Nucleoside diphosphate kinase of *Saccharomyces cerevisiae*, Ynk1p: localization to the mitochondrial intermembrane space

Boominathan AMUTHA and Debkumar PAIN[†]

Department of Pharmacology and Physiology, UMDNJ – New Jersey Medical School, 185 South Orange Avenue, MSB I-669, Newark, NJ 07101-1709, U.S.A.

Nucleoside diphosphate kinase (NDPK) is a highly conserved multifunctional enzyme. It catalyses the transfer of γ phosphates from nucleoside triphosphates to nucleoside diphosphates by a mechanism that involves formation of an autophosphorylated enzyme intermediate. The phosphate is usually supplied by ATP. NDPK activity in different subcellular compartments may regulate the crucial balance between ATP and GTP or other nucleoside triphosphates. NDPKs are homo-oligomeric proteins and are predominantly localized in the cytosol. In this paper, we demonstrate that in *Saccharomyces cerevisiae* a small fraction of total NDPK activity encoded by *YNK1* is present in the intermembrane space (IMS) of mitochondria, and the corresponding protein Ynk1p in the IMS represents approx. 0.005 % of total mitochondrial proteins. Ynk1p, synthesized as a single gene product, must therefore be partitioned between cytoplasm

and mitochondrial IMS fractions. A mechanism for this partitioning is suggested by our observations that interaction with a 40 kDa protein of the translocase of outer mitochondrial membrane (Tom40p), occurs preferentially with unfolded, unphosphorylated forms of Ynk1p. A population of newly translated, but not yet folded or autophosphorylated, Ynk1p intermediates may be imported into the IMS of mitochondria and trapped there by subsequent folding and oligomerization. Within the small volume of the IMS, Ynk1p may be more concentrated and may be required to supply GTP to several important proteins in this compartment.

Key words: autophosphorylation, protein folding, protein–protein interaction, targeting, Tom40p.

INTRODUCTION

Nucleoside diphosphate kinases (NDPKs) are ubiquitous and highly conserved enzymes; they are crucial for the cellular homeostasis of nucleoside triphosphates (NTPs) and nucleoside diphosphates (NDPs). The enzyme catalyses the transfer of a γ phosphate from NTP to NDP [1]. This involves a Ping Pong mechanism: the enzyme transiently phosphorylates itself on a conserved histidine residue at the catalytic site and then transfers the high-energy phosphate to NDP [2]. Although the enzyme has a broad substrate specificity, the high-energy phosphate is usually supplied by ATP, and the enzyme often acts as a highly localized supplier of GTP for specific reactions, such as dynamin-dependent synaptic vesicle recycling [3]. Interestingly, autophosphorylated NDPK intermediates also exhibit a protein phosphotransferase activity, thereby phosphorylating other proteins that participate in metabolism and other cellular activities [4,5]. Furthermore, NDPKs appear to serve as signalling molecules because of their functions in development, cell differentiation, proliferation, cell motility, tumour metastasis and apoptosis [6–10]. Localization of NDPK activity within different cellular compartments may be critical for its functions.

Many (> 60) NDPK primary sequences and a number of crystal structures from prokaryotes to eukaryotes have been determined [11–13]. All NDPKs are homo-oligomeric proteins; although most NDPKs are hexamers, a few bacterial enzymes are tetramers [14]. Nevertheless, they all have a very similar three-dimensional structure; each subunit contains an α/β domain with a four-stranded antiparallel β -sheet. NDPKs have been

found associated with different cellular compartments, including cytosol [1,15], plasma membrane [15], nucleus [16] and mitochondria [17–22]. With regard to mitochondrial localization, the enzyme, depending on the species, may reside in different subcompartments of the organelle. The pigeon NDPK [20] and the human NDPK (Nm23-H4) [18] were shown to contain a cleavable mitochondrial targeting sequence at the N-terminus of the proteins; the former was found in the mitochondrial matrix, the latter in the mitochondrial membrane. NDPK in *Dictyostelium discoideum* contains an N-terminal extension, which is similar to the bipartite presequence of proteins targeted to the intermembrane space (IMS) [21]. On the other hand, NDPKs in plants do not contain an N-terminal extension and have been proposed to be present in the mitochondrial IMS [19,22]. Nevertheless, experimental evidence for IMS localization of the protein is lacking.

In the yeast *Saccharomyces cerevisiae*, NDPK is encoded by a single nuclear gene, *YNK1* [23]. The *YNK1* coding sequence lacks an identifiable mitochondrial targeting sequence. However, subcellular fractionation and enzyme assays show that NDPK activity is predominantly cytoplasmic, with a small portion (approx. 3 %) associated with mitochondria [15]. These results on subcellular localization of NDPK activity have not been confirmed by experiments other than enzyme assays. This is important because the activity assay measures NDP formation and may not be completely specific for NDPK. A haploid yeast strain lacking the *YNK1* gene still shows approx. 10 % of wild-type activity [23], suggesting that other enzymes such as NTPases may interfere with the specificity of the assay [2]. Furthermore, considering such a low abundance of Ynk1p in mitochondria, it

Abbreviations used: CAT, carboxyatractylsoid; HA, haemagglutinin; IMS, intermembrane space; NDP, nucleoside diphosphate; NDPK, NDP kinase; Ni-NTA, Ni²⁺-nitrilotriacetate; NTP, nucleoside triphosphate; ORF, open reading frame; SE, salt-extractable; Tom40p, 40 kDa protein of the translocase of outer mitochondrial membrane.

[†] To whom correspondence should be addressed (e-mail painde@umdnj.edu).

has been very difficult to rule out the possibility that isolated mitochondria were contaminated with cytoplasmic Ynk1p. In this paper, we provide several independent lines of evidence that the Ynk1p found in purified mitochondria is specifically localized to the IMS. Furthermore, we have investigated a mechanism by which Ynk1p synthesized in the cytoplasm may be partitioned between cytoplasm and IMS of mitochondria.

EXPERIMENTAL

Constructs

Wild-type Ynk1p

The gene encoding the open reading frame (ORF) for Ynk1p was amplified by PCR from a yeast genomic library using primers 5'-GCGGATCCCATATGTCTAGTCAAACAGAAAGAAC-TTTTATTG-3' (sense) and 5'-GCGAATTCCTCGAGTTCATAAATCCACTTAGCTTGATTAG-3' (antisense). The PCR product was digested with *NdeI* and *XhoI*, and cloned into the same sites of pET21b (Novagen, Madison, WI, U.S.A.). The plasmid pET21b/Ynk1p introduces a His₆ tag in frame at the C-terminus of Ynk1p, and was used for bacterial expression.

Mutant Ynk1p (H119G)

His¹¹⁹ residue of Ynk1p was mutated to glycine (H119G) using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). The plasmid pET21b/Ynk1p was used as the template and the two oligonucleotides used were 5'-GCGAG-AAACGCTCTGTGGCGGCAGTGATTCTGTTGATAGC-3' (sense) and 5'-GCTATCAACAGAATCACTGCCGCCACAG-ACGTTTCTGCC-3' (antisense). The mutation was confirmed by sequencing.

Ynk1p with three tandemized copies of haemagglutinin (HA) epitopes (Ynk1p-HA₃)

The *YNK1* ORF was amplified by PCR from a yeast genomic library using primers 5'-GCGGATCCCATATGTCTAGTCAAACAGAAAGAACTTTTATTG-3' (sense) and 5'-CGCAGATCTTTCATAAATCCACTTAGCTTG-3' (antisense). The PCR product was digested with *NdeI* and *BglII*, and cloned into the same sites of pBS-Arh1HA3 [24]. The *NdeI*-*SalI* fragment of the resulting plasmid (pBS/Ynk1p-HA₃), containing the *YNK1* ORF and the three copies of tandemized HA tags, was cloned downstream of the constitutive yeast glyceraldehyde-3-phosphate dehydrogenase promoter in the vector pRS416 [25]. The resulting plasmid was referred to as pRS416/Ynk1p-HA₃.

Bacterial expression and purification of proteins

BL21 (DE3) codon plus cells (Stratagene), carrying the plasmid pET21b/Ynk1p or pET21b/Ynk1p (H119G), were cultured in M9 medium, supplemented with 0.1 mg/ml ampicillin and 34 µg/ml chloramphenicol at 37 °C. Protein induction and ³⁵S-radiolabelling were done as described previously [26,27]. Both wild-type and mutant proteins were found to be soluble. Proteins were purified on Ni²⁺-nitrilotriacetate (Ni-NTA)-agarose (Qiagen, Chatsworth, CA, U.S.A.), dialysed against 50 mM Tris/HCl (pH 8.0), containing 10% (v/v) glycerol, and stored at -80 °C. Expression, radiolabelling and purification of a 40 kDa protein of the translocase of outer mitochondrial membrane (Tom40p) and pYfh1p were done as described previously [27].

