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Nucleoside diphosphate kinase III is localized to the inter-membrane space in plant mitochondria

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Abstract Three types of nucleoside diphosphate kinases (NDPKs) are found in plants but the intra-cellular compartmentation of these proteins is not certain, especially the location of the recently identified type III proteins. Through the fractionation of plant mitochondria from potato and *Arabidopsis*, display of protein profiles by 2D gel electrophoresis, and identification by mass spectrometry, we present the first direct evidence that type III proteins are localized in the inter-membrane space of plant mitochondria. The possible metabolic functions of NDPK III are discussed in light of its sub-cellular localization.

Key words: Plant mitochondrion; Nucleoside diphosphate kinase; Proteomic

1. Introduction

Nucleoside diphosphate kinases (NDPKs) are ubiquitous and highly conserved enzymes that catalyze the transfer of γ phosphates from nucleoside triphosphates to nucleoside diphosphates. This activity is crucial for maintaining the balance between cellular adenine triphosphates and other nucleoside triphosphates, particularly GTP [1]. However, in addition to this primary metabolic role, there is increasing evidence that NDPK proteins may be involved in a variety of regulatory functions. For example, the Nm23 gene family in humans, that were initially identified as suppressors of metastasis in carcinoma cell lines, encode NDPK proteins [2]. This suppression function is independent of NDP kinase activity, but instead is associated with a protein phosphotransferase activity that occurs when NDPK forms a complex with the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase [3,4]. As well as this association with glyceraldehyde-3-phosphate dehyrogenase, human NDPKs have also been shown to act as transcription factors [5] and as potential regulators of the HSP class of molecular chaperones [6]. Although most of the animal NDPKs are located in the cytosol, a mitochondrial NDPK has also been identified that is present either in the mitochondrial matrix [7] or in the inter-membrane space (IMS) [8].

In contrast to this wealth of information about animal NDPKs, much less is known about the function and sub-cellular distribution of NDPKs in plants. The induction of plant NDPKs during cellular responses to a variety of insults, including wounding [9], heat shock [10] and UV [11], suggests that plant NDPKs may also have a regulatory role in addition to their primary metabolic function. Genome and cDNA sequencing has revealed three types of NDPK sequences in plants based on similarity [12]. Type I NDPKs contain no identifiable targeting sequences and are thought to be located in the cytosol. Type II NDPKs have an N-terminal extension that suggests targeting to the chloroplast stroma. A third type of NDPK from spinach was fully sequenced by Edman degradation and said to be located in the chloroplast in spinach [13], but no cDNA encoding this protein has yet been cloned. More recently, a mitochondrial NDPK was characterized in pea and EDTA inhibition of the autophosphorylation of this protein in intact mitochondria, indirectly suggesting an IMS localization [14]. When the cDNA for this pea protein was cloned, it had highest similarity to the NDPK III from spinach [12]. This pea cDNA contained an N-terminal extension, suggesting organelle targeting, and import studies of in vitro translated precursor showed import and processing in pea mitochondria.

We have been investigating the IMS components in mitochondria from two plant species: *Solanum tuberosum* and *Arabidopsis thaliana* using proteomic approaches. The IMS contains proteins that fulfil a variety of functions from electron transport, to import and assembly of proteins [15], to induction of apoptosis [16]. While this sub-compartment has been investigated in other organisms, there is very little known about its protein complement in plants. We have both discovered proteins in this compartment identified by mass spectrometry (MS) as NDPKs. The two cDNAs encoding these genes have been identified from genomic databases. Sequence comparisons show that both proteins are type III NDPKs. Use of new targeting prediction programs clearly suggests that three classes of NDPKs are present in plants, one cytosolic, one putative chloroplastic and one putative mitochondrial. This represents the clearest direct evidence that type III NDPKs in higher plants are localized to the mitochondrial IMS.

