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Mechanism of Mitochondrial Import of Adenylate Kinase Isozymes[1]

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Adenylate kinase (AK) is a ubiquitous enzyme that contributes to the homeostasis of the cellular adenine nucleotide composition. Three isozymes, AK1, AK2, and AK3, have so far been characterized in vertebrates. They are located in different tissues, while their primary and tertiary structures are similar. Among them, AK2 and AK3 are located in mitochondria, but unlike most mitochondrial proteins, both proteins lack a cleavable presequence. In this study, we first confirmed that AK2 is distributed in liver cells in both the cytosol and the intermembrane space of mitochondria, while AK3 is localized exclusively in the mitochondrial matrix. Next, we analyzed the process of import of AK2 and AK3 by incubating isolated rat mitochondria with proteins that were synthesized in a reticulocyte lysate translation system. The results indicated that both AK2 (an intermembrane-space-targeting protein) and AK3 (a matrix-targeting protein) require an inner membrane electrochemical potential for their import. This finding for AK2 is in contrast with those of other noncleavable intermembrane-space-targeting proteins such as cytochrome *c* and cytochrome *c* heme lyase, which do not require the membrane potential for their import. In the transport process, AK2 and AK3 competed with the adrenodoxin precursor, which is imported into the matrix through a mechanism common to other mitochondrial matrix proteins. Thus, AK2 and AK3 were thought to be translocated into mitochondria through the same pathway as that for most mitochondrial protein precursors. Neither AK2, that was previously synthesized in reticulocyte lysates, nor AK2, that was purified from an *Escherichia coli* overexpression system, was imported into mitochondria in a post-translational import manner. In contrast, AK3 was imported into mitochondria after completion of protein synthesis. Thus, the import of AK2 is likely to be co-translational, and the co-translational import mechanism might contribute to the bi-topological distribution of AK2 in both the cytosol and mitochondria.

Key words: adenylate kinase, bi-topological distribution, co-translational import, mitochondria, noncleavable protein.

Adenylate kinase (AK) is a ubiquitous enzyme that contributes to the homeostasis of the adenine nucleotide composition in the cell (*1*). In vertebrates, three isozymes, AK1, AK2, and AK3, have been identified (*2-4*). AK1 is present mainly in the cytosol of skeletal muscle, brain, and erythrocytes (*2*). AK2 is present in liver, kidney, and heart (*3*), and has been thought to be a mitochondrial intermembrane-space protein. However, it was reported that AK2 is located in the cytosol as well as in mitochondria (*5, 6*). The other isozyme, AK3, which uses GTP as a phosphate donor in the reaction, is thought to be localized in the mitochondrial matrix of various tissues (*7, 8*). Among these isozymes, AK2 and AK3 are nuclear-encoded, synthesized in the cytosol, and subsequently imported into mitochondria where they are sorted to different submitochondrial locations. Therefore, comparison of the import mechanisms for these two proteins could provide new insight into the targeting and sorting of mitochondrial proteins.

Most mitochondrial proteins are synthesized in the cytosol as precursor forms and imported into mitochondria after cleavage of their N-terminal presequences. For example, adrenodoxin (*9*) and aspartate aminotransferase (AspAT) (*10*), which are imported into the matrix, and cytochrome b_2 and cytochrome c_1 (*11*), which are imported into the intermembrane space, have cleavable N-terminal presequences. They are imported through the general import pathway including Tom and Tim proteins, and need a mitochondrial inner membrane electrochemical potential

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Abbreviations: AK, adenylate kinase; AK1, cytosolic ATP:AMP phosphotransferase or adenylate kinase isozyme 1; AK2, mitochondrial ATP:AMP phosphotransferase or adenylate kinase isozyme 2; AK3, mitochondrial GTP:AMP phosphotransferase or adenylate kinase isozyme 3; AspAT, aspartate α-ketoglutarate aminotransferase; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; PMSF, phenylmethanesulfonyl fluoride.

(*12-14*). In contrast, both AK2 and AK3 have no cleavable presequence (*15, 16*). Noncleavable matrix-targeting proteins (*e.g.* 3-oxoacyl CoA thiolase and rhodanese) are imported through the general import pathway with the inner membrane electrochemical potential (*17*) similar to cleavable mitochondrial proteins. On the other hand, two other noncleavable intermembrane-space-targeting proteins, cytochrome *c* and cytochrome *c* heme lyase, are imported directly into the intermembrane space across the outer membrane without interaction with the inner membrane (*18, 19*). Thus, AK2 was also thought to be directly imported into the mitochondrial intermembrane space like these two proteins. However, since AK2 is homologous to matrix-targeting AK3 with respect to the primary as well as the tertiary structure (*20, 21*), we speculated that both AK isozymes should be imported into mitochondria through the general import pathway with the inner membrane electrochemical potential. Since we previously cloned the cDNAs of these AK isozymes (*15, 16*), we are now able to analyze the import pathway for AK isozymes using *in vitro* translated proteins and isolated mitochondria.

MATERIALS AND METHODS

Materials—Restriction enzymes, SP6 RNA polymerase, and ribonuclease inhibitor were purchased from Takara Shuzo (Kyoto); bovine serum albumin (fatty acid-free) from Sigma (St. Louis, MO, USA); [^{35}S]methionine (185 MBq, >800 Ci/mmol, translation grade) from American Radiolabeled Chemicals (St. Louis, MO, USA); and ECL Western blotting detection reagents from Amersham International (Buckinghamshire, England). All other chemicals were of analytical grade. Bovine AK2 and AK3 were prepared from *Escherichia coli* cells harboring pVEX-AK2B and pUC7-AK3, respectively (Nobumoto, M., in preparation). The homogeneity of each purified protein preparation was confirmed on SDS-PAGE. Rabbit antisera against AK isozymes were prepared using purified AK isozymes (Nobumoto, M., in preparation).