Expression of Ynk1p-HA₃ in yeast

A wild-type yeast strain BY4741 (Research Genetics, Huntsville, AL, U.S.A.) was transformed with the plasmid pRS416/Ynk1p-HA₃. The resulting strain was referred to as BY4741/Ynk1p-HA₃. Expression of Ynk1p-HA₃ was monitored by Western blots using anti-HA antibodies [24].

Fractionation of mitochondria

Mitochondria and cytosol were isolated from *S. cerevisiae* strain D273-10B (A.T.C.C. 24657), the *Δynk1* deletion strain (Research Genetics) the congenic wild-type strain BY4741 or the BY4741/Ynk1p-HA₃ strain. The procedure for purifying mitochondria is described elsewhere [28]. Mitochondrial subfractions [IMS, salt-extractable (SE) IMS, matrix and membrane] were prepared as described previously [29]. Protein concentrations were determined using Micro BCA Protein Assay kit (Pierce, Rockford, IL, U.S.A.).

Autophosphorylation and transphosphorylation

Intact mitochondria (100 µg of proteins) or corresponding mitochondrial subfractions were used in each autophosphorylation assay unless otherwise indicated. For some experiments, (IMS × 10) or SE IMS (SE × 10), corresponding to 1 mg of intact mitochondria was used (see Figures 2C and 3A). Fractions were incubated with 1 µCi of [γ -³²P]ATP (3000 Ci/mmol; Perkin-Elmer, Norwalk, CT, U.S.A.) in the absence or presence of 1 mM EDTA for 5–10 min on ice or at room temperature (25 °C) [22]. Where indicated, intact mitochondria were preincubated with 100 µg/ml carboxyatractylide (CAT) for 5 min on ice before the addition of [γ -³²P]ATP. Purified recombinant Ynk1p was used at 1–50 ng of protein per assay. Samples were precipitated with chilled acetone and analysed by SDS/PAGE. The SDS-loading buffer contained 3.6% SDS, 0.29 M Tris base, 86 mM dithiothreitol and 17% glycerol. Gels were fixed with 40% methanol and processed for autoradiography. Note that phosphohistidine is sensitive to acid and Coomassie Blue staining of proteins in methanol is poor. Therefore, when needed, a duplicate gel with identical samples was processed separately for Coomassie Blue staining in methanol/acetic acid mixture to visualize protein patterns.

For transphosphorylation assays, recombinant Ynk1p was first autophosphorylated in the presence of [γ -³²P]ATP (no EDTA) as described above, and dialysed against 50 mM Tris/HCl (pH 8.0) and 10% glycerol to remove excess of radiolabelled ATP. Autophosphorylated Ynk1p was then incubated with GDP (1 mM) or AMP (1 mM) in buffer containing 1 mM MgCl₂. Samples were analysed by SDS/PAGE followed by autoradiography. Loss of radioactive signal from phosphorylated Ynk1p indicates the transfer of radiolabelled phosphate to its substrates [2].

Enzyme assays

NDPK activity was measured using the coupled pyruvate kinase-lactate dehydrogenase assay as described previously [1]. Assays were performed at room temperature in a total volume of 1 ml, containing 50 mM Tris/HCl (pH 7.5), 50 mM KCl, 6 mM MgCl₂, 1 mM phosphoenolpyruvate, 1 mM ATP, 0.2 mM NADH, 6 units each of pyruvate kinase and lactate dehydrogenase (Sigma, St Louis, MO, U.S.A.) and 0.45 mM dTDP

serving as the acceptor nucleotide. The reaction was initiated by the addition of IMS fractions equivalent to 100 µg of intact mitochondria or purified recombinant Ynk1p (1–10 ng). The decrease in absorbance at 340 nm due to oxidation of NADH reflects the amount of ADP produced. Specific activity was defined as nmol of ADP formed per min per mg of protein.

Osmotic swelling of mitochondria and protease treatment

To examine mitochondrial localization of Ynk1p-HA₃, osmotic swelling of mitochondria was performed as follows. Intact mitochondria in 20 mM Hepes/KOH (pH 7.5), 0.6 M sorbitol and 0.1 mg/ml BSA were subjected to hypotonic shock by diluting (1:5) with the same buffer lacking sorbitol [26]. After incubation on ice for 10 min, samples were centrifuged at 15000 g for 2 min. The supernatant and pellet fractions represent soluble IMS and mitoplast respectively.

For protease treatment, mitochondria were subjected to hypotonic shock but soluble IMS proteins were not separated from the mitoplast pellet ('Broken mitochondria'). Mitochondria (intact or broken; 100 µg of proteins) and cytosol (10 µg of proteins) were treated with increasing concentrations of trypsin or proteinase K in a final volume of 50 µl for 15 min on ice. After inactivation of proteases with PMSF, proteins were precipitated with 10 % (w/v) trichloroacetic acid, and analysed by Western blots.

Antibodies

Following affinity chromatography on Ni-NTA-agarose, Ynk1p-His₆ was further purified on SDS gels and antibodies were raised in rabbits. Mouse monoclonal anti-HA antibodies were obtained from Covance Research Products (Berkeley, CA, U.S.A.). Other antibodies used in the present study, and the procedure for Western blotting, have been described elsewhere [30,31].

Immunoprecipitation

IMS fractions containing radiolabelled phosphorylated proteins were briefly incubated at room temperature with 0.2 % SDS, and diluted 10-fold with immunoprecipitation buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/5 mM EDTA/1 % (v/v) Triton X-100/0.5 mM PMSF]. Samples were centrifuged at 15000 g for 5 min to remove insoluble materials, if any, and the supernatant fractions were transferred to tubes containing anti-Ynk1p antibodies prebound to Protein A-Sepharose. Following incubation with end-over-end mixing, Sepharose beads were extensively washed with the immunoprecipitation buffer. Samples were analysed by SDS/PAGE followed by autoradiography. The corresponding preimmune serum served as a negative control [32].

Tom40p-binding assays

The ability of various forms of Ynk1p to interact with Tom40p was tested essentially as described for other mitochondrial precursor proteins [27]. Briefly, unlabelled Ynk1p-His₆ and ³⁵S-labelled Tom40p (no His₆) were separately incubated for 10 min at room temperature with 50 mM Tris/HCl (pH 8.0), containing 8 M urea. Samples were centrifuged at 400000 g for 10 min. Supernatant fractions (approx. 2–3 µl) containing denatured proteins (1–2 µg of Ynk1p or 0.1–0.2 µg of Tom40p) were mixed and then diluted with 1 ml of binding buffer [50 mM Tris/HCl (pH 8.0)/1 % Triton X-100/ 10 % glycerol/40 mM potassium acetate]. After incubation at room temperature for 10 min,

samples were centrifuged at 15000 g for 10 min to remove aggregates, if any. The supernatant was transferred to an Eppendorf tube containing 25 µl of a 50 % Ni-NTA-agarose suspension and incubated at room temperature for 90 min with end-over-end mixing. The beads were washed with binding buffer and bound protein complexes were eluted with SDS-loading buffer containing 10 mM EDTA. Samples were analysed by SDS/PAGE, followed by Coomassie Blue staining and autoradiography. Where indicated, Ynk1p was preincubated with [γ -³²P]ATP, unlabelled ATP or ADP for 10 min on ice, before denaturation with urea. In a typical binding assay, the final concentration of urea was kept below 40 mM. Urea-denatured pYfh1p served as a positive control [27]. In some experiments, only Tom40p, not Ynk1p, was denatured with urea. Urea-denatured Tom40p was first diluted in binding buffer, followed by addition of recombinant native Ynk1p, and the assay performed as described above.

RESULTS

Bacterial expression and purification of active Ynk1p

NDPK has been previously purified from *S. cerevisiae* total cell extract. It involves an elaborate procedure with an overall yield of approx. 10 % [33]. To investigate Ynk1p biogenesis, we took advantage of the recombinant protein. Wild-type Ynk1p with a C-terminal His₆ tag was expressed in bacteria in soluble form and purified to homogeneity by Ni-NTA-agarose chromatography (Figure 1A). A mutant form of Ynk1p, in which the critical histidine residue at the catalytic site of the protein was replaced by glycine residue (H119G), was also expressed in bacteria with a C-terminal His₆ tag and purified (results not shown). The C-terminal tag was preferred over an N-terminal tag because mitochondrial precursor proteins often contain the targeting sequence at their N-termini, and a C-terminal His₆ tag does not interfere with import or functions of several mitochondrial proteins that we have studied previously [26,27,34,35].

