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Identification of the Mitochondrial NAD$^+$ Transporter in *Saccharomyces cerevisiae*[*]

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The mitochondrial carriers are a family of transport proteins that shuttle metabolites, nucleotides, and cofactors across the inner mitochondrial membrane. In *Saccharomyces cerevisiae*, NAD$^+$ is synthesized outside the mitochondria and must be imported across the permeability barrier of the inner mitochondrial membrane. However, no protein responsible for this transport activity has ever been isolated or identified. In this report, the identification and functional characterization of the mitochondrial NAD$^+$ carrier protein (Ndt1p) is described. The *NDT1* gene was overexpressed in bacteria. The purified protein was reconstituted into liposomes, and its transport properties and kinetic parameters were characterized. It transported NAD$^+$ and, to a lesser extent, (d)AMP and (d)GMP but virtually not α-NAD$^+$, NADH, NADP$^+$, or NADPH. Transport was saturable with an apparent K_m of 0.38 mM for NAD$^+$. The Ndt1p-GFP was found to be targeted to mitochondria. Consistently with Ndt1p localization and its function as a NAD$^+$ transporter, cells lacking *NDT1* had reduced levels of NAD$^+$ and NADH in their mitochondria and reduced activity of mitochondrial NAD$^+$-requiring enzymes. Similar results were also found in the mitochondria of cells lacking *NDT2* that encodes a protein (Ndt2p) displaying 70% homology with Ndt1p. The Δ*ndt1* Δ*ndt2* double mutant exhibited lower mitochondrial NAD$^+$ and NADH levels than the single deletants and a more pronounced delay in growth on nonfermentable carbon sources. The main role of Ndt1p and Ndt2p is to import NAD$^+$ into mitochondria by unidirectional transport or by exchange with intramitochondrially generated (d)AMP and (d)GMP.

Mitochondria contain in their matrix the universal hydrogen transfer coenzyme NAD$^+$, which serves to transfer hydrogen from substrates to the respiratory chain by oxidative phosphorylation. In addition to its well known role as a coenzyme in redox reactions, NAD$^+$ exerts other important functions in mitochondria. In *Saccharomyces cerevisiae*, mitochondrial NADH has been shown to participate in Fe/S protein biogenesis (1) and to be the source for NADPH (2), which is required in mitochondria for oxidative stress protection and for specific biogenesis reactions. NAD$^+$ has been shown also to serve critical regulatory functions in gene transcription, enzyme activity, and other important processes through ADP-ribosylation and deacetylation reactions (3–5). In addition, the increase in the NAD$^+$/NADH ratio in mitochondria seems to be important for the extension of the life-span of yeast by calorie restriction (6).

The enzymes of NAD$^+$ biosynthesis are generally believed to be localized outside the mitochondria (Refs. 7–9, but see Refs. 10 and 11), therefore, NAD$^+$ must be imported into these organelles. For a long time nicotinamide adenine dinucleotides were known to be unable to cross the inner membranes of mitochondria (12). However, NAD$^+$ has been shown to be taken up by intact plant mitochondria, the uptake being concentration- and temperature-dependent and specifically inhibited by an azido derivative of NAD$^+$ (13, 14). Moreover, using human cultured cells harvested under quiescent conditions (6–8 days after medium change), Rustin *et al.* (15) observed a depletion of mitochondrial NAD$^+$ and an influx of NAD$^+$ into the mitochondrial matrix of these cells after adding external NAD$^+$ to digitonin-permeabilized cells. These studies contradicted the notion of mitochondrial inner membrane impermeability to pyridine coenzymes and led to the hypothesis that NAD$^+$ is transported into mitochondria by a carrier-mediated system. However, the one or more proteins responsible for the observed transport activities have not been hitherto isolated or identified.

In this study we provide evidence that the gene products of *YIL006W* and *YEL006W*, named Ndt1p and Ndt2p, respectively, are two isoforms of the mitochondrial NAD$^+$ transporter in *S. cerevisiae*. These proteins are 373 and 335 amino acids long, respectively, possess the characteristics of the MCF,[2] and display a high degree (70%) of homology. Ndt1p was overexpressed in *Escherichia coli*, purified, reconstituted in phospholipid vesicles, and identified from its transport properties as a carrier for NAD$^+$. GFP fused to Ndt1p was found to be targeted to mitochondria. Both Δ*ndt1* and Δ*ndt2* cells exhibited lower levels of NAD$^+$ and NADH in their mitochondria and decreased activity of the NAD$^+$-requiring mitochondrial enzymes PDH and ACDH. The Δ*ndt1* Δ*ndt2* double mutant displayed also a severe delay in growth on nonfermentable carbon sources. This is the first time that proteins responsible for the uptake of NAD$^+$ into mitochondria and their genes have been identified at the molecular level.

MATERIALS AND METHODS

Sequence Search and Analysis—Databases were screened with the sequence of Ndt1p (encoded by *YIL006W*) with BLASTP and TBLASTN. The amino acid sequences were aligned with ClustalW (version 1.7). The phylogenetic tree was constructed using the neighbor-joining method (16), MacVector 7.2 software (Accelrys, Cambridge, UK), and PAM250 matrix.

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[2] The abbreviations used are: MCF, mitochondrial carrier family; ACDH, acetaldehyde dehydrogenase; ADH, alcohol dehydrogenase; GFP, green fluorescence protein; NAAD, deamido-NAD; NAMN, nicotinic acid mononucleotide; Ndt, nicotinamide adenine dinucleotide (NAD$^+$) transporter; NMN, nicotinamide mononucleotide; PDH, pyruvate dehydrogenase; PIPES, 1,4-piperazinediethanesulfonic acid; YP, rich medium; SC, synthetic complete medium; SM, synthetic minimal medium; LC-MS/MS, liquid chromatography tandem mass spectrometry.

Yeast Strains, Media, and Preparation of Mitochondria—BY4742 (wild-type), Δ*ndt1* and Δ*ndt2* yeast strains were provided by the EUROFAN resource center EUROSCARF (Frankfurt, Germany). The *NDT1* (*YIL006W*) or *NDT2* (*YEL006W*) locus of the *S. cerevisiae* strain BY4742 (MATα;*his*3Δ1;*leu*2Δ0;*lys*2Δ0;*ura*3Δ0) was replaced by kanMX4. The double deletion strain Δ*ndt1* Δ*ndt2* was constructed using the PCR-mediated gene disruption technique by replacing the *YEL006W* open reading frame with the hygromycin B resistance cassette (hphMX3) in the Δ*ndt1* mutant (17). All deletions were verified by PCR. The wild-type and deletion strains were grown in rich medium (YP) containing 2% bactopeptone and 1% yeast extract, synthetic complete medium (SC), or synthetic minimal medium (SM) (18). All media were supplemented with a fermentable or a nonfermentable carbon source (2% glucose, 2% ethanol, 3% acetate, 2% DL-lactate, or 2% pyruvate) and with auxotrophic nutrients when required. The final pH was adjusted to 4.5 or, with acetate or pyruvate, to 6.5. Mitochondria and spheroplasts were isolated from cells grown in ethanol-supplemented YP until the early exponential phase (optical density between 1.0 and 1.5) was reached. The mitochondria were further purified on a discontinuous gradient (19).

