile with co-translational import of Aky2p, but does not exclude this possibility. The concomitant occurrence of the modified and the unmodified forms of Aky2p simultaneously in cytoplasm and mitochondria at about the same ratio could indicate that also this second modification is applied to the polypeptide before release from the ribosome although not to all molecules.

Neither modification on Aky2p, *N*-acetylation or the other, not yet identified alteration, affects mitochondrial import indicating that neither has an influence on the velocity of tertiary structure formation. *N*-acetylation was found to increase the half-life of proteins considerably both in mitochondria and in the cytoplasm (data not shown). Presumably *N*-acetylation stabilizes the tertiary structure thereby rendering proteins more resistant to ubiquitination and subsequent degradation by the proteasome [34] in agreement with the N-end rule [35,36]. Indeed, we observed considerable degradation of Aky2p and other proteins in the *nat1* mutant by contrast to the wild type.

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