Enzyme Assay—The enzyme assay was performed as described previously (*8*). AK2 activity was assayed in the reaction: ATP+AMP→2ADP, and ADP formation was coupled with pyruvate kinase and lactate dehydrogenase reactions, leading to NADH oxidation. For the AK3 assay, activity was measured in the reaction: GTP+AMP→GDP+ADP, and ADP formation coupled with pyruvate kinase and lactate dehydrogenase reactions was measured by monitoring NADH oxidation. Kynurenine hydroxylase and fumarase were assayed as described previously (*18, 33*).

Subcellular Localization—Cytosol and mitochondrial fractions were prepared from rat liver as described by Watanabe *et al.* (*5*). The enzyme activities of lactate dehydrogenase (a cytosolic marker enzyme) and succinate dehydrogenase (a mitochondrial marker enzyme) were assayed in both fractions (*22, 23*). Proteins were subjected to 0.1% SDS-12% PAGE and then electrophoretically transferred to a nitrocellulose membrane. The membrane was analyzed with antiserum against AK3 and ECL Western blotting detection reagents.

Preparation of Mitochondria—Rat liver mitochondria were prepared from Wistar rats as described by Murakami *et al.* (*24*). The mitochondria were suspended in the isolation medium at a concentration of 50 mg protein/ml. Protein was determined with a Bio-Rad protein assay dye reagent (*25*) using bovine serum albumin as a standard. The intactness of the isolated mitochondria was confirmed by the respiratory control ratio of each mitochondrial preparation, that was determined to be higher than 4.0 with the oxygen electrode method using succinate as a substrate (*26*).

Submitochondrial Localization—For the preparation of mitoplasts, mitochondria (300 μg) were suspended in 60 μl of 20 mM HEPES-KOH, pH 7.4, and then placed in a test tube on ice for 30 min (*27*). The mitoplasts were recovered by centrifugation at 4,000×*g*, and then resuspended in 50 μl of 10 mM HEPES-KOH, pH 7.4, containing 220 mM mannitol and 70 mM sucrose. The mitochondria and mitoplasts were treated with 250 μg/ml proteinase K with or without 1% Triton X-100 at 4°C for 30 min, followed by the addition of 400 μg/ml PMSF to terminate the protease reaction. The mitochondria (300 μg) were treated with digitonin (final concentrations, 0-0.27 mg/mg protein) in 200 μl of 480 mM mannitol, placed in a 25% sucrose solution containing 20 mM HEPES, pH 7.4, in an Eppendolf tube, and then centrifuged at 12,000×*g* for 10 min. The precipitates were dissolved, and the proteins were subjected to 0.1% SDS-12% PAGE and then electrophoretically transferred to a nitrocellulose membrane. The membrane was analyzed with antiserum against AK3 and ECL Western blotting detection reagents.

Construction of Plasmids—The plasmids used as DNA templates for *in vitro* transcription were constructed by inserting DNA sequences after the SP6 promoter in pSP6 vectors (*28*). The AK2-encoding sequence was excised from pUC-AK2B (*15*) at the *Eco*RI and *Pst*I sites, and the AK3-encoding sequence from pUC7-AK3 at the *Hinc*II and *Eco*RI sites. These sequences were inserted in the polylinker sites of pSP64 and pSP65 (*28*). Thus, pSP6-AK2B and pSP6-AK3-1 were obtained. pSP6-AK3-1 encodes a protein having an additional dipeptide Met-Ala to the initiation Met at the N terminus of AK3.

In Vitro Transcription—mRNA was synthesized *in vitro* under reaction conditions modified from those of Melton *et al.* (*28*). Nonlinearized DNAs (4 μg) were transcribed at 37°C for 1.5 h in a transcription reaction mixture containing 40 units SP6 RNA polymerase, 100 units ribonuclease inhibitor, and 750 μM of each rNTP, in a final volume of 100 μl. After phenol/chloroform extraction and ethanol precipitation, each transcription product was dissolved in 40 μl of distilled water.

In Vitro Import Accompanied by Translation—Nuclease-treated rabbit reticulocyte lysates were prepared as described by Pelham and Jackson (*29*). *In vitro* translation was carried out according to the procedure of Mori *et al.* (*30*) with some modifications. The final potassium concentrations in the reaction mixture were 70 mM for the synthesis of AK isozymes and 120 mM for AspAT. The amount of the reticulocyte lysate was increased to 40% the volume of the translation reaction mixture. AK1 mRNA (2.0 μg), AK2 mRNA (3.0 μg), AK3 mRNA (4.0 μg), or AspAT mRNA (4.0 μg) was used for translation in the presence of [^{35}S]methionine. At the beginning of translation, 25 μg mitochondria were added to the 25-μl reaction mixture for the AK3 and AspAT import experiments, and

100 μg mitochondria to the 100-μl reaction mixture for the AK2 import experiments. Each mixture was incubated at 28°C for 3 h. In this way, both the translation and import reactions proceeded simultaneously. After the addition of a 9-fold volume of suspension buffer (10 mM HEPES-KOH, pH 7.4, containing 1 mM EDTA and 300 mM sucrose) to the reaction mixture, mitochondria were precipitated by centrifugation at 4,000 × *g* for 10 min at 4°C. After washing with 100 μl of the suspension buffer, the mitochondria were resuspended in 50 μl of the same buffer. Mitochondria were combined after the AK2 and AK3 import reactions, and mitoplasts were prepared as described above. After the mitochondria and mitoplasts had been treated with proteinase K (100-250 μg/ml), proteins were resolved by 0.1% SDS-12% PAGE for AK isozymes or by 0.1% SDS-8.5% PAGE for AspAT, followed by determination with an imaging analyzer (BAS 2000; Fuji Photo Film, Tokyo).