Construction of Expression Plasmids—The coding sequence of *NDT1* (*YIL006W*) or *NDT2* (*YEL006W*) was cloned into the pMW7 vector for expression in *E. coli*. Both open reading frames were amplified from *S. cerevisiae* genomic DNA by PCR using primers corresponding to the extremities of the coding sequences with additional BamHI and HindIII sites for *NDT1* and NdeI and HindIII sites for *NDT2*. The pRS416-*NDT1* and pRS416-*NDT2* plasmids were constructed by cloning a DNA fragment consisting of the *NDT1* or *NDT2* open reading frame, of 450-bp upstream and 250-bp downstream of the open reading frame into the low copy centromeric vector pRS416 (20). The latter DNA fragments were amplified from *S. cerevisiae* genomic DNA by PCR using primers with additional BamHI and XhoI sites. Transformants of *E. coli* DH5α cells were selected on ampicillin (100 μg/ml) and screened by direct colony PCR and by restriction digestion of purified plasmids. The sequences of the inserts were verified.

Bacterial Expression and Purification of Ndt1p and Ndt2p—The overproduction of Ndt1p or Ndt2p as inclusion bodies in the cytosol of *E. coli* was accomplished as previously described (21), except that the host cells were *E. coli* C0214(DE3) (22). Control cultures with the empty vector were processed in parallel. Inclusion bodies were purified on a sucrose density gradient (21) and washed at 4 °C, first with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.0), then twice with a buffer containing Triton X-114 (3%, w/v), 1 mM EDTA, and 10 mM PIPES, pH 7.0, and finally with 10 mM PIPES, pH 7.0. Ndt1p or Ndt2p was solubilized in 1.6% Sarkosyl (w/v). Small residues were removed by centrifugation (258,000 × *g* for 20 min at 4 °C).

Reconstitution into Liposomes and Transport Assays—The recombinant protein in Sarkosyl was reconstituted into liposomes in the presence of substrates, as described previously (23). External substrate was removed from proteoliposomes on Sephadex G-75 columns, pre-equilibrated with 50 mM NaCl and 10 mM PIPES-NaOH at pH 7.0 (buffer A). Transport at 25 °C was started by adding [^{3}H]NAD$^+$ (Moravek Biochemicals, Brea, CA) or the indicated radioactive substrate to proteoliposomes and terminated by the addition of 20 mM bathophenanthroline and 30 mM pyridoxal 5′-phosphate (the "inhibitor-stop" method (23)). In controls, the inhibitors were added at the beginning together with the radioactive substrate. All transport measurements were carried out in the presence of 10 mM PIPES at pH 7.0 in the internal and external compartments. The external substrate was removed, and the radioactivity in the liposomes was measured (23). The experimental values were corrected by subtracting control values. The initial transport rate was calculated from the radioactivity taken up by proteoliposomes after 45 s (in the initial linear range of substrate uptake). For efflux measurements, proteoliposomes containing 2 mM NAD$^+$ were labeled with 20 μM [^{3}H]NAD$^+$ by carrier-mediated exchange equilibration (23). After 40 min, external radioactivity was removed by passing the proteoliposomes through Sephadex G-75 columns. Efflux was started by adding unlabeled external substrate or buffer A alone and terminated by adding the inhibitors indicated above.

Subcellular Localization of Ndt1p—The yeast strain BY4742 was transformed with the pUG35 vector containing the coding sequence for Ndt1p in-frame with the yEGFP (yeast-enhanced green fluorescent protein) coding sequence under the control of the MET25 promoter. Cells were grown in ethanol-supplemented SC medium until mid-logarithmic phase and transferred to the same medium without methionine. After 4 h the cells were incubated for 1 h at 30 °C in the presence of 50 nM MitoTracker Red CMXRos (Molecular Probes), washed twice with fresh medium, and imaged at the microscope as described previously (22).

NAD$^+$ and NADH Determination by Mass Spectrometry—For mass spectrometry analysis of NAD$^+$ and NADH, mitochondria were extracted as described (24). A Quattro Premier mass spectrometer interfaced with an Alliance 2695 high-performance liquid chromatography system (Waters, Milford, MA) was employed for electrospray ionization LC-MS/MS analysis in the positive ion mode. The multiple reaction monitoring transition monitored for NAD$^+$ was m/z 664.2 > 428.2 and for NADH m/z 666.2 > 649.1. Chromatographic resolution of NAD$^+$ and NADH was achieved using an Atlantis dC$_{18}$ column (2.1 × 150 mm, 5-μm particle size, Waters) eluted with a linear gradient from 100% 10 mM ammonium formate (initial phase) to 10% 10 mM ammonium formate/90% methanol. The flow was set at 0.3 ml/min. Calibration curves were established using standards, processed in the same conditions as the samples, at four concentrations. The lines of best fit were determined using regression analysis based on the peak area of the analytes.

Other Methods—NAD$^+$ synthetase and NAMN/NMN adenylyltransferase activities were assayed according to published protocols (25, 26), except that NAD$^+$ produced after 1 h was quantified by LC-MS/MS analysis. The activities of PDH and ACDH were measured as described in Refs. 27 and 28, respectively, except that only the NAD$^+$ present in the mitochondrial extracts was added to the assay mixtures. In these assays, NAD$^+$ was regenerated by the diaphorase-coupled cycling reaction (29) and the increase in fluorescence due to resorufin production was monitored by an LS 50B luminescence spectrometer (PerkinElmer Life Sciences). Citrate synthase was measured as described before (30). Proteins were separated by SDS-PAGE and stained with Coomassie Blue dye. The N termini were sequenced, and the amount of purified proteins was estimated by laser densitometry of stained samples using carbonic anhydrase as a protein standard (31). The amount of protein incorporated into liposomes was measured as described (31) and was ~25% of the protein added to the reconstitution mixture.