Purified recombinant wild-type Ynk1p was found to be active based on three criteria. First, when Ynk1p (50 ng) was supplemented with [γ -³²P]ATP, it could rapidly phosphorylate itself as determined by SDS/PAGE and autoradiography (Figure 1B, lane 1). As little as 1 ng of Ynk1p can be detected by autophosphorylation assays. In contrast, a large excess (1 µg) of mutant Ynk1p (H119G) failed to exhibit any detectable autophosphorylation, and served as a negative control (lane 2). Secondly, the high-energy radiolabelled phosphate from preformed autophosphorylated Ynk1p was specifically removed by NDPs (e.g. GDP) but not by NMPs (e.g. AMP) (Figure 1C). The phosphate that was removed in the presence of NDPs was used to generate the corresponding NTPs; this transphosphorylation process required the presence of Mg²⁺ and was blocked by chelation of metals with EDTA (results not shown) [2]. Finally, purified Ynk1p was also found to be active by spectrophotometric enzyme assays (Figure 1D), and the specific activity calculated to be 2.3×10^6 nmol of ADP formed $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹. Ynk1p (H119G) did not show any detectable activity. These results confirm that Ynk1p is a NDPK of *S. cerevisiae*.

Subcellular localization of Ynk1p

To examine Ynk1p localization, we raised antibodies against purified Ynk1p. These antibodies were able to detect easily as little as 50 ng of purified protein on Western blots (Figure 2A, lane 2). We then tested the presence of Ynk1p in cytosol and mitochondria purified from a wild-type yeast strain. Ynk1p was easily detected in the cytosol (lane 4) but not in mitochondria

Figure 1 Characterization of recombinant Ynk1p

(A) BL21 (DE3) codon plus cells carrying the plasmid pET21b/Ynk1p were incubated in the absence ('Uninduced') or presence ('Induced') of isopropyl-1-thio- β -D-galactopyranoside. The overexpressed protein with a C-terminal His₆ tag was found soluble and purified on Ni-NTA-agarose ('Purified'). Samples were analysed by SDS/PAGE and Coomassie Blue staining. The positions of molecular-mass standards in kDa are indicated. (B) Purified wild-type Ynk1p (50 ng) or mutant Ynk1p (H119G) (1 μ g) was incubated with [γ -³²P]ATP for 10 min on ice, and analysed by SDS/PAGE and autoradiography. (C) Purified wild-type Ynk1p was incubated with [γ -³²P]ATP as in (B), dialysed, incubated with GDP (1 mM) or AMP (1 mM) in the presence of 1 mM MgCl₂ for 5 min at room temperature, and analysed by SDS/PAGE and autoradiography. 'Std' (lane 1) indicates the amount of autophosphorylated Ynk1p ([³²P]Ynk1p) used per assay. (D) NDPK activity of wild-type (2.5 ng) and mutant (10 ng) Ynk1p was measured as described in [1]; see the Experimental section for details.

(lane 5) in samples containing 100 μ g of total proteins. Fractions from the Δ ynk1 deletion strain served as negative controls (lanes 6 and 7). These results suggest that Ynk1p is quite abundant in the cytosol. Should Ynk1p exist in mitochondria, it is present below the detection limit by Western blots, and may represent < 0.05 % of total mitochondrial proteins.

Because autophosphorylation assays were 50–100 times more sensitive than Western blots, we looked for an 18 kDa phosphorylated protein in mitochondria that might represent Ynk1p. We knew from results on the NDPK reaction cycle that the autophosphorylated intermediate might be only transiently visible because of rapid transfer of the phosphate to target substrates. This transfer process requires Mg²⁺ and is blocked in

Figure 2 Dual localization of Ynk1p

(A) Mitochondria ('Mito') and cytosol ('Cyto') were purified from wild-type ('WT') and Δ ynk1 strains. Samples (100 μ g of proteins) were analysed by Western blots using anti-Ynk1p antibodies. Different amounts of recombinant Ynk1p served as positive controls. Note that the recombinant Ynk1p containing the His₆ tag migrates slightly slower than the endogenous yeast Ynk1p. (B) Purified recombinant Ynk1p (5 ng) or purified intact mitochondria ('Mito', 100 μ g of proteins) were incubated with [γ -³²P]ATP and 1 mM EDTA for 10 min on ice, and analysed by SDS/PAGE and autoradiography. (C) Intact mitochondria or mitochondrial subfractions were incubated with [γ -³²P]ATP plus EDTA and analysed as in (B). Mito, intact mitochondria (100 μ g of proteins); Mplast, mitoplasts corresponding to 100 μ g of intact mitochondria.

the presence of EDTA. Intact mitochondria (100 μ g of proteins) were therefore briefly incubated with [γ -³²P]ATP in the presence of EDTA and analysed by SDS/PAGE, followed by autoradiography. A major radiolabelled protein (approx. 33 kDa) was easily detected (Figure 2B, lane 2). The 33 kDa phosphorylated protein was present in Δ ynk1 mitochondria and was apparently unrelated to NDPK activity. Upon longer exposure of the autoradiography film, other proteins were detected but none in the vicinity of 18 kDa. Similar results were obtained when the assay was performed in the absence of EDTA or in the presence of added Mg²⁺ (results not shown).

Ynk1p does not appear to contain a typical N-terminal mitochondrial matrix targeting signal. Since Ynk1p is a soluble protein, we suspected that the protein, if at all present in mitochondria, should be in the IMS. Together with the other obvious possibility that only a small portion (approx. 3%) of total cellular Ynk1p might be associated with mitochondria [15], we investigated the presence of Ynk1p in an enriched IMS fraction in our assays. A technical difficulty was that more than 100 μ g of total mitochondrial proteins per lane in our SDS/PAGE system leads to overloading and distortion of protein bands. However, the protein content of the IMS is low compared

Figure 3 Mitochondrial localization of Ynk1p by Western blots and autophosphorylation

(A) Mitochondria and cytosol were purified from a wild-type strain, and various fractions were analysed by Western blots using different antibodies. Cyto, cytosol (100 μg of proteins); Mito, intact mitochondria (100 μg of proteins); Mplast, mitoplasts corresponding to 100 μg of intact mitochondria. (B) Intact mitochondria (100–200 μg of proteins) were incubated with 1 mM EDTA in the absence or presence of CAT for 5 min on ice. Following addition of [γ - ^{32}P]ATP, incubation continued for another 5 min on ice. An aliquot was subjected to hypotonic shock, and released IMS proteins were subjected to immunoprecipitation. Samples were analysed by SDS/PAGE and autoradiography. Lanes 1–3, 100 μg of intact mitochondria; lanes 4 and 5, IMS fraction corresponding to 200 μg of intact mitochondria. Iso, mitochondria at isotonic conditions; Hypo, mitochondria subjected to hypotonic shock; T, total sample; S, IMS-soluble proteins released as a result of hypotonic shock to intact mitochondria; IP, immunoprecipitation; IS, immune serum containing anti-Ynk1p antibodies; PI, preimmune serum.

with that of the membrane or the matrix fractions (results not shown). This allowed us to use ten times more protein for the IMS fraction in our assays. Mitochondria were subjected to hypotonic shock under conditions that rupture the outer membrane but leave the inner membrane intact. Soluble proteins (IMS) that were released as a result of the outer membrane rupture were separated by centrifugation. The resulting pellet was resuspended in buffer containing high salt, and centrifuged to separate salt-extracted supernatant from the mitoplast (Mplast) pellet [29].

Fractions were incubated with [γ - ^{32}P]ATP in the presence of EDTA, and analysed by SDS/PAGE, followed by autoradiography. As expected from Figure 2(B), a 33 kDa protein was labelled in total mitochondria, and most of this protein was found in the matrix fraction (results not shown). To our surprise, however, an 18 kDa phosphorylated protein was found to be radiolabelled almost exclusively in the enriched IMS fraction with a minor amount in the salt extract (Figure 2C, lanes 2 and 3). The 18 kDa radiolabelled protein was not detected in the enriched IMS fraction of mitochondria isolated from the *Δynk1* strain (lane 5). These results strongly suggest that the 18 kDa protein in wild-type mitochondria is Ynk1p. At a first glance, Ynk1p appears to be the major phosphorylated protein in the mitochondrial IMS (not shown in Figure 2C but see Figure 3B). However, this may or may not be the case. We performed phosphorylation assays in the presence of EDTA to trap the autophosphorylated Ynk1p intermediate. Some proteins, however, may require Mg^{2+} for phosphorylation and may remain phosphorylated only transiently. These proteins will not be detected under our assay conditions. Furthermore, it should be emphasized that we analysed our samples by SDS/PAGE under alkaline conditions. Phosphohistidine is acid-labile and base-stable. In contrast, phosphotyrosine, phosphoserine and phosphothreonine are base-labile and acid-stable, whereas phosphoaspartic acid and phosphoglutamic acids are both acid- and base-labile [36]. Therefore, proteins phosphorylated at residues Tyr, Ser, Thr, Asp or Glu may not be efficiently detected in our alkaline gel system.