Inhibition of Import by CCCP and Valinomycin—CCCP and valinomycin were dissolved in ethanol. Various concentrations of CCCP and valinomycin (final ethanol concentration in the import mixture, 0.5%) were added to the translation mixture (100 μl) containing 100 μg mitochondria, and the transport was carried out simultaneously with the translation reaction at 28°C for 3 h. The import of AK isozymes and AspAT was analyzed as described above.

Competition of AK Import by Adrenodoxin Precursor—After AK3 and AspAT had been synthesized *in vitro* at 28°C for 30 min in the translation mixture (25 μl), an adrenodoxin precursor (final concentration, 0.1-1.0 μM) and mitochondria (25 μg protein) were added, and the mixture was further incubated at 28°C for 2.5 h for mitochondrial import. For AK2 import, the adrenodoxin precursor (final concentration, 75-750 nM) and mitochondria (100 μg protein) were added to the translation mixture (100 μl) at the beginning of translation, followed by incubation at 28°C for 3 h.

Purification, Denaturation, Reduction, and Alkylation of AK Isozymes—Purified bovine AK2 and AK3 were prepared from *E. coli* cells harboring pVEX-AK2B and pUC7-AK3, respectively. For denaturation, the purified AK isozymes were dialyzed against 7 M urea. For reduction, the purified AK2 was dialyzed against 0.5 M Tris-HCl, pH 8.5, containing 7 M urea and 1 mM EDTA, and then the sample was incubated with 20 mM DTT at 25°C for 2 h (*31*). An aliquot of the DTT-reduced sample was treated with 45 mM iodoacetate in 7 M urea for 30 min in a dark room at 25°C (*32*). The alkylated AK2 was dialyzed against a 7-M urea solution to remove free iodoacetate.

Competition of AspAT Import by AK Isozymes—After translation of AspAT for 30 min, the purified AK isozymes (final concentration, 1.0 μM) and mitochondria were added to the translation mixture of AspAT, followed by incubation for 2.5 h. In other experiments, the denatured, reduced, and alkylated isozymes (final concentrations, 0.1-0.4 μM) were added to the translation mixture of AspAT, and then the import of AspAT was analyzed as described above.

RESULTS

Subcellular Localization of AK Isozymes—AK2 is often used as a marker enzyme of the mitochondrial intermembrane space (*33*). However, previous analysis of AK activity showed that AK2 is also distributed in the cytosol of liver cells (*5*). To check the subcellular localization of AK isozymes, we prepared total cell extracts of rat liver, and separated the cytosolic and mitochondrial fractions. Aliquots were analyzed by Western blot analysis using antiserum against AK3, which cross-reacts with AK2 (Fig. 1). AK3 (25.6 kDa) was detected almost exclusively in the mitochondrial fraction, while AK2 (26.0 kDa) was detected in both the mitochondrial and cytosolic fractions, confirming the previous reports (*5, 6*). The amount of AK2 protein in the mitochondrial fraction comprised about 50% of the total cell extract protein.

Submitochondrial Localization of AK Isozymes—The location of AK3 in the mitochondrial matrix was not definitely determined. Thus, we analyzed the submitochondrial locations of AK2 and AK3. Mitochondria and mitoplasts were prepared from rat liver, and proteins were subjected to SDS-PAGE. Western blot analysis using the same antiserum revealed that AK2 existed in mitochondria (Fig. 2A, lane 1), but not in mitoplasts (Fig. 2A, lane 2). AK3 was detected in both mitochondria and mitoplasts, and the amount of AK3 in mitoplasts was the same as that in mitochondria (Fig. 2A, lanes 1 and 2). AK3 in mitoplasts was resistant to proteinase K digestion (Fig. 2A, lane 3), whereas it was completely destroyed by the protease after rupturing of the mitoplasts with Triton X-100 (Fig. 2A, lane 4). Thus, we concluded that AK2 is located in the intermembrane space, and AK3 in the matrix of mitochondria.

Mitochondria prepared from rat liver were treated with various concentrations of digitonin and then precipitated by centrifugation. The precipitates were subjected to SDS-PAGE, and AK proteins were analyzed by Western blot analysis (Fig. 2B). The bands of AK2 decreased gradually after treatment with increasing concentrations of digitonin, and disappeared with 0.30 mg/ml of the detergent. The enzyme activity of kynurenine hydroxylase, an outer membrane enzyme, disappeared completely with 0.15 mg/ml digitonin, respectively. These results were consistent with the data previously reported (*33*). AK3 remained in mitochondria unless they were treated with a digitonin concentration of more than 0.40 mg/ml. This pattern of

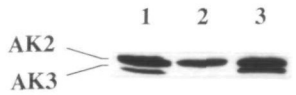

Fig. 1. **Subcellular localization of AK2 and AK3 in rat liver.** The total cell extract, and cytosolic and mitochondrial fractions were prepared from rat liver as described under "MATERIALS AND METHODS." The amounts of protein in the cytosolic and mitochondrial fractions were 100 and 40 mg, respectively. The total and specific enzyme activities of lactate dehydrogenase were 1,300 and 13 U/mg protein, respectively, for the cytosolic fraction, and 6 and 0.15 U/mg protein, respectively, for the mitochondrial fraction. The total and specific enzyme activities of succinate dehydrogenase were 0.22 and 0.002 U/mg protein, respectively, for the cytosolic fraction, and 13 and 0.32 U/mg protein, respectively, for the mitochondrial fraction. A part (1/1,300) of each fraction was subjected to 0.1% SDS-12% PAGE, and the proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was analyzed using antiserum against AK3 and ECL Western blotting detection reagents. Lane 1, rat liver extract; lane 2, cytosolic fraction; lane 3, mitochondrial fraction.