RESULTS

Sequence Features of Ndt1p and Ndt2p—The proteins encoded by *YIL006W* and *YEL006W*, named Ndt1p and Ndt2p, respectively, belong to the MCF, because their amino acid sequences are composed of 3 tandem repeats of about 100 amino acids, each containing two transmembrane α-helices, linked by an extensive loop, and a conserved signature motif (see Ref. 32 for a review). Compared with other MCF members, Ndt1p and Ndt2p exhibit a tryptophan instead of an acidic

residue in the signature motif of the second repeat (PIWVVK). This characteristic feature is found in a mitochondrial carrier subfamily, which in *S. cerevisiae* comprises Flx1p (encoded by *YIL134W*) and Rim2p (encoded by *YBR192W*). A major difference between Ndt1p and Ndt2p is that the N-terminal extension of the former is 39 amino acids longer than the extension of the latter. Ndt1p is therefore longer than the great majority of mitochondrial carriers (molecular mass, 30–34 kDa) and shorter than the Ca^{2+}-activated mitochondrial carriers (molecular mass, 50–60 kDa) (see Ref. 32 for a review and Ref. 33).

Bacterial Expression of Ndt1p and Ndt2p—The *NDT1* and *NDT2* genes were overexpressed in *E. coli* C0214(DE3). The respective proteins were accumulated as inclusion bodies and purified by centrifugation and washing. The purified proteins gave a single band by SDS-PAGE with apparent molecular masses of ~42.0 and 37.5 kDa for Ndt1p and Ndt2p, respectively (data not shown), in agreement with the calculated values with initiator methionine (41,951 and 36,975 Da, respectively). Their identities were confirmed by N-terminal sequencing. The proteins were not detected in bacteria harvested immediately before the induction of expression nor in cells harvested after induction but lacking the coding sequence in the expression vector. Approximately 100 mg of Ndt1p and 75 mg of Ndt2p per liter of culture were obtained.

Functional Characterization of Ndt1p—In the search for potential substrates of Ndt1p and Ndt2p, we based our choice on the fact that these proteins are most closely related to Flx1p (34) and Rim2p (35), which transport coenzyme FAD and pyrimidine nucleotides, respectively. Proteoliposomes reconstituted with recombinant Ndt1p catalyzed an active [^{3}H]NAD$^+$/NAD$^+$ exchange that was completely inhibited by a mixture of bathophenanthroline and pyridoxal 5′-phosphate. They did not catalyze homo-exchanges for pyruvate, nicotinamide, folate, citrate, carnitine, and aspartate (internal concentration, 10 mM; external concentration, 1 mM) (results not shown). Importantly, no [^{3}H]NAD$^+$/NAD$^+$ exchange activity was detected if Ndt1p had been boiled before incorporation into liposomes or if proteoliposomes were reconstituted with Sarkosyl-solubilized material from bacterial cells either lacking the expression vector for Ndt1p or harvested immediately before induction of expression. Likewise, no such activity was detected in liposomes reconstituted with six unrelated mitochondrial carriers, Ort1p (36), Sfc1p (37), Sam5p (22), Ggc1p (38), APC (33), and GC1 (39) and the closely related carrier Rim2p (35), which had been expressed and purified from *E. coli*. At variance with Ndt1p, recombinant and reconstituted Ndt2p showed no activity under any of the experimental conditions tested, which include variation of the parameters that influence solubilization of the inclusion bodies and reconstitution of the protein into liposomes.

To investigate the substrate specificity of Ndt1p in more detail, we measured the uptake of [^{3}H]NAD$^+$ into proteoliposomes that had been preloaded with a variety of potential substrates (Fig. 1). The highest activity of [^{3}H]NAD$^+$ uptake into proteoliposomes was with internal NAD$^+$. [^{3}H]NAD$^+$ also exchanged substantially with internal NAAD, (d)AMP, and (d)GMP. Notably, internal α-NAD$^+$, NADH, NMN, NAMN, and ADP-ribose were very poor counter-substrates. In addition, NADP$^+$, NADPH, nicotinamide, and nicotinic acid were virtually not exchanged.

Among nucleotides, those of A and G exchanged with [^{3}H]NAD$^+$ more effectively than those of U, T, and C. In all cases, internal nucleoside diphosphate and nucleoside triphosphate were less effective than nucleoside monophosphate, and the deoxynucleotides were nearly as effective as the corresponding nucleotides. Interestingly, [^{3}H]NAD$^+$ also exchanged, to some extent, with internal FMN, FAD, and 3′-AMP. In contrast, the uptake of [^{3}H]NAD$^+$ was negligible in the presence of

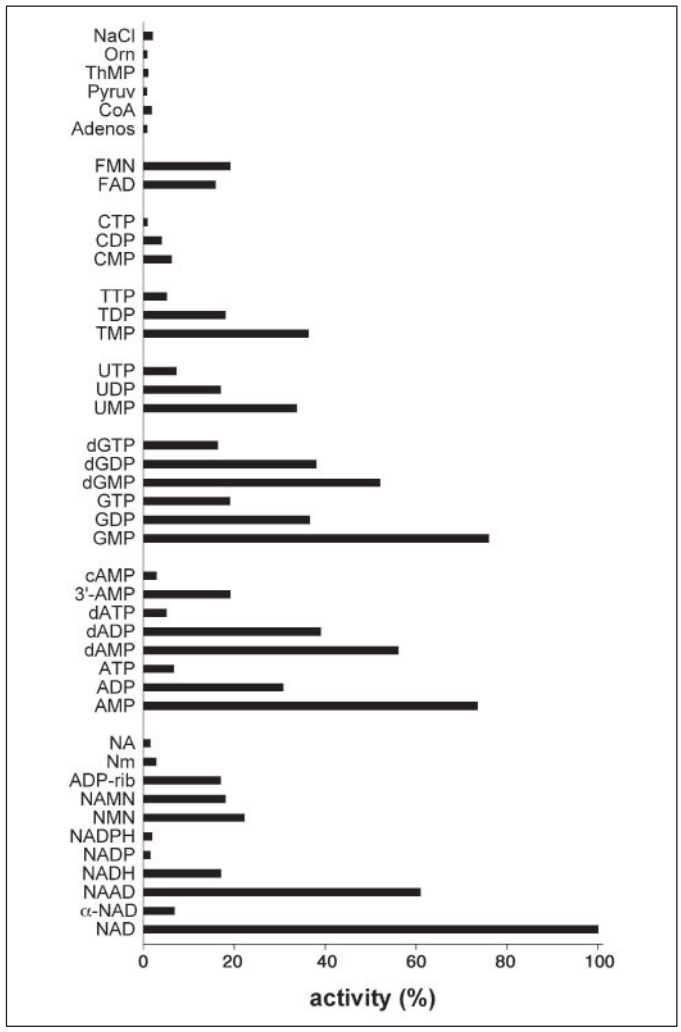

FIGURE 1. **Substrate specificity of Ndt1p.** Proteoliposomes were preloaded internally with various substrates (concentration 10 mM). Transport was started by addition of 0.38 mM [^{3}H]NAD$^+$ and stopped after 45 s. The values are expressed as a percentage of the [^{3}H]NAD$^+$/NAD$^+$ exchange activity, which was 0.32 ± 0.04 mmol/min per gram of protein. Similar results were obtained in at least three independent experiments. *Adenos*, adenosine; *ADP-rib*, ADP-ribose; *NA*, nicotinic acid; *Nm*, nicotinamide; *Orn*, ornithine; *Pyruv*, pyruvate; *ThMP*, thiamine monophosphate.