The intensity of phosphorylated Ynk1p in IMS $\times$ 10 (i.e. IMS prepared from 1 mg of mitochondria; Figure 2C, lane 2) corresponded to an equivalent signal expected for 50 ng of purified recombinant Ynk1p. We therefore estimate that Ynk1p represents approx. 0.005 % of total mitochondrial proteins. This is in good agreement with our spectrophotometric data for NDPK activity in the mitochondrial IMS. We measured the activity using the coupled pyruvate kinase–lactate dehydrogenase assay system [1], and specific activities [nmol of ADP formed $\cdot \text{min}^{-1} \cdot (\text{mg}$ of total mitochondrial proteins) $^{-1}$] for wild-type and *Δynk1* IMS fractions were found to be 144 and 9 respectively. Based on specific activity of purified Ynk1p [2.3×10^6 nmol of ADP formed $\cdot \text{min}^{-1} \cdot (\text{mg}$ of protein) $^{-1}$], Ynk1p in the IMS represents approx. 0.006 % of total mitochondrial proteins.

One of our concerns was that the IMS $\times$ 10 fraction might be contaminated with proteins of the cytosol, mitochondrial matrix or mitochondrial membranes. To rule out this possibility, we tested various fractions for the absence or presence of marker proteins: Cyc1p (cytochrome *c* in SE IMS), Pgk1p (phosphoglycerate kinase in the cytosol), Put2p (δ -1-pyrroline-5-carboxylate dehydrogenase of the mitochondrial matrix) and Tom40p. Identical amounts of proteins as in Figure 2(C) were used for Western-blot analysis. As shown in Figure 3(A), Ynk1p was detected in the cytosol and enriched IMS $\times$ 10 fractions. The IMS $\times$ 10 fraction did not contain any detectable Pgk1p, Put2p or Tom40p. After a long exposure of autoradiography films, these proteins

still remained undetectable and only a small fraction of Cyc1p, as expected, was detected.

Another concern was the fact that we failed to detect autophosphorylated Ynk1p in intact mitochondria (Figures 2B and 2C). One would have expected to see a signal for Ynk1p in intact mitochondria (100 µg of proteins), particularly considering the estimated abundance (approx. 0.005%) of Ynk1p in mitochondria and the sensitivity of autophosphorylation assays for purified Ynk1p (1 ng). To resolve this issue, we modified our assays based on the following rationale. We have demonstrated previously that ATP, added to isolated intact mitochondria, rapidly (within seconds) enters into the matrix via the ADP/ATP carrier located at the inner mitochondrial membrane [26]. However, when the ADP/ATP carrier is blocked with a specific inhibitor CAT, ATP entry into the matrix is completely inhibited, and under these conditions, added ATP resides outside of the inner membrane. We took advantage of this system to demonstrate that Ynk1p in the IMS of intact mitochondria is radiolabelled with its autophosphorylation only when the ADP/ATP carrier is blocked with CAT.

When intact mitochondria were supplemented with [γ - 32 P]ATP plus EDTA, the 33 kDa matrix protein was radiolabelled (Figure 3B, lane 1). However, when the ADP/ATP carrier of the inner mitochondrial membrane was blocked with CAT before the addition of [γ - 32 P]ATP, the labelling of 33 kDa matrix protein was strongly inhibited. More importantly, an 18 kDa protein was found to be labelled in the presence of CAT (lane 2). This 18 kDa protein was released by hypotonic shock (lane 3) that specifically releases IMS-soluble proteins but not matrix proteins, and could be immunoprecipitated using anti-Ynk1p antibodies (lane 5). Together, these results strongly suggest that a small portion of total cellular Ynk1p is present in the IMS of mitochondria.

Tagged Ynk1p protected from protease digestion in intact mitochondria

Since the amount of Ynk1p in the mitochondrial IMS is very small compared with that in the cytosol, the results presented above cannot rule out the possibility of cytoplasmic contamination. We did not observe any difference in the mobility of cytosolic and mitochondrial Ynk1p on Western blots (Figure 3A). This suggests that Ynk1p, like many other proteins in the mitochondrial IMS, does not contain a cleavable mitochondrial targeting sequence. Ideally, it must be demonstrated that Ynk1p, by virtue of its localization in the IMS of intact mitochondria, remains protected from external proteases. However, purified Ynk1p was found to be highly resistant to trypsin even in the absence of mitochondria; non-phosphorylated and autophosphorylated forms of Ynk1p were equally resistant (results not shown). These results suggest that purified and active Ynk1p is tightly folded. Protection from trypsin degradation therefore cannot be used to distinguish intramitochondrial Ynk1p from cytoplasmic Ynk1p. Another problem was that anti-Ynk1p antibodies recognized Ynk1p in an enriched IMS fraction but not in total mitochondria (100 µg of proteins) (Figure 3A).

Both these problems can be overcome by attachment of a peptide tag that is sensitive to protease digestion and by the use of commercially available high titre antibodies against the peptide tag. We therefore generated a construct in which three HA tags in tandem were introduced at the C-terminus of Ynk1p, and the resulting chimaeric protein, Ynk1p-HA₃, was expressed in a wild-type yeast strain. We first tested the protease sensitivity of cytosolic Ynk1p-HA₃. A cytosolic fraction (10 µg of proteins) was treated with increasing concentrations of trypsin (Figure 4A)

or proteinase K (Figure 4B), and analysed by Western blots using anti-HA antibodies. Although cytosolic Ynk1p-HA₃ was readily digested by proteinase K in a dose-dependent manner (5–25 µg/ml), it was not digested even at the highest concentration of trypsin that we tested (0.2 mg/ml). The ability of proteinase K to digest Ynk1p-HA₃ therefore allowed us to examine mitochondrial localization of Ynk1p-HA₃ with confidence. Purified intact mitochondria (100 µg of proteins) were treated with increasing concentrations of proteinase K and analysed by Western blots using anti-Tom70p antibodies (Figure 4C) or anti-HA antibodies (Figure 4D). Tom70p is a protein of the outer mitochondrial membrane with a major portion of the protein exposed on the cytosolic side [37]. As expected, Tom70p was rapidly degraded by proteinase K. In contrast with Tom70p (and cytosolic Ynk1p-HA₃), mitochondrial Ynk1p-HA₃ remained quantitatively protected even at the highest concentration of proteinase K that we tested (Figure 4D). When intact mitochondria were subjected to hypotonic shock, mitochondrial Ynk1p-HA₃ was released with the soluble IMS proteins (Figure 4E). More importantly, hypotonically released mitochondrial Ynk1p-HA₃ now behaved like the cytosolic Ynk1p-HA₃, as evidenced by degradation of the protein by proteinase K (Figure 4F). Note that proteinase K digestion was not performed with isolated soluble IMS fraction containing Ynk1p-HA₃, instead it was done with total 'Broken mitochondria' (Figure 4F). In this way, all mitochondrial samples that were treated with proteinase K in Figures 4(C), 4(D) and 4(F) had 100 µg of proteins, thereby allowing a direct comparison. These results indicate that Ynk1p found in purified mitochondria is specifically localized to the IMS. It should be emphasized that Ynk1p-HA₃ was able to assemble with wild-type Ynk1p (containing no HA tag) both in the cytosol and in the mitochondrial IMS, as evidenced by co-immunoprecipitation of tagged and untagged forms of Ynk1p by anti-HA antibodies. Furthermore, Ynk1p-HA₃ was found to be as active as the wild-type Ynk1p (results not shown). These results suggest that Ynk1p-HA₃ behaves like the wild-type protein in terms of protein folding, assembly and activity. Mitochondrial localization of Ynk1p-HA₃ is therefore not due to any folding or assembly defect resulting from the C-terminal HA tags.