disappearance of AK3 from mitochondria upon digitonin treatment was similar to that of fumarase, a soluble matrix enzyme (*18*). Proteins integrated into the inner membrane, such as cytochrome *c* heme lyase, are not released by digitonin (*18*). Thus, we speculated that AK3 is in a soluble state in the matrix.

In Vitro Import Accompanied by Translation of AK Isozymes—To analyze the import mechanism, isolated rat liver mitochondria were added to the *in vitro* translation reaction mixture of reticulocyte lysates. When the import experiments were carried out after completion of translation, as usually done for mitochondrial protein import (*10, 24*), we could detect the import products of AK3, but not those of AK2. Thus, we chose a system in which the import and translation reactions proceeded simultaneously to analyze protein import (Fig. 3). When the AspAT import into mitochondria was carried out as a control, the mature AspAT (44.6 kDa) migrated between the precursor (47.4 kDa) and a minor product (41.4 kDa) (Fig. 3A, lanes 1 and 2), as reported by Nishi *et al.* (*10*), and it was resistant to proteinase K digestion (Fig. 3A, lane 4). Without mitochondria, the AspAT precursor was completely digested by proteinase K (Fig. 3A, lane 3). More than 50% of the translated AspAT product was imported into mitochondria in our system (Fig. 3A, lane 2).

In the AK3 import reaction, a discrete band corresponding to the authentic size of AK3 was detected for mitochondria after proteinase K treatment (Fig. 3B, lane 2). AK3 was completely digested by the protease after disruption of the mitochondria with Triton X-100 (Fig. 3B, lane 3). About 40% of the translated AK3 product was imported into mitochondria (Fig. 3B, lanes 1 and 2). AK2 of the authentic size was also detected in the mitochondrial fraction after proteinase K digestion (Fig. 3C, lane 2). The AK2 in mitochondria became sensitive to the protease after the addition of Triton X-100 (Fig. 3C, lane 3). The yield of AK2 in the import comprised about 10% of the translation product (Fig. 3C, lanes 1 and 2).

To determine the submitochondrial locations of the imported AK isozymes, mitoplasts were prepared and the imported proteins were analyzed by SDS-PAGE (Fig. 3D). While the AK2 band disappeared for the mitoplasts, the AK3 band remained (Fig. 3D, lane 2). The AK3 in the mitoplasts was resistant to protease digestion (Fig. 3D, lane 3), but was sensitive to the digestion in the presence of Triton X-100 (Fig. 3D, lane 4). Thus, we concluded that AK2 was imported into the intermembrane space, and AK3 into the matrix.

Inhibition of AK2 Import by Cycloheximide—Our obser-

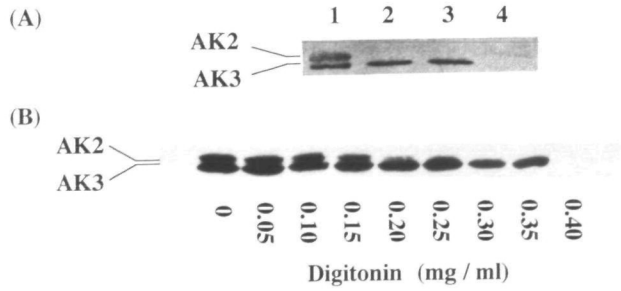

Fig. 2. **Submitochondrial localization of AK2 and AK3 in rat liver mitochondria.** Mitochondria and mitoplasts were prepared as described under "MATERIALS AND METHODS." The samples were subjected to 0.1% SDS-12% PAGE, followed by analysis with antiserum against AK3 and ECL Western blotting detection reagents. (A) AK isozymes in mitochondria and mitoplasts. Lane 1, mitochondria (300 μg of protein); lane 2, mitoplasts prepared from the mitochondria (300 μg protein); lane 3, mitoplasts treated with 250 μg/ml proteinase K; lane 4, mitoplasts treated with 250 μg/ml proteinase K and 1% Triton X-100. (B) AK isozymes in mitochondria treated with various concentrations of digitonin. The mitochondria (300 μg) were treated with digitonin (final concentration, 0-0.40 mg/ml) and then precipitated by centrifugation. The enzyme activity of kynurenine hydroxylase that was released from mitochondria was 7, 82, and 100% of the total activity (specific activity, 0.012 U/mg protein) with 0.05, 0.10, and 0.15 mg/ml digitonin, respectively. The activity of fumarase that was released from mitochondria was 8, 13, and 94% of the total activity (specific activity, 92 U/mg protein) with 0.30, 0.35, and 0.40 mg/ml digitonin, respectively.