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Abbreviations: IEF, isoelectric focusing; IMS, inter-membrane space; NDPK, nucleoside diphosphate kinase

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2. Materials and methods

2.1. Potato mitochondria isolation and fractionation

Potato tubers (*S. tuberosum* cv Desiree) mitochondria were isolated according to [17] using a 28% (v/v) Percoll density gradient. Sub-fractionation of mitochondria was performed by a modification of [18]. Mitochondria (100 mg) were resuspended in a 50 mM sucrose, 2 mM EDTA, 10 mM MOPS–KOH (pH 6.7) stirred on ice for 15 min. Osmotic strength was adjusted to 0.3 M sucrose and the suspension centrifuged for 15 min at $20\,800\times g$. The pellet was resuspended in standard wash medium supplemented with 250 mM KCl, incubated for 15 min and again centrifuged for 15 min at $20\,800\times g$. The IMS/outer membrane supernatants were centrifuged for 1 h at $128\,000\times g$ to yield a soluble protein supernatant of IMS proteins and a membrane pellet. The KCl-washed pellet containing inner-membrane and matrix proteins was ruptured by repeated freeze–thawing, and centrifugation for 15 min at $20\,800\times g$ yielded separate matrix and membrane fractions. IMS soluble proteins were concentrated for analysis on 5 kDa centrifugal filters (Millipore, Sydney).

2.2. Arabidopsis mitochondria isolation and fractionation

Mitochondria were isolated from dark-grown *Arabidopsis* cell suspension cultures according to [19] using Percoll density gradient centrifugation. Sub-fractionation of mitochondria was done as follows: Mitochondria were resuspended to a final protein concentration of 10 mg/ml in 70 mM sucrose, 10 mM Tes–KOH (pH 7.5) and incubated on ice with occasional mixing for 15 min. The osmotic strength was adjusted to 0.3 M sucrose, and after a further 15 min incubation on ice, the suspension was centrifuged for 10 min at $18\,000\times g$, yielding a supernatant containing IMS and outer membrane and a mitoplast pellet. The IMS/outer membrane supernatant was centrifuged for 10 min at $200\,000\times g$ to yield a soluble protein supernatant of IMS proteins and an outer membrane pellet. The mitoplast pellet was ruptured by repeated freeze–thawing, and centrifugation for 15 min at $18\,000\times g$ yielded separate matrix and inner membrane fractions.

2.3. Electrophoresis and immunodetection

1D SDS–PAGE was performed according to standard protocols using 14% (w/v) polyacrylamide gels and a Tris/Tricine split buffer system [20]. For immunodetection, proteins were transferred onto a nitrocellulose membrane and incubated with primary antibodies raised to UCP or F_1F_0 ATP-synthase α subunit (inner membrane markers), cytochrome *c* (IMS marker), porin, (outer membrane marker) and HSP70 or fumarase (matrix markers). Antibodies were from the following sources: UCP (Mr. Michael Considine and Dr. Jim Whelan, The University of Western Australia, Australia [21]), F_1F_0 ATP synthase α subunit [22], cytochrome *c* (Pharmingen, San Diego, CA, USA), porin (Dr. Tom Elthon, University of Nebraska, Lincoln, NE, USA), HSP70 (Prof. Elzbieta Glaser, Stockholm University, Sweden), fumarase (Dr. Bernd Müller-Röber, Max-Planck-Institut für Molekulare Pflanzenphysiologie, Golm, Germany). A chemiluminescence detection system linked to horseradish peroxidase was used as a secondary antibody, and quantitative light emission was recorded using a Luminescent Image Analyser (LAS 100, Fuji). 2D isoelectric focusing (IEF)/SDS–PAGE was performed according to [19] also using the Tris/Tricine buffer system for SDS–PAGE.