internal cAMP, adenosine, coenzyme A, pyruvate, thiamine monophosphate, ornithine, NaCl (Fig. 1), and cGMP, thiamine, thiamine diphosphate, *S*-adenosylmethionine, succinate, malate, malonate, oxoglutarate, oxalacetate, phosphoenolpyruvate, citrate, carnitine, aspartate, glutamate, glutamine, lysine, arginine, histidine, proline, threonine, glutathione, choline, and spermine (data not shown). In contrast with the data shown for Ndt1p in Fig. 1, no uptake of [^{3}H]NAD$^+$ was observed in liposomes reconstituted with Ort1p, Sfc1p, Sam5p, Ggc1p, APC, GC1, or Rim2p containing even their best substrate, *i.e.* ornithine, fumarate, *S*-adenosylmethionine, GTP, ATP-Mg, glutamate, or TTP, respectively.

The [^{3}H]NAD$^+$/NAD$^+$ exchange reaction catalyzed by reconstituted Ndt1p was inhibited markedly by mercurials (*p*-hydroxymercuribenzoate, mersalyl, and mercuric chloride), pyridoxal 5′-phosphate and bathophenanthroline (inhibitors of several mitochondrial carriers), as well as by bromcresol purple and tannic acid (inhibitors of the glutamate carrier) (Fig. 2). In contrast, little inhibition was observed with *N*-ethylmaleimide, α-cyano-4-hydroxycinnamate, 1,2,3-benzenetricarboxylate, butylmalonate, carboxyatractyloside, and bongkrekic acid (inhibitors of other mitochondrial carriers).

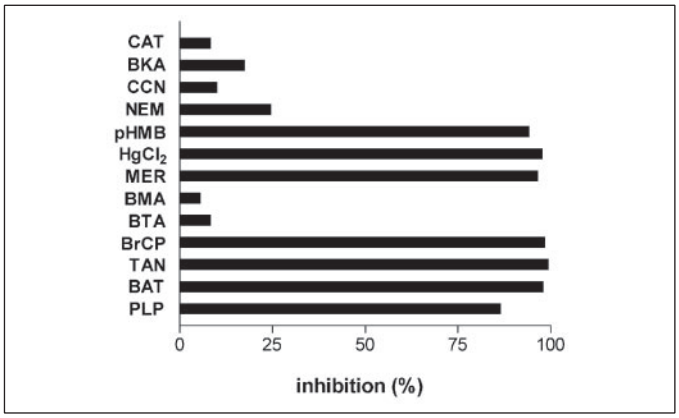

FIGURE 2. **Effect of inhibitors on the [^{3}H]NAD$^+$/NAD$^+$ exchange by Ndt1p.** Proteoliposomes were preloaded internally with 10 mM NAD$^+$ and transport was initiated by adding 0.38 mM [^{3}H]NAD$^+$. The incubation time was 45 s. Thiol reagents and α-cyanocinnamate were added 2 min before the labeled substrate; the other inhibitors were added together with [^{3}H]NAD$^+$. The final concentrations of the inhibitors were: 10 μM (*CAT*, carboxyatractyloside; *BKA*, bongkrekic acid), 0.2 mM (*pHMB*, *p*-hydroxymercuribenzoate; *HgCl$_2$*, mercuric chloride; *MER*, mersalyl), 0.3 mM (*BrCP*, bromcresol purple), 1 mM (*CCN*, α-cyanocinnamate), 2 mM (*NEM*, *N*-ethylmaleimide; *BTA*, benzene-1,2,3-tricarboxylate; *BMA*, butylmalonate), 20 mM (*BAT*, bathophenanthroline; *PLP*, pyridoxal 5′-phosphate), and 0.2% (*TAN*, tannic acid). The extents of inhibition (%) from a representative experiment are reported.

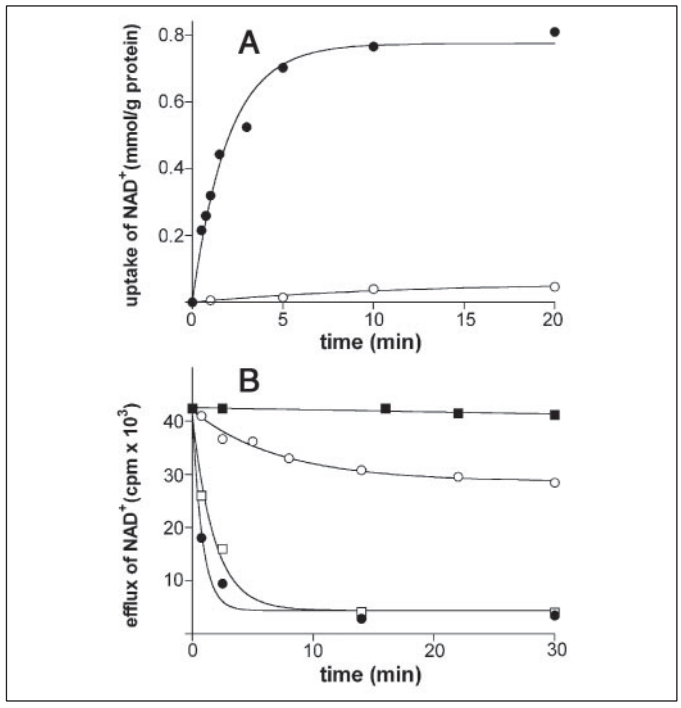

FIGURE 3. **Kinetics of [^{3}H]NAD$^+$ transport in proteoliposomes reconstituted with Ndt1p.** *A*, uptake of NAD$^+$. 1 mM [^{3}H]NAD$^+$ was added to proteoliposomes containing 10 mM NAD$^+$ (exchange, ●) or 10 mM NaCl and no substrate (uniport, ○). Similar results were obtained in three independent experiments. *B*, efflux of [^{3}H]NAD$^+$ from proteoliposomes reconstituted in the presence of 2 mM NAD$^+$. The internal substrate pool was labeled with [^{3}H]NAD$^+$ by carrier-mediated exchange equilibration. Then the proteoliposomes were passed through Sephadex G-75. [^{3}H]NAD$^+$ efflux was initiated by adding buffer A alone (○), 2 mM NAD$^+$ in buffer A (●), 2 mM AMP in buffer A (□), or 20 mM bathophenanthroline and 30 mM pyridoxal 5′-phosphate in buffer A (■). Similar results were obtained in three independent experiments.