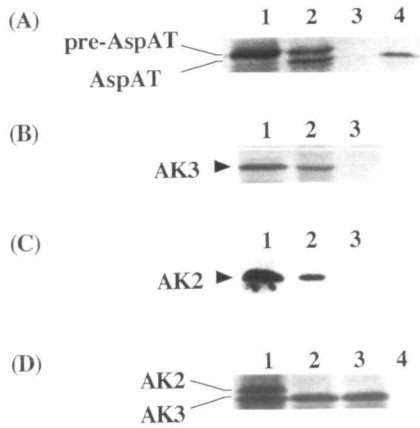

Fig. 3. **Import of AspAT, AK2, and AK3 into rat liver mitochondria.** The preparation of mitochondria, *in vitro* translation, and import reactions were carried out as described under "MATERIALS AND METHODS." Proteins were resolved by 0.1% SDS-12% PAGE. (A) Import of AspAT. Lane 1, *in vitro* translation product (10 μl of the translation mixture); lane 2, product of import accompanied by translation (25 μl of the translation mixture containing 25 μg mitochondria); lane 3, *in vitro* translation product (10 μl of translation mixture) treated with proteinase K (150 μg/ml); lane 4, product of import accompanied by translation (25 μl of the translation mixture containing 25 μg mitochondria) treated with proteinase K (150 μg/ml). The positions of the precursor (pre-AspAT) and mature form (AspAT) of AspAT are indicated on the left. (B) Import of AK3. Lane 1, product of import accompanied by translation (25 μl of the translation mixture containing 25 μg mitochondria); lane 2, mitochondria (25 μg) were recovered after import and treated with proteinase K (150 μg/ml); lane 3, mitochondria (25 μg) were recovered after import and treated with proteinase K (150 μg/ml) and Triton X-100 (1%). (C) Import of AK2. Lane 1, product of import accompanied by translation (50 μl of the translation mixture containing 50 μg mitochondria); lane 2, mitochondria (100 μg) were recovered after import and treated with proteinase K (250 μg/ml); lane 3, mitochondria (100 μg) were recovered and treated with both proteinase K (250 μg/ml) and Triton X-100 (1%). (D) Submitochondrial location of the imported AK isozymes. The import reaction was carried out separately for AK2 and AK3 with 25 μg and 100 μg mitochondria, respectively. Mitochondria were recovered from each reaction mixture and then combined. Mitoplasts were prepared as described under "MATERIALS AND METHODS." Lane 1, mitochondria (125 μg) treated with proteinase K (250 μg/ml); lane 2, mitoplasts prepared from mitochondria (125 μg); lane 3, mitoplasts prepared from mitochondria (125 μg) and treated with proteinase K (250 μg/ml); lane 4, mitoplasts prepared from mitochondria (125 μg) and treated with both proteinase K (250 μg/ml) and Triton X-100 (1%). The positions of AK2 and AK3 are indicated on the left.

vation that AK2 was not imported into mitochondria after completion of translation suggested that the import of AK2 might be a co-translational event. To analyze the import mechanism further, we compared the AK3 and AK2 import in the presence of cycloheximide, which inhibits protein synthesis. Since the efficiency of translation decreased in the presence of mitochondria, which might be due to nucleases and proteases in the mitochondrial preparation, three procedures were used for import experiments (Fig. 4). On the addition of mitochondria after 30 min of translation (procedure 3), the translation product of AK3 increased about twofold in 3 h (Fig. 4A, lanes 1 and 3), and the AK3 import increased concomitantly (Fig. 4B, lanes 1 and 3). When cycloheximide was added to the translation mixture together with mitochondria after the translation had proceeded for 30 min (procedure 2), the amount of imported AK3 was found to have increased about 1.5-fold as compared with that obtained with procedure 1 (Fig. 4B, lanes 1 and 2), which reflected the amounts of the translation product of AK3 in these procedures (Fig. 4A, lanes 1 and 2). About 40% of the translated AK3 product was imported with each procedure. The observations that AK3 product accumulated during the first 30-min of incubation for translation was imported into mitochondria and that the amount of imported AK3 paralleled that of the translation product indicated that AK3 was imported post-translationally.

In contrast to AK3, on the addition of cycloheximide together with mitochondria after 30 min, the amount of imported AK2 was less than 5% as compared with that with procedure 1 (Fig. 4D, lanes 1 and 2), although the amount of the translation product was greater with procedure 2 (Fig. 4C, lanes 1 and 2). Similarly, the imported AK2 with procedure 3 did not increase compared with that with procedure 1 (Fig. 4D, lanes 1 and 3), even though the amount of the translation product was the greatest among three procedures (Fig. 4C, lane 3). The import efficiency of AK2 was about 10, 1, and 3% of the translated product with procedure 1, 2, and 3, respectively. These results indicated that AK2 was not imported efficiently into mitochondria post-translationally.

Requirement of Membrane Electrochemical Potential for Import of AK Isozymes—We examined the requirement of a membrane electrochemical potential for the import of AK isozymes (Fig. 5). In a control experiment, the import of AspAT was almost completely inhibited by both 50 μM CCCP, an uncoupler, and 5 μM valinomycin, a potassium ionophore (Fig. 5A). The import of AK3 and AK2 was also inhibited by both CCCP and valinomycin (Fig. 5, B and C). The inhibition pattern of AK2 with increasing concentrations of the inhibitors resembled that of AK3. These findings indicated that both AK2 and AK3 require the inner membrane electrochemical potential for their import.

Competition of AK Import by Adrenodoxin Precursor—Both the precursors of adrenodoxin and AspAT were reported to be translocated into the mitochondrial matrix through the general import pathway (*9, 10*), and it is known that the adrenodoxin precursor becomes import-competent when denatured in 7 M urea (*9*). We carried out import competition experiments with the urea-denatured protein. The urea-denatured adrenodoxin precursor competed with both AK2 and AK3 as well as with the AspAT precursor for the mitochondrial import (Fig. 6, lanes 3 and 4). The same concentration of denatured bovine serum albumin (with the same final concentration of urea) did not inhibit the import of the AspAT precursor or AK isozymes (Fig. 6, lane 2). These results suggested that AK2 and AK3 were imported into mitochondria through the general import pathway like the adrenodoxin precursor and the AspAT precursor.