2.4. Quadrupole time-of-flight (Q-TOF) MS and data analysis

For potato proteins, Q-TOF MS/MS was performed on an Applied Biosystems Q-STAR Pulsar (Q-TOF MS) using an IonSpray source. Proteins to be analyzed were cut from the 2D PAGE gel, dried at 50°C in a dry block heater and stored at −70°C. For the sequencing analysis, the proteins were digested with trypsin according to [19], desalted with C18-ZipTips (Millipore, Sydney), injected in 50% acetonitrile, and selected doubly charged peptides were fragmented by N_2 collision and analyzed by MS/MS. Mass spectra and collision MS/MS data were analyzed with BioAnalyst software (Applied Biosystems, Sydney). For *Arabidopsis* proteins, mass spectrometric analysis was carried out using LC-MS/MS according to [23,24]. Proteins to be analyzed were cut from the 2D PAGE gel and stored in 15 μl of H_2O at −20°C. The HPLC (CapLC, Waters, Milford, MA, USA) was coupled via a Nano-LC inlet to the Q-TOF mass spectrometer (Micromass UK Ltd., Manchester, UK) equipped with a nanoelectrospray Z-spray source. The tryptic peptides were concentrated and desalted on a 300 mm ID/5 mm length C18 PepMap column (LC Packings, San Francisco, CA, USA). Elution of peptides was carried out with 80% acetonitrile over 13 min at a flow rate of 200 nl/min. The eluted peptide mixture was analyzed by tandem mass spectrometric sequencing with an automated MS-to-MS/MS switching protocol. Online determination of precursor-ion masses was performed over the *m/z* range from 300 to 1200 atomic mass units in the positive charge detection mode with a cone voltage of 30 V. The collision-induced decomposition (CID) for peptide sequencing by MS/MS was performed with argon gas at 20–40 eV and a 3 Da quadrupole resolution. Mass spectra were analyzed with the ProteinLynx software (Micromass). The complex CID MS/MS spectra containing fragment ions in multiple charge states were replotted into a simple spectrum of monoisotopic ions using MaxEnt-3 software.

Sub-cellular targeting of predicted protein sequences were performed with TargetP (http://www.cbs.dtu.dk/services/targetp/) as directed by [25] and by Predotar (http://www.inra.fr/internet/produits/predotar/) as directed on this website. Multisequence alignment and dendograms were made using a combination of ClustalW, Protdist, Neighbor and DrawGram in the Bionavigator software suite (http://www.bionavigator.com).

3. Results and discussion

3.1. Fractionation of potato mitochondria

A controlled osmotically induced swelling caused breakage of the mitochondrial outer membrane and released IMS proteins from potato and *Arabidopsis* mitochondria. Outer membrane fragments were removed and collected by centrifugation. The mitochondrial pellet was then separated into membrane and matrix fractions by freeze–thawing and centrifugation. From 100 mg of potato mitochondrial protein as starting material, approximately 55% of the protein was in the membrane fraction, 40% in the matrix fraction and 3 and 1% respectively in the IMS and IMS-salt fractions.

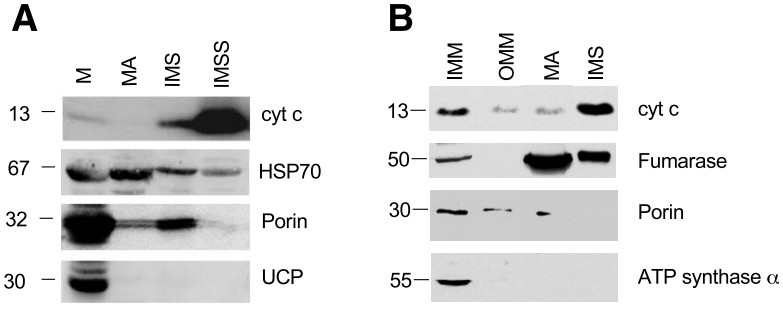


Fig. 1. Fractionation of potato (A) and *Arabidopsis* (B) mitochondria. Mitochondria were fractionated into membrane (M), inner mitochondrial membrane (IMM), outer mitochondrial membrane (OMM), matrix (MA), IMS and salt extracted inter-membrane space (IMSS) components. The specific abundance of marker enzymes for different sub-mitochondrial compartments was determined by immunoblotting: IMS (cytochrome *c*), matrix (HSP70 or fumarase), outer membrane (porin) and inner membrane (UCP or ATP synthase α subunit).