Interaction of Ynk1p with Tom40p

Nuclear-encoded and cytoplasmically synthesized mitochondrial precursor proteins, regardless of their final submitochondrial localization, are expected to interact with Tom40p, which is the core component of the protein-conducting channel of the outer mitochondrial membrane [38–40]. To investigate the interaction of Ynk1p with Tom40p, we used an assay that we recently developed for this purpose [27]. Briefly, the principle of our assay is as follows. One protein contains a His₆ tag at its C-terminus and is unlabelled (e.g. Protein X-His₆), whereas the other component does not contain a His₆ tag but is radiolabelled (e.g. 35 S-labelled Protein Y). Protein X-His₆ by virtue of its His₆ tag binds to Ni-NTA-agarose. On the other hand, 35 S-labelled Protein Y lacking a His₆ tag does not bind to Ni-NTA-agarose. When the two proteins are mixed together, 35 S-labelled Protein Y becomes associated with Ni-NTA-agarose only through an interaction with Protein X-His₆. One advantage of this assay is that both components can be denatured first with urea; unfolded proteins are mixed together and then diluted in buffer containing a non-ionic detergent for them to interact. Tom40p, expressed in bacteria, is found sequestered in inclusion bodies, which can be solubilized with 8 M urea. More importantly, urea-denatured Tom40p interacts specifically with mitochondrial precursor

Figure 4 Mitochondrial localization of Ynk1p by protease protection

Mitochondria and cytosol were purified from a wild-type strain expressing Ynk1p-HA₃. Cytosol (10 μg of proteins) was incubated for 15 min on ice in the absence or presence of increasing concentrations of trypsin (**A**) or proteinase K (**B**). Samples were analysed by Western blots using anti-HA antibodies. Purified intact mitochondria (100 μg of proteins) were incubated for 15 min on ice in the absence or presence of increasing concentrations of proteinase K. Samples were analysed by Western blots using anti-Tom70p antibodies (**C**) or anti-HA antibodies (**D**). Intact mitochondria (100 μg of proteins) were subjected to osmotic swelling, centrifuged to separate soluble IMS and 'Mplast' fractions. Samples were analysed by Western blots using anti-HA antibodies (**E**). Intact mitochondria (100 μg of proteins) were subjected to osmotic swelling as in (**E**) but released intermembrane space proteins were not separated. Total samples ('Broken mitochondria') were incubated for 15 min on ice in the absence or presence of increasing concentrations of proteinase K, and analysed by Western blots using anti-HA antibodies (**F**). The degraded fragments of Ynk1p-HA₃ in panels (**B**) and (**F**) are indicated; the bands with * or ** represent Ynk1p with one or two HA tags respectively. Note that the calculated molecular mass of Ynk1p-HA₃ from its amino acid composition is approx. 22 kDa. However, on SDS gels the protein migrates with an estimated molecular mass of approx. 25 kDa. The molecular mass of Ynk1p on SDS gels is approx. 18 kDa.

proteins and it can be functionally reconstituted into liposomes [27,40].

Urea-denatured ³⁵S-labelled Tom40p (no His₆) was mixed with urea-denatured unlabelled Ynk1p-His₆, and the mixture was diluted in buffer containing Triton X-100. Samples were then subjected to Ni-NTA-agarose chromatography, and proteins

bound to the resin were analysed by SDS/PAGE followed by Coomassie Blue staining for unlabelled Ynk1p and autoradiography for ³⁵S-labelled Tom40p (Figure 5, middle and upper panels respectively). The affinity eluate contained not only Ynk1p-His₆ (middle panel, lane 3) but also a substantial amount of ³⁵S-labelled Tom40p used in the assay (upper panel, lane 3);

Figure 5 Interaction of Ynk1p with Tom40p

Binding of unlabelled Ynk1p-His₆ to ³⁵S-labelled Tom40p was performed as described in the Experimental section. Samples include both urea-denatured (UD) and non-denatured (ND) Ynk1p-His₆. pYfh1p represents the precursor form of Yfh1p, and was used as a positive control. Proteins bound to Ni-NTA-agarose were analysed by SDS/PAGE followed by Coomassie Blue staining (middle panel) and autoradiography (upper panel). 'Std' (lane 1) indicates the amount of ³⁵S-labelled Tom40p used per binding assay. Association of ³⁵S-labelled Tom40p with Ni-NTA-agarose occurs through an interaction with denatured unlabelled Ynk1p-His₆ (lane 3), and this is schematically shown in the lower panel.

see the lower panel for a schematic presentation. Tom40p associated with Ynk1p (upper panel, lane 3) was comparable with that observed with pYfh1p (lane 5); the latter served as a positive control. pYfh1p (yeast frataxin homologue) is a nuclear-encoded mitochondrial precursor protein; its interaction with Tom40p and its import into mitochondria are well characterized [27,34,35]. In the absence of Ynk1p-His₆ and pYfh1p-His₆, no detectable ³⁵S-labelled Tom40p was found to be associated with Ni-NTA-agarose, thereby serving as a negative control (upper panel, lane 2). When native or non-denatured Ynk1p (instead of urea-denatured Ynk1p) was used in the assay (lane 4), Ynk1p was able to bind to Ni-NTA agarose (middle panel) and yet no ³⁵S-labelled Tom40p was detected in the affinity eluate (upper panel). These results suggest that Ynk1p must be unfolded to interact with Tom40p.

Effect of Ynk1p autophosphorylation on Tom40p recognition

To investigate the effect of autophosphorylation of Ynk1p on its recognition by Tom40p, we performed two sets of experiments. In the first set (Figure 6A), purified Ynk1p-His₆ was briefly preincubated with or without [γ -³²P]ATP, denatured with urea, mixed with urea-denatured ³⁵S-labelled Tom40p (no His₆), diluted in buffer containing Triton X-100 and then subjected to Ni-NTA-agarose chromatography. Samples were analysed by SDS/PAGE followed by Coomassie Blue staining (bottom panel, Ynk1p-His₆) or autoradiography (middle panel, ³²P-labelled Ynk1p-His₆; upper panel, ³⁵S-labelled Tom40p). As expected, Ynk1p was efficiently autophosphorylated and remained autophosphorylated under the assay conditions. Auto-

Figure 6 Effect of Ynk1p autophosphorylation on its binding to Tom40p

(A) Purified Ynk1p-His₆ was incubated in the absence or presence of [γ -³²P]ATP for 5 min on ice. Samples were denatured with urea and binding to ³⁵S-labelled Tom40p was performed. Following Ni-NTA agarose chromatography, identical samples were analysed by two SDS gels. One gel was fixed with methanol (no acetic acid) and processed for autoradiography; the other gel was stained with Coomassie Blue in methanol/acetic acid mixture. Note that the autoradiograph shows two radiolabelled bands, ³⁵S-labelled Tom40p and ³²P-labelled Ynk1p. The regions showing these bands are shown in the upper and middle panels respectively. The lower panel shows Coomassie Blue staining of Ynk1p; the amount of Tom40p used in binding reactions is low and is not detected by Coomassie Blue staining. For details, see the Experimental section. (B) Purified wild-type or mutant Ynk1p-His₆ was incubated in the presence or absence of unlabelled ATP or ADP, denatured with urea and binding to ³⁵S-labelled Tom40p performed. Samples were analysed as in (A). The amount of ³⁵S-labelled Tom40p used per binding assay is shown in lane 1 ('Std').

phosphorylation did not interfere with the quantitative binding of Ynk1p-His₆ to Ni-NTA-agarose (Figure 6A, middle and lower panels, cf. lanes 3 and 4). Surprisingly, however, Tom40p association with phosphorylated Ynk1p was reduced > 70 % compared with the corresponding non-phosphorylated Ynk1p control (upper panel, cf. lanes 4 and 5).

To confirm these results further, we performed a second set of experiments with additional controls (Figure 6B). Purified wild-type or H119G mutant Ynk1p-His₆ was incubated with unlabelled ATP or ADP, and denatured with urea. Samples were then assessed for their ability to bind to ³⁵S-labelled Tom40p (no His₆) as described above. Preincubation of Ynk1p with ATP, but not with ADP, significantly reduced its interaction with Tom40p (Figure 6B, upper panel, cf. lanes 3–5). On the other hand, preincubation of mutant Ynk1p with ATP or ADP did not have any effect on its interaction with Tom40p (cf. lanes 6–8). The mutant protein that had been preincubated with ATP (lane 7) was recognized by Tom40p as efficiently as the wild-type protein that had not been preincubated with ATP (lane 3). The lower panel in Figure 6(B) (Coomassie Blue staining) shows that comparable amounts of wild-type and mutant proteins were bound to Ni-NTA-agarose regardless of the absence or presence

of variable components in the assay. These results strongly suggest that phosphorylated Ynk1p, compared with its non-phosphorylated form, is poorly recognized by Tom40p, and this inhibitory effect is specifically due to autophosphorylation of the protein and not due to non-specific effects of nucleotides in the assay.