Kinetic Characteristics of Recombinant Ndt1p—The kinetics were compared for the uptake of 1 mM [^{3}H]NAD$^+$ into proteoliposomes either as uniport (in the absence of internal NAD$^+$) or as exchange (in the presence of 10 mM NAD$^+$) (Fig. 3). The uptake of NAD$^+$ by exchange followed a first-order kinetics (rate constant, 0.06 min^{-1}; initial rate, 442 nmol/min per mg of protein), isotopic equilibrium being approached exponentially. In contrast, the uniport uptake of NAD$^+$ was very low. The uniport mode of transport was further investigated by measuring the efflux of [^{3}H]NAD$^+$ from prelabeled active proteoliposomes, because it provides a more convenient assay for unidirectional transport (23). In these experiments, little yet significant efflux of [^{3}H]NAD$^+$ from prelabeled proteoliposomes was observed in the absence of external substrate, whereas extensive efflux occurred upon addition of external NAD$^+$ or AMP (Fig. 3). Both effluxes, *i.e.* with and without external substrate, were prevented completely if the inhibitors bathophenanthroline and pyridoxal 5′-phosphate were present (data not shown). Therefore, Ndt1p is able to catalyze a low unidirectional transport of NAD$^+$ besides a fast exchange reaction of substrates.

The kinetic constants of the recombinant purified Ndt1p were determined by measuring the initial transport rate at various external [^{3}H]NAD$^+$ concentrations in the presence of a constant saturating internal concentration of 10 mM NAD$^+$. The transport affinity (K_m) and specific activity (V_{max}) values for NAD$^+$/NAD$^+$ exchange at 25 °C were 0.38 ± 0.04 mM and 617 ± 97 nmol/min per mg of protein, respectively, in 40 experiments. The K_m value of Ndt1p for NAD$^+$ reported here is virtually the same as that found for the uptake of [^{14}C]NAD$^+$ into potato mitochondria (13). This value is lower than the intracellular concentrations of 1–3 mM of NAD$^+$ plus NADH found in yeast (40 and references therein). However, it should be noted that most of the total cellular NAD$^+$ and NADH is bound to proteins and that only ~10% has been estimated to be in the free form in mammalian cells (41). Therefore, the determined K_m of 0.38 mM for NAD$^+$ transport by Ndt1p should be rather close to the physiological concentrations of cytosolic free NAD$^+$. Several external substrates were competitive inhibitors of [^{3}H]NAD$^+$ uptake (Table 1), because they increased the apparent K_m without changing the V_{max} (not shown). These results confirm that the affinity of Ndt1p for (d)AMP and (d)GMP is higher than that for the corresponding nucleoside diphosphates. Furthermore, the K_i values of AMP and GMP are about three times lower than those of TMP and UMP.

TABLE 1

Competitive inhibition by various substrates of [^{3}H]NAD$^+$ uptake in proteoliposomes containing recombinant Ndt1p

The values were calculated from Lineweaver-Burk plots of the rate of [^{3}H]NAD$^+$ *versus* substrate concentrations. The competing substrates at appropriate constant concentrations were added together with 0.05–1.25 mM [^{3}H]NAD$^+$ to proteoliposomes containing 10 mM NAD$^+$. The data represent the means ± S.D. of at least three different experiments.

Substrate	K_i
	mM
NAD$^+$	0.33 ± 0.03
AMP	0.98 ± 0.09
GMP	0.95 ± 0.08
TMP	3.01 ± 0.34
UMP	3.12 ± 0.29
ADP	1.95 ± 0.24
GDP	2.14 ± 0.23
dAMP	1.08 ± 0.14
dGMP	1.09 ± 0.15
dADP	2.03 ± 0.25
NAAD	1.29 ± 0.17

Subcellular Localization of Ndt1p—Because some members of the MCF are localized in non-mitochondrial membranes (42, 43), the intracellular localization of Ndt1p was investigated using a GFP-tagged protein. Cells expressing Ndt1p-GFP showed a green fluorescence typical of the mitochondrial network (Fig. 4). Furthermore, the fluorescence of Ndt1p-GFP completely overlapped with the red fluorescence displayed by the mitochondrial-specific dye, MitoTracker Red (Fig. 4). The structural integrity of the cells was documented by phase contrast micros-

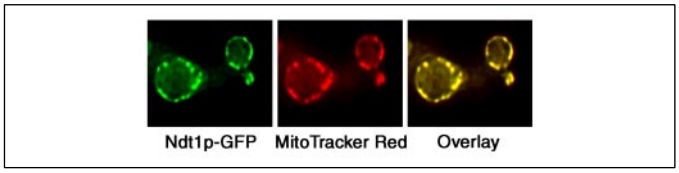

FIGURE 4. **Subcellular localization of Ndt1p-GFP fusion protein after expression in *S. cerevisiae* cells.** Wild-type cells were transfected with the Ndt1p-GFP construct, and MitoTracker Red was used to locate mitochondria in the cells. The same cells were photographed first with a GFP filter set and then with the MitoTracker Red filter set. Identical fields are presented. *Ndt1p-GFP*, fluorescence of GFP fused to Ndt1p; *MitoTracker Red*, fluorescence of MitoTracker Red; *Overlay*, merged image of MitoTracker Red fluorescence with Ndt1p-GFP fluorescence.

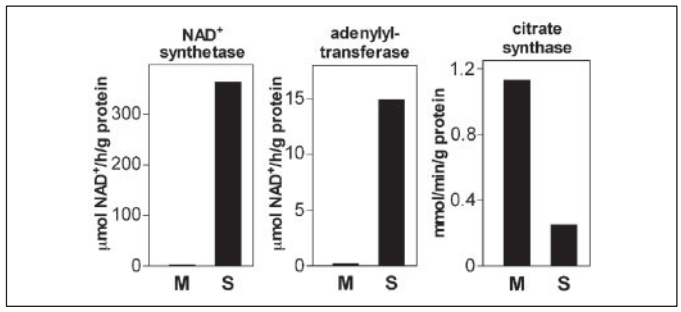

FIGURE 5. **NAD$^+$ synthesis in mitochondria and spheroplasts.** Purified mitochondria (*M*) or spheroplasts (*S*) were lysed in a mixture containing 0.05% Triton X-100 and the appropriate substrate-less assay buffer. The reaction was started by addition of glutamine plus ATP plus NAAD (to test NAD$^+$ synthetase), NMN plus ATP (to test NAMN/NMN adenylyltransferase), and oxaloacetate (to test citrate synthase).