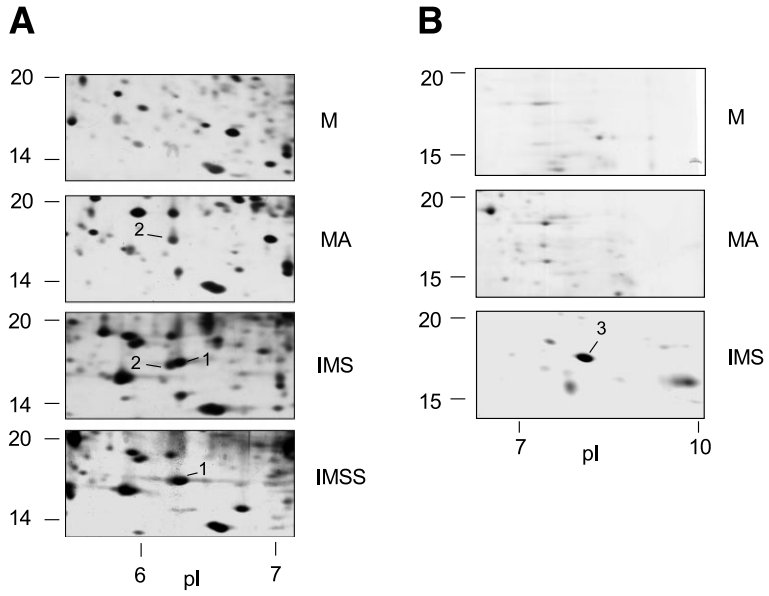


Fig. 2. Identification of IMS-specific proteins from potato (A) and *Arabidopsis* (B) mitochondria using 2D gel electrophoresis. Proteins from membrane (M), matrix (MA), IMS and salt extracted inter-membrane space (IMSS) fractions separated by IEF/SDS–PAGE were compared to identify proteins found only in the IMS fractions. Spot 1 highlights a potato IMS-specific protein and spot 2 a similar-sized potato matrix contaminant. Spot 3 highlights an *Arabidopsis* IMS-specific protein.

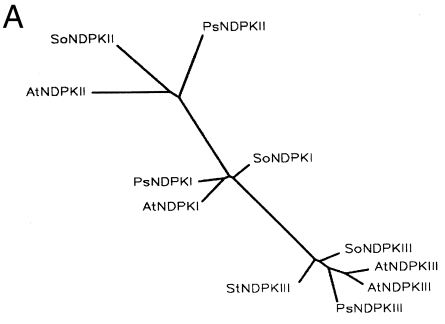
A similar protein distribution was also seen in *Arabidopsis* mitochondria: membrane, 48%; matrix, 42%; IMS, 10%. Marker antibodies for the separate fractions were then used to confirm the purity achieved by this separation technique (Fig. 1). Similar results were obtained with both potato and *Arabidopsis* mitochondria. The IMS marker (cytochrome *c*) has the greatest specific abundance in the IMS fractions and was absent from matrix and membrane fractions. The inner membrane markers, UCP and ATP synthase α subunit, were found exclusively in the membrane fraction. The outer membrane marker, porin, was most abundant in the membrane fraction, while some outer membrane proteins were observed in the IMS and matrix fractions. HSP70 and fumarase, soluble matrix markers, were most abundant in the matrix fraction, although some contamination of other fractions was evident. Quantitation of immunoreactions showed that the IMS fractions were contaminated from 10 to 30% with outer membrane and matrix markers (Fig. 1).

3.2. 2D Gel electrophoresis and comparison of the 14–20 kDa proteins between fractions

Separation of 1 mg of each fraction from either potato or *Arabidopsis* mitochondria by IEF followed by SDS–PAGE revealed a 2D profile of each fraction that was compared to identify putative IMS-specific proteins. In the potato fractions at an apparent molecular mass of 15 kDa and a p*I* value of 6.3, a protein spot was observed that was found exclusively in the IMS and IMS-salt fractions and was absent from matrix and membrane fractions (Fig. 2A, spot 1). Another protein with a very similar position on the gels (apparent molecular mass of 15 kDa and a p*I* value of 6.25) was observed in matrix fraction, IMS and IMS-salt fractions (Fig. 2A, spot 2). In the *Arabidopsis* fractions, a IMS-specific spot was observed at an apparent molecular mass of 18 kDa and a p*I* value of 8.1 (Fig. 2B, spot 3).