DISCUSSION

NDPKs are ubiquitous enzymes with multiple functions. In eukaryotic cells, NDPK is a hexamer of identical subunits and is largely found in the cytosol. As early as 1953, NDPK was first discovered in yeast [41] and in pigeon breast muscle [42]. Although the former is widely used as a model eukaryotic system, the yeast enzyme has not received much attention. Here we have studied the biogenesis of *S. cerevisiae* NDPK (Ynk1p) with particular emphasis on its mitochondrial targeting. We have demonstrated that Ynk1p is present in the IMS of purified mitochondria in small amounts. This conclusion is based on several independent lines of evidence. In addition to mitochondrial subfractionation, Western-blot analysis and spectrophotometric enzyme assays, we have identified an autophosphorylated Ynk1p intermediate in the IMS. When intact mitochondria were incubated with [γ - 32 P]ATP plus EDTA, phosphorylated Ynk1p was not detected; instead a matrix protein (approx. 33 kDa) was labelled (Figures 2B and 3B). Probably, this was due to rapid entry of ATP through the inner membrane ADP/ATP carrier into the matrix leaving little, if any, ATP available in the IMS [26]. When the ADP/ATP carrier was blocked, ATP entry into the matrix was inhibited and it remained available in the IMS. Consequently, on the one hand, labelling of the matrix-localized 33 kDa protein was inhibited; on the other, IMS-localized Ynk1p was labelled (Figure 3B). We estimate that Ynk1p represents approx. 0.005 % of total mitochondrial proteins. Note that we used purified active Ynk1p as control for Western blots, autophosphorylation and enzyme assays. This allowed us to validate our quantification for such a low abundant protein by three independent methods. Although the amount of Ynk1p in the IMS is low, it should be emphasized that the volume of the IMS is very small [43]; therefore Ynk1p may actually be more concentrated than it appears.

Ynk1p does not contain a cleavable mitochondrial targeting sequence and, therefore, its mitochondrial localization must be confirmed by protease protection, particularly to rule out the possibility of cytoplasmic contamination. However, purified recombinant Ynk1p (or cytosolic Ynk1p) was found resistant to degradation by trypsin, suggesting that the protein is tightly folded. Protease resistance of Ynk1p made it technically difficult to distinguish between Ynk1p in the mitochondrial IMS from contaminating cytosolic Ynk1p that may be associated with the outside of the mitochondrial outer membrane. To overcome this problem, we introduced three HA tags in tandem at the C-terminus of Ynk1p since we knew that HA tags are usually digested by the non-specific protease, proteinase K, at low concentrations (approx. 5–10 μ g/ml). Cytosol and mitochondria were purified from a wild-type yeast strain expressing the chimaeric protein Ynk1p-HA₃, and were analysed by Western blots using anti-HA antibodies. Cytosolic Ynk1p-HA₃ was degraded by proteinase K; in contrast, mitochondrial Ynk1p-HA₃ remained quantitatively protected. More importantly, when the outer mitochondrial membrane was ruptured by osmotic swelling, mitochondrial Ynk1p-HA₃ was released with the soluble IMS proteins and degraded by proteinase K (Figure 4).

Trafficking of most cytoplasmically synthesized proteins to target organelles is usually highly specific and efficient; this keeps

the cytoplasmic pool of precursor proteins low and also ensures that a particular gene product resides at a particular subcellular location where it functions. However, like Ynk1p, at least three other proteins in *S. cerevisiae* are found predominantly in cytosol with a small fraction (approx. 1–5 %) in the mitochondrial IMS. These proteins are Cu, Zn superoxide dismutase (Sod1p), copper chaperone for superoxide dismutase and adenylate kinase (Adk1p/Aky2p). Insertion of copper cofactor into Sod1p requires copper chaperone for superoxide dismutase, and Sod1p helps in protecting mitochondria from oxidative damage. Interestingly, mitochondrial localization of Sod1p is greatly enhanced by the mitochondrial localization of copper chaperone for superoxide dismutase [43]. By analogy, it is possible that the mitochondrial import of Ynk1p *in vivo* is controlled by interactions with a partner protein, and the partner protein may also have a dual localization in mitochondria and cytosol. Adk1p/Aky2p is the major adenylate kinase that provides ADP for oxidative phosphorylation [44]. Similar to Ynk1p, Adk1p/Aky2p is also tightly folded [45]; protein folding therefore seems to be a common factor that determines subcellular partitioning of these proteins between cytosol and mitochondria *in vivo*. Another protein, Cox17p, which functions as a mitochondrial copper shuttle was also localized in the mitochondrial IMS and cytosol [46]. A major portion (60 %) of Cox17p, unlike in the case of Ynk1p (< 5 %), was found associated with mitochondria. The dual localization of Cox17p appears to correlate with the state of oligomerization of the protein; Cox17p exists as dimers in the cytosol, and forms tetramers in the mitochondrial IMS [47]. However, the mechanism for mitochondrial targeting of Cox17p remains unclear.

When purified Ynk1p was incubated with isolated mitochondria, little, if any, Ynk1p was associated with the organelle. This suggests that the mitochondrial targeting signal of Ynk1p is buried in a folded structure and not available for its recognition by the mitochondrial import machinery. Upon unfolding with urea, a portion of Ynk1p was found associated with mitochondria (results not shown). These results suggest that Ynk1p must be unfolded to be recognized by the outer mitochondrial import machinery. To understand mitochondrial targeting of Ynk1p better, we investigated its interaction directly with Tom40p, the core component of the protein-conducting channel in the outer mitochondrial membrane. Native (non-denatured) Ynk1p was not recognized by Tom40p but urea-denatured Ynk1p was efficiently recognized (Figure 5). During the course of the present study, we discovered a novel phenomenon: autophosphorylation of Ynk1p greatly inhibits its recognition by Tom40p (Figure 6). Together, these results suggest that not only the folding status but also the phosphorylation status of Ynk1p control its import into mitochondria.

To our knowledge, this is the first example where targeting of a protein to mitochondria is at least partly controlled by autophosphorylation of the protein. Of particular interest to this finding is our earlier report showing that some cytochrome P450 isoforms contain an N-terminal chimaeric mitochondrial and endoplasmic reticulum targeting signal [48,49]. Differential targeting is regulated through cAMP-dependent phosphorylation of these proteins by protein kinase A: phosphorylated proteins are preferentially targeted to mitochondria. We also demonstrated that phosphorylation of cytochrome P450 isoforms increases their affinity for binding to Tom40p, perhaps due to a structural alteration in the proteins that leads to exposure or activation of a cryptic mitochondrial targeting signal [48,49]. This is quite opposite to what we report here for Ynk1p: phosphorylation of Ynk1p lowers its binding to Tom40p, and this poor binding is unlikely due to structural alterations resulting

from phosphorylation because of two reasons. First, we used urea-denatured Ynk1p for Tom40p-binding assays and, secondly, phosphorylation of Ynk1p has no effect on its resistance towards trypsin. It is, however, possible that the Ynk1p targeting signal is located close to, or overlaps with, the catalytic site and introduction of negatively charged phosphate on to the histidine residue at the catalytic site interferes with the recognition of the targeting signal by Tom40p. Much work is needed to determine how phosphorylation affects these protein–protein interactions. Nevertheless, inefficient recognition of Ynk1p by Tom40p due to Ynk1p autophosphorylation is unique and provides a novel mechanism of mitochondrial sorting.

How is cytoplasmically synthesized Ynk1p *in vivo* partitioned between cytoplasmic and mitochondrial pools? We propose that immediately after completion of Ynk1p synthesis on cytoplasmic ribosomes, most of the molecules are rapidly folded and assembled into active form. This may involve folding of the monomer and assembly into homo-oligomers that are enzymically active. The folded enzyme complex interacts poorly with the mitochondrial import machinery, and phosphorylation of Ynk1p may further inhibit this interaction. However, a small fraction of newly synthesized Ynk1p before rapid folding and phosphorylation remains translocation-competent, and these Ynk1p molecules are imported into the IMS of mitochondria. Subsequent folding/oligomerization traps Ynk1p in the IMS and makes the import process unidirectional. It should be noted that most mitochondrial precursor proteins are imported into isolated mitochondria post-translationally (for review see [50,51]). However, it is possible that some proteins are *in vivo* imported co-translationally [52]. For example, cytosolic polysomes remain attached to mitochondria particularly when the cytosolic protein synthesis is blocked, and these polysomes are enriched in mRNAs for imported mitochondrial proteins [53,54]. Furthermore, import of a few proteins appears to be strictly co-translational both *in vitro* and *in vivo* [55,56]. A population of Ynk1p might be co-translationally imported and in this way maintain an unfolded translocation-competent conformation.