Competition of AspAT Import by AK Isozymes—Next, competition experiments on AspAT import with the purified AK2 and AK3 were performed. Neither the purified AK3 (Fig. 7A, lane 2) nor the purified AK2 (Fig. 7B, lane 2) competed with the AspAT import when they were added as native forms to the post-translational import system. These results suggested that once the protein folding of AK

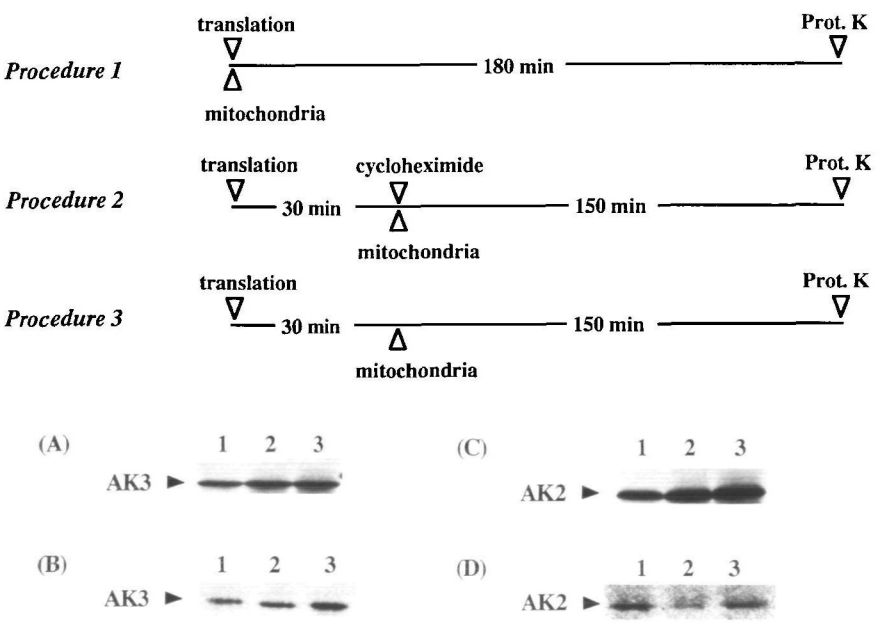

Fig. 4. **Effect of a protein synthesis inhibitor on the import of AK2 and AK3.** The preparation of mitochondria and import reactions were carried out as described under "MATERIALS AND METHODS." The upper panel shows the protocol for incubation for the translation and import reactions. Samples were resolved by 0.1% SDS-12% PAGE. The translation was carried out in the reaction mixture (100 μl for AK2 import and 25 μl for AK3 import). Mitochondria (100 μg for AK2 import and 25 μg for AK3 import) were added as indicated. Cycloheximide (10 mM) was added for Procedure 2. After the reactions, the mitochondria were recovered by centrifugation and then treated with proteinase K (250 μg/ml for AK2 import and 150 μg/ml for AK3 import). (A) Translation products of AK3 (25 μl of the reaction mixture). (B) Mitochondria (25 μg) recovered after AK3 import. (C) Translation products of AK2 (25 μl of the reaction mixture). (D) Mitochondria (100 μg) recovered after AK2 import. Lanes 1-3 are imported proteins with Procedures 1-3, respectively. The positions of AK2 and AK3 are indicated on the left.

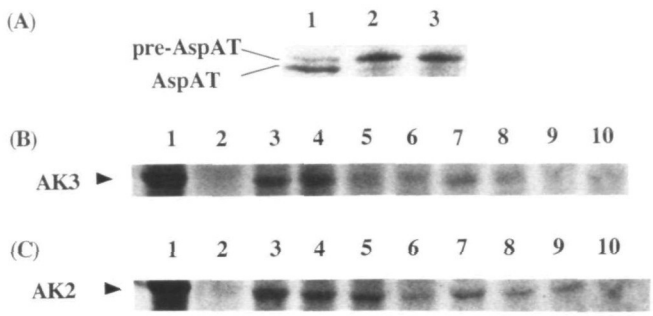

Fig. 5. **Dependence of AspAT import and AK isozyme import on the inner membrane electrochemical potential.** The *in vitro* import of AspAT and AK isozymes was performed as described under "MATERIALS AND METHODS," and the imported proteins after treatment of the mitochondria with proteinase K were resolved by SDS-PAGE. (A) AspAT import. The product of import accompanied by translation (25 μl translation mixture containing 25 μg mitochondria) was subjected to 0.1% SDS-8.5% PAGE. Lane 1, product of import in the absence of an inhibitor; lane 2, product of import in the presence of CCCP (50 μM); lane 3, product of import in the presence of valinomycin (5 μM). The positions of the precursor (pre-AspAT) and mature form (AspAT) are indicated on the left. (B) AK3 import. Mitochondria (100 μg) were recovered after import accompanied by translation and treated with 250 μg/ml proteinase K. Lane 1, product of import in the absence of an inhibitor; lane 2, same sample as in lane 1 with 1% Triton X-100 added before proteinase K treatment; lanes 3-6, product of import in the presence of 5, 10, 20, and 50 μM CCCP, respectively; lanes 7-10, product of import in the presence of 0.1, 1, 5, and 10 μM valinomycin, respectively. (C) AK2 import. Mitochondria (100 μg) were recovered after import accompanied by translation and treated with 250 μg/ml proteinase K. Lane 1, product of import in the absence of an inhibitor; lane 2, same sample as in lane 1 with 1% Triton X-100 added before proteinase K treatment; lanes 3-6, product of import in the presence of 5, 10, 20, and 50 μM CCCP, respectively; lanes 7-10, product of import in the presence of 0.1, 1, 5, and 10 μM valinomycin, respectively. The positions of AK2 and AK3 are indicated on the left.