3.3. MS identification

These three proteins were excised from the gels, trypsin-digested in gel, extracted and subjected to MS/MS analysis to determine the peptide sequence of specific multiply charged



B

			Target P			Predotar		
	Accession	Length AA	Chl	Mito	Pred	Mito	Plastid	Pred
AtNDPKI	SP P39207	149	0.045	0.111	N1	0.01	0.23	-
PsNDPKI	SP P47922	149	0.026	0.154	N2	0.01	0.15	-
SoNDPKII	SP Q02254	148	0.035	0.123	N1	0.01	0.30	-
PsNDPKII	SP P47923	230	0.887	0.073	C1	0.04	0.97	P
AtNDPKII	SP O64903	231	0.954	0.036	C 1	0.15	0.90	P
SoNDPKII	SP Q01402	233	0.963	0.085	C 1	0.00	0.94	P
SoNDPKIII	SP P81766	153	0.029	0.220	N2	-	-	-
StNDPKIII	TR TC5371	221	0.787	0.562	C4	0.99	0.00	M
PsNDPKIII	TE Q9SP13	233	0.212	0.585	M4	0.95	0.01	M
AtNDPKIII	SP O49203	238	0.412	0.157	C4	0.90	0.00	M
AtNDPKIII	TE Q9T0A5	237	0.226	0.596	M4	0.96	0.00	M

Fig. 3. A: Dendogram of alignment of NDPK I, II, and III sequences from *Arabidopsis* (At), pea (Ps), and spinach (So) with the potato (St) NDPK III. B: Prediction of sub-cellular targeting for the same set of NDPKs using TargetP and Predotar. N: non-organellar, M: mitochondrial, C/P: chloroplastic/plastidic. TargetP prediction, 1–5 = high to low likelihood. Accession numbers from SwissProt (SP), TrEMBL (TE) and TIGR (TR).

Table 1
Sequences of peptides derived from IMS-specific proteins by MS/MS analysis.

Spot No.	MM	p*I*	*m/z*	Sequence	Identification
1	15 000	6.3	841.91 $[M+2H]^{2+}$	LLGATDPQKSE	NDP kinase
			561.61 $[M+3H]^{3+}$	LLGATDPQKSEPGTIR	TIGR TC5371
			669.81 $[M+2H]^{2+}$	NLLHGSDGPETAK	
			443.30 $[M+2H]^{2+}$	GDLAVVGR	MM/p*I* = 23761/9.54
					*MM/p*I* = 18276.9/6.52
2	15 000	6.2	880.65 $[M+2H]^{2+}$	PFKVLWMKKVKLVK	ribosomal protein
			758.51 $[M+2H]^{2+}$	KVLWMKKVKLVK	PIR T01426
3	18 000	8.1	448.87 $[M+3H]^{3+}$	TFIAIKPDGVQR	NDP kinase
			672.82 $[M+2H]^{2+}$	TFIAIKPDGVQR	SP O49203
					MM/p*I* = 25734.42/9.2
					*MM/p*I* = 21820.93/8.02

Protein spots from Fig. 2 were excised, digested with trypsin and multiply charged peptides fragmented and analyzed by MS/MS. The *m/z* ratio and the derived sequence are shown along with match assignments based on homology with database sequences. Leucine and isoleucine residues have identical mass and are shown for simplicity as leucine (L) in each case. *MM (molecular mass) and p*I* calculated following cleavage of the predicted presequence from the precursor protein sequence.

peptides (Table 1). Derivation of amino acid sequences from *m/z* data of fragment ion series and database searching was done using BioAnalyst software (Applied Biosystems) or Mascot (http://www.matrixscience.com/). The sequences of all four peptides derived from the potato spot 1 were identical to regions from a translated mRNA from *S. tuberosum*. The sequence has a high degree of similarity to the spinach NDPK III protein. These four peptides cover a continuous region of 39 aa in the sequence of the potato protein. Spot 2 was not conclusively identified, but the two peptide sequences have significant similarity to an L19 ribosomal protein from *A. thaliana*, suggesting it might be the potato *ortho*log of this protein. The amino acid sequences from spot 2 were not found in any NDPK gene identified in plants. The amino acid sequence of the two peptides from the *Arabidopsis* spot 3 were identical to *Arabidopsis* NDPK III.