A key question is: what does Ynk1p do in the mitochondrial IMS? ATP synthesized in the mitochondrial matrix is exchanged for ADP across the inner mitochondrial membrane by an ADP/ATP carrier; ATP thus exported to the IMS can then diffuse across the outer mitochondrial membrane into the cytosol. Ynk1p being localized in the IMS may utilize some of the exported ATP to provide compartmentalized synthesis of other NTPs, particularly GTP that in turn participates in mitochondrial biogenesis. This notion is supported by our earlier observation that GTP in the IMS is generally required for efficient import of precursor proteins into the mitochondrial matrix [26]. Because GTP is hydrolysed during the import process, Ynk1p activity may help in maintaining a critical concentration of GTP in the IMS. Likewise, Ynk1p may supply GTP to the dynamin-related GTPase, Mgm1p, which is an IMS protein required for maintenance of fusion-competent mitochondria [57]. This would be reminiscent of a recent finding that cytosolic NDPK-dependent supply of GTP determines the rate of endocytosis mediated by the GTPase dynamin [3]. NDPK has also been shown to interact directly or indirectly with other GTPases and to modulate their activity through GTP loading and/or GTP hydrolysis [58–61]. By analogy to other NDPKs, Ynk1p may also have a protein phosphotransferase activity, thereby serving as a protein kinase in the mitochondrial IMS. Our assays described here will certainly help in the identification of Ynk1p substrate protein(s).

We thank Donna M. Gordon for helpful discussions during this work and Andrew Dancis and Andrew Harris for critical comments on the manuscript. This work was

supported by the NIH grant no. GM57067 to D.P. and a postdoctoral fellowship from the American Heart Association to B.A.

REFERENCES

- Parks, Jr, R. E. and Agarwal, R. P. (1973) Nucleoside diphosphokinases. In *The Enzymes*, vol. 8 (Boyer, P. D., ed.), pp. 307–333, Academic Press, New York
- Lascu, I. and Gonin, P. (2000) The catalytic mechanism of nucleoside diphosphate kinases. *J. Bioenerg. Biomembr.* **32**, 237–246
- Krishnan, K. S., Rikhy, R., Rao, S., Shivalkar, M., Mosko, M., Narayanan, R., Etter, P., Estees, P. S. and Ramaswami, M. (2001) Nucleoside diphosphate kinase, a source of GTP, is required for dynamin-dependent synaptic vesicle recycling. *Neuron* **30**, 197–210
- Wagner, P. D. and Vu, N.-D. (2000) Histidine to aspartate phosphotransferase activity of nm23 proteins: phosphorylation of aldolase C on Asp-319. *Biochem. J.* **346**, 623–630
- Engel, M., Seifert, M., Theisinger, B., Seyfert, U. and Welter, C. (1998) Glyceraldehyde-3-phosphate dehydrogenase and Nm23-H1/nucleoside diphosphate kinase A. Two old enzymes combine for the novel Nm23 protein phosphotransferase function. *J. Biol. Chem.* **273**, 20058–20065
- MacDonald, N. J., De la Rosa, A., Benedict, M. A., Freije, J. M., Kruttsch, H. and Steeg, P. S. (1993) A serine phosphorylation of Nm23, and not its nucleoside diphosphate kinase activity, correlates with suppression of tumor metastatic potential. *J. Biol. Chem.* **268**, 25780–25789
- Biggs, J., Hersperger, E., Steeg, P. S., Liotta, L. A. and Shearn, A. (1990) A *Drosophila* gene that is homologous to a mammalian gene associated with tumor metastasis codes for a nucleoside diphosphate kinase. *Cell (Cambridge, Mass.)* **63**, 933–940
- Kantor, J. D., McCormick, B., Steeg, P. S. and Zetter, B. R. (1993) Inhibition of cell motility after nm23 transfection of human and murine tumor cells. *Cancer Res.* **53**, 1971–1973
- Postel, E. H., Berberich, S. J., Flint, S. J. and Ferrone, C. A. (1993) Human *c-myc* transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. *Science* **261**, 478–480
- Rosengard, A. M., Kruttsch, H. C., Shearn, A., Biggs, J. R., Barker, E., Margulies, I. M., King, C. R., Liotta, L. A. and Steeg, P. S. (1989) Reduced Nm23/Awd protein in tumor metastasis and aberrant *Drosophila* development. *Nature (London)* **342**, 177–180
- Dumas, C., Lascu, I., Morera, S., Glaser, P., Fourme, R., Wallet, V., Lacombe, M. L., Veron, M. and Janin, J. (1992) X-ray structure of nucleoside diphosphate kinase. *EMBO J.* **11**, 3203–3208
- Strelkov, S. V., Perisic, O., Webb, P. A. and Williams, R. L. (1995) The 1.9 Å crystal structure of a nucleoside diphosphate kinase complex with adenosine 3', 5'-cyclic monophosphate: evidence for competitive inhibition. *J. Mol. Biol.* **249**, 665–674
- Janin, J., Dumas, C., Morera, S., Xu, Y., Meyer, P., Chiadmi, M. and Cherfils, J. (2000) Three-dimensional structure of nucleoside diphosphate kinase. *J. Bioenerg. Biomembr.* **32**, 215–225
- Lascu, I., Giartosio, A., Ransac, S. and Erent, M. (2000) Quaternary structure of nucleoside diphosphate kinases. *J. Bioenerg. Biomembr.* **32**, 227–236
- Zhang, S. Q., Hu, Y. and Jong, A. Y. (1995) Temporal and spatial distributions of yeast nucleoside diphosphate kinase activities and its association with the Cdc8p. *Cell. Mol. Biol. Res.* **41**, 333–346
- Phung-Ba Pinon, V., Millot, G., Munier, A., Vassy, J., Linares-Cruz, G., Capeau, J., Calvo, F. and Lacombe, M.-L. (1999) Cytoskeletal association of the A and B nucleoside diphosphate kinases of interphasic but not mitotic human carcinoma cell lines: specific nuclear localization of the B subunit. *Exp. Cell Res.* **246**, 355–367
- Tsuiki, H., Nitta, M., Furuya, A., Hanai, N., Fujiwara, T., Inagaki, M., Kochi, M., Ushio, Y., Saya, H. and Nakamura, H. (1999) A novel human nucleoside diphosphate (NDP) kinase, Nm23-H6, localizes in mitochondria and affects cytokinesis. *J. Cell. Biochem.* **76**, 254–269
- Milon, L., Meyer, P., Chiadmi, M., Munier, A., Johansson, M., Karlsson, A., Lascu, I., Capeau, J., Janin, J. and Lacombe, M.-L. (2000) The human nm23-H4 gene product is a mitochondrial nucleoside diphosphate kinase. *J. Biol. Chem.* **275**, 14264–14272
- Sweetlove, L. J., Mowday, B., Hebestreit, H. F., Leaver, C. J. and Millar, A. H. (2001) Nucleoside diphosphate kinase III is localized to the inter-membrane space in plant mitochondria. *FEBS Lett.* **508**, 272–276
- Lambeth, D. O., Mehus, J. G., Ivey, M. A. and Milavetz, B. I. (1997) Characterization and cloning of a nucleoside diphosphate kinase targeted to matrix of mitochondria in pigeon. *J. Biol. Chem.* **272**, 24604–24611
- Troll, H., Winckler, T., Lascu, I., Müller, N., Saurin, W., Veron, M. and Mutzel, R. (1993) Separate nuclear genes encode cytosolic and mitochondrial nucleoside diphosphate kinase in *Dictyostelium discoideum*. *J. Biol. Chem.* **268**, 25469–25475
- Struglics, A. and Hakansson, G. (1999) Purification of a serine and histidine phosphorylated mitochondrial nucleoside diphosphate kinase from *Pisum sativum*. *Eur. J. Biochem.* **262**, 765–773