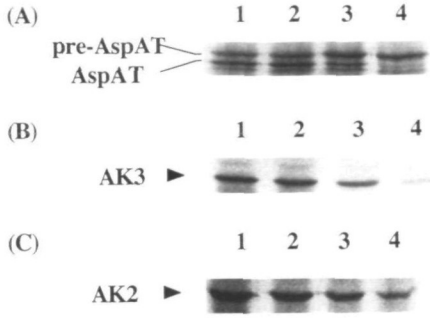

Fig. 6. **Competition of AspAT and AK isozyme import with the urea-denatured adrenodoxin precursor.** The competition assay was performed as described under "MATERIALS AND METHODS." (A) AspAT import. The products of import (25 μl of translation mixture containing 25 μg mitochondria) were resolved by 0.1% SDS-8.5% PAGE. Lane 1, product of import without a competitor; lane 2, product of import competed with 1 μM urea-denatured bovine serum albumin; lanes 3 and 4, product of import competed with 0.1 μM and 1 μM urea-denatured adrenodoxin precursor, respectively. The positions of the precursor (pre-AspAT) and mature form (AspAT) are indicated on the left. (B) AK3 import. Mitochondria (25 μg) were recovered by centrifugation after import (25 μl of translation mixture) and treated with proteinase K (150 μg/ml). The proteins were resolved by 0.1% SDS-12% PAGE. Lane 1, product of import without a competitor; lane 2, product of import competed with 1 μM urea-denatured bovine serum albumin; lanes 3 and 4, product of import competed with 0.1 μM and 1 μM urea-denatured adrenodoxin precursor, respectively. (C) AK2 import. Mitochondria (100 μg) were recovered after import (100 μl of translation mixture) and treated with proteinase K (250 μg/ml). The mitochondrial proteins were resolved by 0.1% SDS-12% PAGE. Lane 1, product of import without a competitor; lane 2, product of import competed with 750 nM urea-denatured bovine serum albumin; lanes 3 and 4, product of import competed with the 75 and 750 nM urea-denatured adrenodoxin precursor, respectively. The positions of AK2 and AK3 are indicated on the left.

molecules is completed, they can not be imported into mitochondria, as has often been seen for other mitochondrial proteins (*13*). Urea-denatured AK3 (Fig. 7A, lanes 3-5) competed with AspAT in its mitochondrial import. The results indicated that AK3 was imported through the general import pathway post-translationally in an unfolded state like the adrenodoxin precursor.

In contrast, urea-denatured AK2 did not compete with AspAT (Fig. 7B, lane 3). It was possible that urea-denatured AK2 was immediately refolded after dilution, thereby regaining its native conformation, in the transport experiments and therefore became incompetent as to import inhibition. In fact, the enzyme activity of urea-denatured AK2 was restored on dilution much more quickly than that of urea-denatured AK3 (data not shown). The rapid refolding of urea-denatured AK2 is probably caused by the stable conformation due to an intramolecular disulfide bond which is absent in AK3 (*34*). To test this possibility, we tried to prevent disulfide bond formation in AK2 by incubating it with 20 mM DTT in a 7-M urea solution. Furthermore, we treated the reduced AK2 with iodoacetate to prepare AK2 alkylated at the -SH groups. Neither the reduced nor the alkylated AK2 competed with AspAT for mitochondrial import (Fig. 7B, lanes 4 and 5). The enzyme activity of urea-denatured and reduced AK2 was slowly restored on dilution, but the denatured alkylated AK2 showed no restoration of its activity on dilution. These data indicated that AK2 could not be imported into mitochondria post-translationally even though it was in an unfolded conformation under our import conditions.

DISCUSSION

The three AK isozymes, AK1, AK2, and AK3, are synthesized in the cytosol and go to different destinations in the cell without undergoing proteolytic cleavage. In this study, the mechanisms of targeting and sorting to different mitochondrial compartments of AK2 and AK3 were analyzed. Since AK2 and AK3 are very similar to each other in the primary as well as the tertiary structure, we expected that both isozymes should be imported into the mitochondria through a common route. We have shown that both matrix-targeting AK3 and intermembrane-space-targeting AK2 require an inner membrane electrochemical potential (Fig. 5), and compete with the adrenodoxin precursor (Fig. 6) in the translocation process. Therefore, it is thought that both AK2 and AK3 are commonly translocated into mitochondria through the general import pathway consisting of Tom and Tim proteins, like other cleavable mitochondrial proteins, at least at a certain step. Thus the route used for AK2 import is different from those previously reported for noncleavable intermembrane-space-targeting proteins, cytochrome *c* and cytochrome *c* heme lyase, which