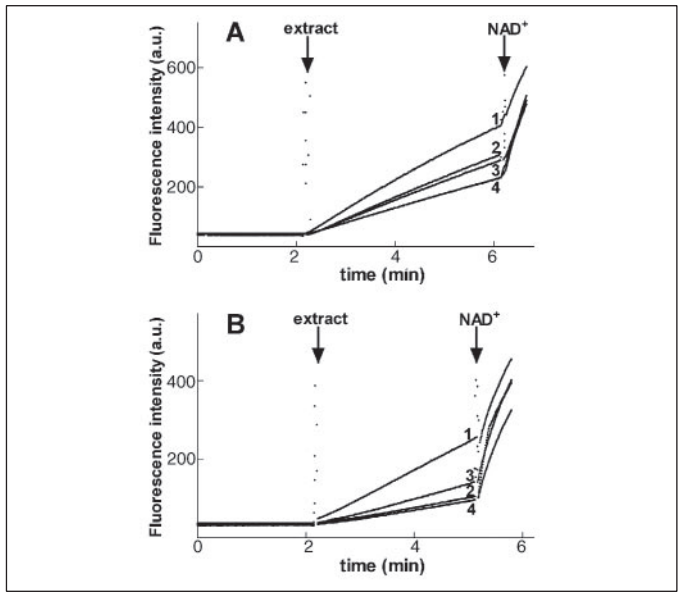

FIGURE 6. **Mitochondria from cells lacking *NDT1* and/or *NDT2* are defective in PDH and ACDH activities.** The reagents of the diaphorase-coupled reaction (0.1 ml) were added to the NAD$^+$-less assay mixture (0.85 ml) for PDH (*panel A*) or ACDH (*panel B*). In both cases, the reaction was initiated by adding the mitochondrial extract (50 μg of protein in 50 μl) from wild-type (*1*), Δ*ndt2* (*2*), Δ*ndt1* (*3*), or Δ*ndt1* Δ*ndt2* (*4*) cells.

copy (data not shown). Using the same tagged-protein technique, Ndt2p also was previously shown to be localized to mitochondria (9).

NAD$^+$ Is Not Synthesized in the Mitochondria—Having established that Ndt1p is a mitochondrial NAD$^+$ transporter, it is essential to ascertain whether mitochondria are capable of synthesizing NAD$^+$ to define the physiological role of Ndt1p in the cell. Although in *S. cerevisiae* evidence exists for an extramitochondrial localization of the NAD$^+$-synthesizing enzymes from NAAD (NAD$^+$ synthetase, Qns1p) and from NAMN or NMN (NAMN/NMN adenylyltransferases, Nma1p and Nma2p) (7–9), in humans a third isoform of Nma (named hNMNAT3) has been reported to reside within mitochondria (10, 11). We have therefore assessed the two NAD$^+$-synthesizing activities, NAD$^+$ synthetase and NAMN/NMN adenylyltransferase, in mitochondria and spheroplasts of wild-type yeast cells over a long period of incubation (1 h). In both assays, no NAD$^+$ was produced in mitochondrial extracts, whereas a substantial amount of NAD$^+$ was found in spheroplast extracts (Fig. 5). This result contrasts with the distribution of citrate synthase which is predominantly localized in mitochondria (Fig. 5). Therefore, NAD$^+$ is not synthesized in the mitochondrial matrix and must be imported.

Ndt1p and Ndt2p Are Required for the Entry of NAD$^+$ into Mitochondria—To confirm the transport function of Ndt1p *in vivo*, we measured the activities of the NAD$^+$-requiring mitochondrial enzymes PDH and ACDH. In these measurements, only the NAD$^+$ present in the mitochondrial extracts was added to the assay mixture. Therefore, under conditions of no NAD$^+$ saturation, one would expect different enzyme activities depending on the NAD$^+$ concentration present in the reaction medium. Fig. 6*A* shows that PDH activity was lower in mitochondria (mitochondrial extracts) from the Δ*ndt1* and Δ*ndt2* single deletants, and even more so from the Δ*ndt1* Δ*ndt2* double mutant, than the activity in mitochondria from the wild-type strain. The addition of 0.1 mM NAD$^+$ to the assay mixture increased PDH activity to similar values in all cases. Under the same conditions, the activity of ACDH was also lower in the knock-out cells than in the wild-type cells, and restoration was observed when NAD$^+$ was added to the assay mixture (Fig. 6*B*). In contrast, the activity of the NAD$^+$-requiring ADH, measured in the post-mitochondrial supernatant, was not significantly different in Δ*ndt1* and Δ*ndt2* single and double deletants as compared with wild-type cells (data not shown). These results indicate that the lower activity of PDH and ACDH in the knock-out strains may be caused by lack of NAD$^+$ in the mitochondria of the mutants.

We then assayed the NAD$^+$ content in mitochondria isolated from the wild-type strain *versus* strains lacking one or both the Ndt1p and Ndt2p isoforms (Fig. 7). The Δ*ndt1* and Δ*ndt2* single deletants displayed a marked decrease in mitochondrial NAD$^+$ content compared with the control, demonstrating that both isoforms are relevant for NAD$^+$ transport into mitochondria. NAD$^+$ was re-instated to the wild-type levels when the single mutants were complemented with the missing gene. In the Δ*ndt1* Δ*ndt2* strain, the NAD$^+$ content in mitochondria was significantly lower than that of the single deletants ($p < 0.05$). In the Δ*ndt1* Δ*ndt2* mutant complemented with *NDT1* or *NDT2*, the content of NAD$^+$ increased with respect to that of the double deletant and reached ~40% higher levels than those measured for the Δ*ndt1* or Δ*ndt2* single mutants, respectively.

The NADH levels assayed in the same strains showed changes similar to those observed for NAD$^+$ (Fig. 7). Because NADH levels were remarkably lower as compared with those of NAD$^+$, we measured the content of NAD$^+$ and NADH in mitochondria incubated with TMPD/ascorbate, a treatment known to increase the NADH/NAD$^+$ ratio (44). In the presence of TMPD/ascorbate, the NADH levels were much higher in both wild-type and Δ*ndt1* Δ*ndt2* strains than in the respective untreated strains. However, the total mitochondrial nicotinamide dinucleotide pool (NAD$^+$ plus NADH) was similar (Fig. 7). Taken together, these results indicate that the low levels of NADH in untreated strains are dependent on the state of mitochondria and not on NADH loss during sample preparation.