- 23 Fukuchi, T., Nikawa, J., Kimura, N. and Watanabe, K. (1993) Isolation, overexpression and disruption of a *Saccharomyces cerevisiae* *YNK* gene encoding nucleoside diphosphate kinase. *Gene* **129**, 141–146
- 24 Li, J., Saxena, S., Pain, D. and Dancis, A. (2001) Adrenodoxin reductase homolog (Ard1p) of yeast mitochondria required for iron homeostasis. *J. Biol. Chem.* **276**, 1503–1509
- 25 Schena, M., Picard, D. and Yamamoto, K. R. (1991) Vectors for constitutive and inducible expression in yeast. *Methods Enzymol.* **194**, 389–398
- 26 Sepuri, N. B. V., Gordon, D. M. and Pain, D. (1998) A GTP-dependent 'push' is generally required for efficient protein translocation across the mitochondrial inner membrane into the matrix. *J. Biol. Chem.* **273**, 20941–20950
- 27 Gordon, D. M., Wang, J., Amutha, B. and Pain, D. (2001) Self-association and precursor protein binding of *Saccharomyces cerevisiae* Tom40p, the core component of the protein translocation channel of the outer mitochondrial membrane. *Biochem. J.* **356**, 207–215
- 28 Murakami, H., Pain, D. and Blobel, G. (1988) 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. *J. Cell Biol.* **107**, 2051–2057
- 29 Martin, H., Eckerskorn, C., Gärtner, F., Rassow, J., Lottspeich, F. and Pfanner, N. (1998) The yeast mitochondrial intermembrane space: purification and analysis of two distinct fractions. *Anal. Biochem.* **265**, 123–128
- 30 Schülke, N., Sepuri, N. B. V., Gordon, D. M., Saxena, S., Dancis, A. and Pain, D. (1999) A multisubunit complex of outer and inner mitochondrial membrane protein translocases stabilized *in vivo* by translocation intermediates. *J. Biol. Chem.* **274**, 22847–22854
- 31 Li, J., Kogan, M., Knight, S. A. B., Pain, D. and Dancis, A. (1999) Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution. *J. Biol. Chem.* **274**, 33025–33034
- 32 Anderson, D. J. and Blobel, G. (1983) Immunoprecipitation of proteins from cell-free translations. *Methods Enzymol.* **96**, 111–120
- 33 Jong, A. Y. and Ma, J. J. (1991) *Saccharomyces cerevisiae* nucleoside-diphosphate kinase: purification, characterization, and substrate specificity. *Arch. Biochem. Biophys.* **291**, 241–246
- 34 Gordon, D. M., Kogan, M., Knight, S. A. B., Dancis, A. and Pain, D. (2001) Distinct roles for two N-terminal cleaved domains in mitochondrial import of the yeast frataxin homolog, Yfh1p. *Hum. Mol. Genet.* **10**, 259–269
- 35 Knight, S. A. B., Sepuri, N. B. V., Pain, D. and Dancis, A. (1998) Mt-Hsp70 homolog, Ssc2p, required for maturation of yeast frataxin and mitochondrial iron homeostasis. *J. Biol. Chem.* **273**, 18389–18393
- 36 Fujitaki, J. M. and Smith, R. A. (1984) Techniques in the detection and characterization of phosphoramidate-containing proteins. *Methods Enzymol.* **107**, 23–36
- 37 Millar, D. G. and Shore, G. C. (1994) Mitochondrial Mas70p signal anchor sequence. Mutations in the transmembrane domain that disrupt dimerization but not targeting or membrane insertion. *J. Biol. Chem.* **269**, 12229–12232
- 38 Vestweber, D., Brunner, J., Baker, A. and Schatz, G. (1989) A 42K outer membrane protein is a component of the yeast mitochondrial import site. *Nature (London)* **341**, 205–209
- 39 Kiebler, M., Pfaller, R., Söllner, T., Griffiths, G., Horstmann, H., Pfanner, N. and Neupert, W. (1990) Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins. *Nature (London)* **348**, 610–616
- 40 Hill, K., Model, K., Ryan, M. T., Dietmeier, K., Martin, F., Wagner, R. and Pfanner, N. (1998) Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins. *Nature (London)* **395**, 516–521
- 41 Berg, P. and Joklik, W. K. (1953) Transphosphorylation between nucleoside polyphosphates. *Nature (London)* **172**, 1008–1009
- 42 Krebs, H. A. and Hems, R. (1953) Some reactions of adenosine and inosine phosphates in animal tissues. *Biochim. Biophys. Acta* **12**, 172–180
- 43 Sturtz, L. A., Diekert, K., Jensen, L. T., Lill, R. and Culotta, V. C. (2001) A fraction of yeast Cu, Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage. *J. Biol. Chem.* **276**, 38084–38089
- 44 Noda, L. (1973) Adenylate kinase. In *The Enzymes*, vol. 8, pp. 279–305, Academic Press, New York
- 45 Strobel, G., Zollner, A., Angermayr, M. and Bandlow, W. (2002) Competition of spontaneous protein folding and mitochondrial import causes dual subcellular location of major adenylate kinase. *Mol. Biol. Cell* **13**, 1439–1448
- 46 Beers, J., Moira Glerum, D. and Tzagoloff, A. (1997) Purification, characterization, and localization of yeast Cox17p, a mitochondrial copper shuttle. *J. Biol. Chem.* **272**, 33191–33196
- 47 Heaton, D. N., Geprge, G. N., Garrison, G. and Winge, D. R. (2001) The mitochondrial copper metallochaperone Cox17 exists as an oligomeric, polycopper complex. *Biochemistry* **40**, 743–751
- 48 Anandatheerthavarada, H. K., Biswas, G., Mullick, J., Sepuri, N. B. V., Otvos, L., Pain, D. and Avadhani, N. G. (1999) Dual targeting of cytochrome P4502B1 to endoplasmic reticulum and mitochondria involves a novel signal activation by cyclic AMP-dependent phosphorylation at Ser128. *EMBO J.* **18**, 5494–5504
- 49 Robin, M.-A., Anandatheerthavarada, H. K., Biswas, G., Sepuri, N. B. V., Gordon, D. M., Pain, D. and Avadhani, N. G. (2002) Bimodal targeting of microsomal CYP2E1 to mitochondria through activation of an N-terminal chimeric signal by cAMP mediated phosphorylation. *J. Biol. Chem.* **277**, 40583–40593
- 50 Gordon, D. M., Dancis, A. and Pain, D. (2000) Mechanisms of mitochondrial protein import. *Essays Biochem.* **36**, 31–43
- 51 Neupert, W. (1997) Protein import into mitochondria. *Annu. Rev. Biochem.* **66**, 863–917
- 52 Fujiki, M. and Verner, K. (1993) Coupling of cytosolic protein synthesis and mitochondrial protein import in yeast: evidence for cotranslational import *in vivo*. *J. Biol. Chem.* **268**, 1914–1920
- 53 Kellem, R. E., Allison, V. F. and Butow, R. A. (1975) Cytoplasmic type 80S ribosomes associated with yeast mitochondria. IV. Attachment of ribosomes to the outer membrane of isolated mitochondria. *J. Cell Biol.* **65**, 1–14
- 54 Suissa, M. and Schatz, G. (1982) Import of proteins into mitochondria. Translatable mRNAs for imported mitochondrial proteins are present in free as well as mitochondria-bound cytoplasmic polysomes. *J. Biol. Chem.* **257**, 13048–13055
- 55 Knox, C., Saas, E., Neupert, W. and Pines, O. (1998) Import into mitochondria, folding and retrograde movement of fumarate in yeast. *J. Biol. Chem.* **273**, 25587–25593
- 56 Nobumoto, M., Yamada, M., Song, S., Inouye, S. and Nakazawa, A. (1998) Mechanism of mitochondrial import of adenylate kinase isoenzymes. *J. Biochem. (Tokyo)* **123**, 128–135
- 57 Wong, E. D., Wagner, J. A., Gorsich, S. W., McCaffery, J. M. and Shaw, J. M. (2000) The dynamin-related GTPase, Mgm1p, is an intermembrane space protein required for maintenance of fusion competent mitochondria. *J. Cell Biol.* **151**, 341–352
- 58 Bominaar, A. A., Molijn, A. C., Pestel, M., Veron, M. and Van Haastert, P. J. M. (1993) Activation of G-proteins by receptor-stimulated nucleoside diphosphate kinase in *Dictyostellium*. *EMBO J.* **12**, 2275–2279
- 59 Chopade, B. A., Shankar, S., Sundin, G. W., Mukhopadhyay, S. and Chakrabarty, A. M. (1997) Characterization of membrane-associated *Pseudomonas aeruginosa* Ras-like protein Pra, a GTP-binding protein that forms complexes with truncated nucleoside diphosphate kinase and pyruvate kinase to modulate GTP synthesis. *J. Bacteriol.* **179**, 2181–2188
- 60 Zhu, J., Tseng, Y.-H., Kantor, J. D., Rhodes, C. J., Zetter, B. R., Moyers, J. S. and Kahn, C. R. (1999) Interaction of the Ras-related protein associated with diabetes Rad and the putative tumor metastasis suppressor NM23 provides a novel mechanism of GTPase regulation. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 14911–14918
- 61 Otsuki, Y., Tanaka, M., Yoshii, S., Kawazoe, N., Nakaya, K. and Sugimura, H. (2001) Tumor metastasis suppressor nm23H1 regulates Rac1 GTPase by interaction with Tiam1. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4385–4390