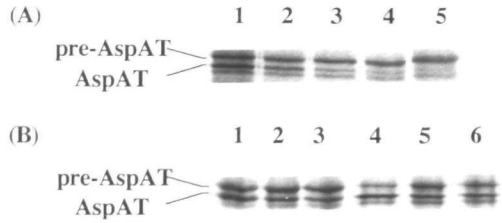

Fig. 7. **Competition of AspAT import with AK isozymes.** The competition assay was performed in the same manner as in Fig. 5A. The positions of the precursor (pre-AspAT) and mature form (AspAT) are indicated on the left. The import product (25 μl of the translation mixture containing 25 μg of mitochondria) was resolved by 0.1% SDS-8.5% PAGE. (A) AspAT import competed with AK3. Lane 1, product of import without a competitor; lane 2, product of import competed with 1 μM native AK3; lanes 3-5, product of import competed with 0.1, 0.2, and 0.4 μM urea-denatured AK3, respectively. (B) AspAT import competed with AK2. Lane 1, product of import without a competitor; lane 2, product of import competed with 1 μM native AK2; lane 3, product of import competed with 0.4 μM urea-denatured AK2; lane 4, product of import competed with 0.4 μM urea-denatured 20 mM DTT-treated AK2; lane 5, product of import competed with 0.4 μM urea-denatured iodoacetate-treated AK2; lane 6, product of import competed with 0.4 μM urea-denatured iodoacetate-treated BSA.

are directly translocated to the compartment without interaction with the inner membrane (*18, 19*).

Cytochrome b_2 and cytochrome c_1 are cleavable intermembrane-space-targeting proteins that use the general import pathway (*11*). These two proteins possess bipartite presequences comprising the targeting and sorting signals, comprising an N-terminal, highly charged sequence followed by a more hydrophobic sequence (*11*). It is proposed that during the import across the outer and inner membranes these proteins stop when the hydrophobic sorting sequence interacts with the inner membrane after the N-terminal targeting sequence has entered the matrix. The N-terminal portion is cleaved by the matrix processing protease, and subsequently the polypeptide is laterally transferred away from the general import pathway in the intermembrane space where the precursor stuck on the outer face of the inner membrane is cleaved to release the mature protein in this submitochondrial compartment (*27*). In the case of AK2 and AK3, the N-terminal regions (residues 16-33 in AK2 and residues 6-23 in AK3) have the ability to form positively charged amphipathic helices, which might serve as targeting sequences in the transport across the mitochondrial membranes (*16*). It is believed that the positive charges of the import proteins are electrophoretically pulled by the electrochemical potential of the inner membrane of mitochondria ($\Delta\psi$, positive outside). Moreover, the ATP-driven import motor would further pull the polypeptides into the matrix across the inner membrane. Since an inner membrane electrochemical potential was needed for the transport of AK2 and AK3, the sequences mentioned above would be the targeting signals for AK2 and AK3, although they are not cleaved during the transport process. Overlapping with the presumed targeting sequence there is a short and weak hydrophobic stretch (residues 20-29) in AK2 which might be regarded as a sorting sequence, but it is thought to be unsuitable for involvement in the stop-transfer mechanism in the inner membrane.

Most mitochondrial proteins that have been tested so far are imported into isolated mitochondria post-translationally. However, AK2 was not imported into mitochondria in a post-translational manner (Fig. 4, Procedure 2). It was imported into mitochondria only when the transport reaction was accompanied by translation (Fig. 4, Procedure 1). The inherent quick folding nature of the AK2 protein could have inhibited its mitochondrial import. However, even completely unfolded AK2, which was obtained by either reduction of a disulfide bond or alkylation of the -SH groups, was not import-competent (Fig. 7). Molecular chaperones that keep the AK2 polypeptide import-competent may be further necessary for our *in vitro* transport system. The possibility that AK2 is imported post-translationally *in vivo* can not be ruled out. However, the following interpretation seems to be compatible with the results obtained in this study. The nascent AK2 polypeptide proceeds along the general import pathway in a co-translational manner. Since the nascent polypeptide is anchored by ribosome on the outer surface of the outer membrane, the short and weak hydrophobic stretch of the N-terminal region of AK2 can interact with the inner membrane when it reaches that location. AK2 moves from the protein-conductive channel of the general import pathway to the intermembrane space as the polypeptide elongation progresses. The process of sorting of AK2 might benefit from the quick folding of the polypeptide into the native conformation. Previous *in vivo* and *in vitro* experiments demonstrated that mitochondrial import is tightly coupled with protein synthesis in the cases of the majority of yeast mitochondrial proteins (*35, 36*). Therefore, it is attractive to assume as above that AK2 import to isolated rat mitochondria is an example of the co-translational import of mitochondrial proteins. In contrast to AK2, AK3 is imported in a post-translational manner like other mitochondrial proteins. It can pass through the outer and inner membranes into the matrix, because the N-terminal hydrophobic stretch of AK3 is not strong enough to stop at the inner membrane, and the matrix import motor can pull the protein across the inner membrane.

AK2 is unique in that it is bi-topologically distributed in the cell; about half of the protein is present in the cytosol, whereas the remaining half is located in the mitochondria (Fig. 1). In general, a protein which has an organella-targeting signal in its molecule is almost exclusively located in that particular organella. An exception is PAI-2, which is a member of the SERPIN family of serine-protease inhibitors (*37*), and translocated in both the cytosol and endoplasmic reticulum (*38*). Some PAI-2 molecules are recognized by the machinery for co-translational import into the endoplasmic reticulum. The rest of the molecules remain unrecognized by the import machinery and thus stay in the cytosol. Our results suggested that AK2 uses a co-translational import mechanism by which the protein can be localized in both the mitochondrial intermembrane space and the cytosol. On the basis of the results of analysis of AK activity in intact tissues involving measuring ^{18}O incorporation into adenine nucleotides, Zeleznikar *et al.* proposed that a high energy β-phosphoryl transfer chain involving multiples of two AK molecules operates in energy-requiring tissues (*39*). The existence of AK2 molecules in different subcellular compartments may be useful for the formation of such a β-phosphoryl transfer

chain in the cell.

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