Growth Characteristics of the Wild-type and Mutant Strains—In comparison to wild-type cells, mutant cells lacking either *NDT1* or *NDT2* showed similar growth patterns in YP and SM media containing

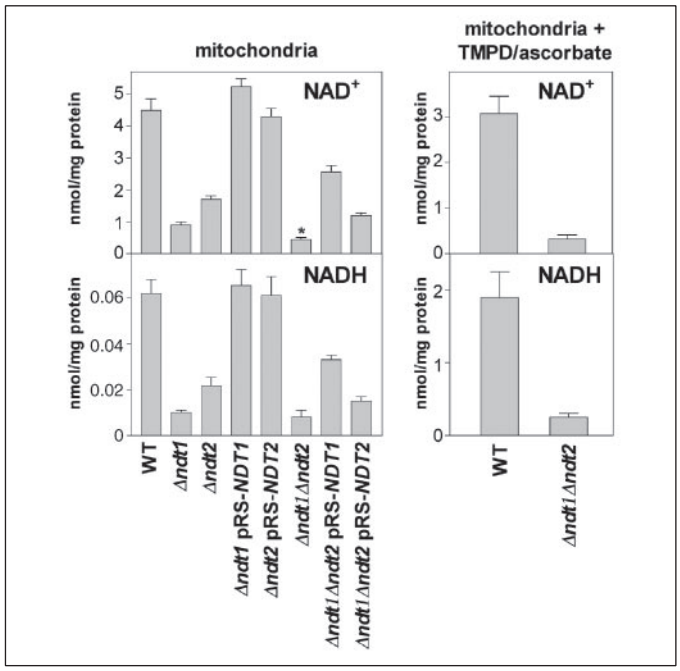

FIGURE 7. **Ndt1p and Ndt2p are required for the entry of NAD+ into mitochondria.** Mitochondrial extracts were assayed for NAD+ and NADH content by LC-MS/MS analysis. Data represent means ± S.E. of at least three independent experiments. The significance of the difference between the intramitochondrial NAD+ level of Δ*ndt1* Δ*ndt2* compared with that of Δ*ndt1* is indicated (*, $p < 0.05$, one-way analysis of variance). In the *right-hand panel*, the mitochondria were incubated with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD)/ascorbate for 1 min before being extracted.

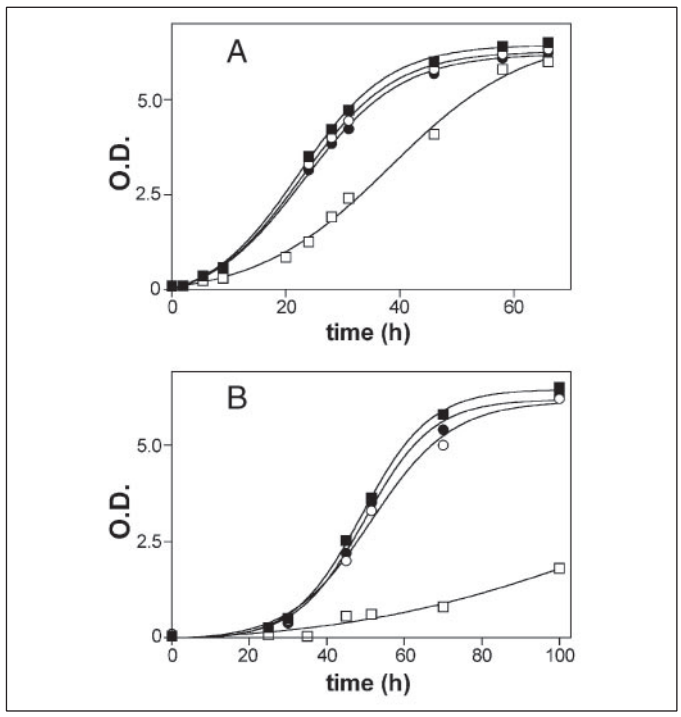

FIGURE 8. **Effect of *NDT1* and *NDT2* single and double deletion on the growth of yeast cells on ethanol.** Wild-type (■), Δ*ndt1* Δ*ndt2* (□), Δ*ndt1* Δ*ndt2* pRS416-*NDT1* (●), and Δ*ndt1* Δ*ndt2* pRS416-*NDT2* (○) were inoculated in YP (*A*) or SM (*B*) medium supplemented with 2% ethanol. The values of optical density at 600 nm refer to cell cultures after the indicated periods of growth. Data from a representative experiment are reported. Similar results were obtained in three independent experiments.

glucose or a nonfermentable carbon source (lactate, pyruvate, acetate, or ethanol; data not shown). The Δ*ndt1* Δ*ndt2* double mutant also exhibited similar growth in the same media supplemented with glucose. However, this mutant displayed a growth delay in the YP medium supplemented with ethanol (Fig. 8*A*) as well as with lactate, pyruvate, or acetate (data not shown). In the SM medium, growth of Δ*ndt1* Δ*ndt2* on ethanol was very poor even after 100 h (Fig. 8*B*); on the other nonfermentable substrates tested, a more pronounced lag-phase of growth was observed in the SM than in the YP medium, although maximal growth was reached after ~100 h (data not shown). The growth phenotype of the Δ*ndt1* Δ*ndt2* cells was recovered when these cells were transformed with either pRS416-*NDT1* or pRS416-*NDT2* (data not shown), demonstrating that the impaired phenotype is the result of the absence of Ndt1p and Ndt2p and not of a secondary effect.

DISCUSSION

The transport properties and kinetic characteristics of recombinant and reconstituted Ndt1p from *S. cerevisiae*, together with its targeting to mitochondria, demonstrate that this protein is the mitochondrial transporter for NAD+. For the closely related sequence of Ndt2p, which was also cloned and expressed in *E. coli* in this study, no biochemical data are available, because we were incapable of renaturing and/or reconstituting it functionally. However, because of the high degree of homology between Ndt1p and Ndt2p (70% homology and 55% identical amino acids), which is similar to that found for other mitochondrial carrier isoforms (33, 39, 45), it is likely that Ndt2p is an isoform of the NAD+ transporter in *S. cerevisiae*. The percentage of homology and identical amino acids is based on the sequence of Ndt1p without the first 54 residues of its long N-terminal extension. In fact, without the first 54 residues Ndt1p exhibits the same transport properties as extended Ndt1p.[3] This is the first time that a mitochondrial carrier for NAD+ has been identified from any organism. Until now, only another transporter for intact NAD+ molecules was known in biological membranes, *i.e.* the NAD+/ADP exchanger of an intracellular bacterial symbiont related to Chlamydiae (46). This exchanger, however, does not belong to the MCF.