3.4. Sequence similarity and targeting prediction of NDPKs in plants

A phylogenetic tree based on alignment of NDPK protein sequences from *Arabidopsis*, spinach and pea reveals three groups corresponding to assignments as type I, II and III (Fig. 3). Currently, there are type III sequences cloned and available in databases from spinach, pea, potato, canola and *Arabidopsis*. The region covered by the peptides sequences obtained for spot 1 in Table 1 are identical across all known plant type III sequences, except for a single Glu-to-Ala change in the pea NDPK III sequence. In contrast, the type I and II sequences were significantly different with only 50–80% identity over the 39 aa region. The peptide sequences derived from the *Arabidopsis* IMS (spot 3) were also identical across all the type III sequences, but this region was only 92% identical to the aligning region in type I sequences and less than 75% identical to the region in type II sequences. Two recent targeting prediction programs that specialize in the distinction between mitochondrial and plastid/chloroplast targeting sequences in plants (TargetP and Predotar) were used to analyze the putative localization of the three types of NDPKs. Type I sequences did not contain any putative targeting sequences. Type II were strongly predicted to be chloroplastic or plastidic by both programs. In contrast, type III proteins were weakly predicted to be either chloroplastic or mitochondria by TargetP, but all strongly predicted to be mitochondrial by Predotar.

3.5. Location of NDPKs in plants

A type III NDPK was first observed in spinach as the third NDPK identified in this species. The authors of this report suggested this NDPK protein was located in the chloroplast, although no data was presented to justify this localization. Unfortunately, no cDNA has ever been cloned encoding this protein so presequence information in unavailable. The discovery of a mitochondrial isoform in pea with similarity to this protein, and its absence from chloroplasts in pea [12], cast some doubt on this localization in spinach. Our localization of similar type III NDPKs to the mitochondrial IMS in *Arabidopsis* and potato clearly show that NDPKs are commonly observed in this organelle in plants. We propose that the simplest explanation of NDPK sub-cellular localization in plants is that each of the three NDPK proteins is located in a different sub-cellular compartment: one located in the cytosol (type I), one in the chloroplast stroma (type II) and one in the mitochondrial IMS (type III). We emphasize, however, that this bioinformatic classification does not preclude the possibility that NDPKs may also be found in other sub-cellular compartments.

3.6. Potential functions of the mitochondrial IMS NDPK

The location of the type III plant mitochondrial NDPK in the IMS raises some interesting questions as to its function. In animals, there is a matrix form of NDPK that functions to metabolize GTP produced by the Krebs cycle enzyme, succinyl CoA ligase [26]. However, since the plant succinyl CoA ligase produces ATP, not GTP [27], this function of NDPK is not required in plants. It would seem logical that an NDPK localized in the mitochondrial IMS, and thus close to the source of cellular ATP exported from the mitochondrial matrix, could play a role in the synthesis of GTP for export to the cytosol or equilibration of NTP pools to sustain DNA synthesis in the matrix.

In addition to this primary role of NDPK in nucleotide metabolism, NDPKs in both animals and plants have been proposed to have a variety of regulatory functions [4–6,10,11]. First, NDPKs can function as transcription factors. For example, *Arabidopsis* NDPK1a can specifically bind to the promotor of a UV-responsive gene, and induces its transcription [11]. However, for NDPK to function as a transcriptional activator, it would clearly have to be present in a DNA-containing compartment, and this would therefore seem an un-

likely role for NDPK III. Secondly, NDPKs can interact with other metabolic proteins, either directly to regulate their function [6] or to form a complex with a modified enzymatic activity [4]. Recently, it was shown that during heat stress, the mitochondrial NDPK III from pea interacts with a 86 kDa protein of unknown function [10]. Clearly there is much work to be done if we are to fully understand the potentially manifold functions of NDPKs in plant metabolism. In clarifying the sub-cellular location of type III NDPKs in plants using a proteomic approach, this work establishes a basic foundation for this research effort and provides some intriguing research directions for unravelling the functional role of IMS components in plant mitochondria.

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