Ndt1p (encoded by *YIL006W*) has been previously reported to be the mitochondrial pyruvate carrier of *S. cerevisiae* (47). This conclusion was based on the observation that among the mitochondria isolated from 18 different *S. cerevisiae* mutants, each lacking an unattributed member of the MCF, only those from the Δ*ndt1* mutant exhibited no inhibitor-sensitive transport of pyruvate (47). However, recombinant and reconstituted Ndt1p does not transport pyruvate either as [^{14}C]pyruvate/pyruvate or as [^{3}H]NAD+/pyruvate exchange. Furthermore, external pyruvate (20 mM) does not enhance loss of [^{3}H]NAD+ from preloaded proteoliposomes and, when added together with [^{3}H]NAD+, does not inhibit the reconstituted NAD+/NAD+ exchange. In addition, in a phylogenetic tree of the *S. cerevisiae* members of the MCF (48), Ndt1p and Ndt2p cluster together with transporters for nucleotides (Flx1p (34) and Rim2p (35), which transport FAD and pyrimidine nucleotides, respectively).

Apart from the relatively low homology that Ndt1p and Ndt2p display with Flx1p and Rim2p (25–29% of identical amino acids), Ndt1p and Ndt2p do not exhibit significant sequence homology with any other mitochondrial carrier functionally identified so far above the basic homology existing between the different members of the MCF. However, several protein sequences available in databases are or may be orthologs of these transporters in other organisms. These sequences include: XP_453688.1 from *Kluyveromyces lactis* (47% of identical

[3] S. Todisco, G. Agrimi, and F. Palmieri, unpublished observations.

amino acids with Ndt1p); EAK95611.1 from *Candida albicans* (45%); XP_661991.1 from *Aspergillus nidulans* (34%); EAA31572.1 from *Neurospora crassa* (30%); AAH90770.1 from *Danio rerio* (34%); AAM68821.1 from *Drosophila melanogaster* (31%); NP_564233.1 from *Arabidopsis thaliana* (31%); AAH87370.1 from *Xenopus laevis* (31%); NP_765990.1 from *Mus musculus* (33%) and NP_110407.2 from *Homo sapiens* (31%). To our knowledge, none of these proteins has been characterized biochemically. However, NP_110407.2 (named hMFT), the closest human relative of Ndt1p and Ndt2p (31 and 30% identical amino acids, respectively), has been reported to be the mitochondrial carrier for folate (49). This conclusion was based on the finding that hMFT re-instates folate in the mitochondria of transfected glyB cells (49). Although recombinant Ndt1p does not transport folate, at present it cannot be excluded that the human protein transports both folate and NAD$^+$.

Besides transporting NAD$^+$, Ndt1p transports NAAD, (d)AMP, (d)GMP, and, to a lesser extent, UMP, TMP, and (d)ADP and (d)GDP. The substrate specificity of Ndt1p is distinct from that of any other previously characterized member of the MCF. In particular, Ndt1p differs markedly from the closely related protein in *S. cerevisiae*, Rim2p, recently identified as the mitochondrial transporter for pyrimidine nucleotides (35), because Rim2p transports pyrimidine nucleoside triphosphates much more efficiently than pyrimidine nucleoside monophosphates and does not transport NAD$^+$ and adenine nucleotides. Because NAD$^+$ is produced exclusively outside the mitochondria, a primary function of Ndt1p is to catalyze the uptake of NAD$^+$ into the mitochondria. For the exchange mode of NAD$^+$ transport, our measurements in reconstituted liposomes suggest that (d)AMP and (d)GMP are the most likely counter-substrates of Ndt1p for NAD$^+$. The rate of the exchange is much higher than that of the uniport. However, the uniport reaction can be essential in special conditions, for example when cells divide or must meet higher energetic demands associated with specific metabolic states and growth conditions.

The findings that mitochondria from Δ*ndt1* cells exhibit a lower level of NAD$^+$ and NADH and a decreased activity of the NAD$^+$-dependent enzymes PDH and ACDH, which is restored on adding the coenzyme to the assay medium, are consistent and strongly support Ndt1p controlling the uptake of NAD$^+$ into the organelles. Because similar effects are caused by the deletion of either *NDT1* or *NDT2*, we conclude that both Ndt1p and Ndt2p are capable of transporting NAD$^+$ and both contribute to the uptake of this coenzyme into mitochondria. This conclusion is substantiated by the fact that normal intramitochondrial levels of NAD$^+$ and NADH in *S. cerevisiae* are restored completely by complementation of the Δ*ndt1* or Δ*ndt2* single deletant with the missing gene and partially by complementation of the Δ*ndt1* Δ*ndt2* double mutant with only one gene. Furthermore, it cannot be excluded that NAD$^+$ is transported into mitochondria by some other transport system, but with lower efficiency, because mitochondria of the Δ*ndt1* Δ*ndt2* mutant strain still contain a small amount of NAD$^+$. This is not unrealistic, because many mitochondrial carriers display some substrate overlapping. For example, pyrimidine nucleotides are transported poorly by Ndt1p and quite well by Rim2p. Similarly, sulfate, citrate, and ATP are transported poorly by Dic1p, Sfc1p, and Tpc1p, respectively, and quite well by Oac1p, Ctp1p, and Aac2p, respectively. The marked decrease in the mitochondrial NAD$^+$ and NADH content in the Δ*ndt1* Δ*ndt2* strain results in growth delay, probably for lack of metabolic energy due to inactivation of NAD$^+$-dependent enzymes. One such defective enzyme is NAD$^+$-dependent ACDH that is known to catalyze the rate-limiting step of yeast growth on ethanol (50). Our results suggest that there is a threshold for the NAD$^+$ intramitochondrial content above which metabolic energy is sufficient for the growth of yeast cells. This hypothesis is consistent with the earlier observation that after depletion of mitochondrial NAD$^+$ in human cells grown under quiescent conditions, catalytic amounts of NAD$^+$ entering the mitochondria are sufficient to stimulate matrix dehydrogenases (15). The threshold value of NAD$^+$ must be between 0.43 ± 0.07 and 0.90 ± 0.09 nmol/mg of protein (*i.e.* the NAD$^+$ content of Δ*ndt1* and Δ*ndt1* Δ*ndt2* mutants, respectively), because the single deletants Δ*ndt1* and Δ*ndt2*, in contrast with the double mutant, do not exhibit growth delay in either the YP or SM medium. Likewise, the Δ*ndt1* Δ*ndt2* mutant, when complemented with Ndt1p or Ndt2p, grows normally though its intramitochondrial NAD$^+$ level is lower than that of the wild-type strain. The observation that the growth defect is more severe in SM than in YP, especially when ethanol is the sole carbon source, can be accounted for by the need of yeast cells to synthesize biomolecules unavailable in SM medium at the expense of the energy provided by NAD$^+$-dependent redox processes.

The findings of the present report add two new members, Ndt1p and Ndt2p, to the group of already identified mitochondrial transporters (Flx1p (34), Tpc1p (51), and human MFT (49)) that import essential coenzymes (FAD, thiamine pyrophosphate, and folate, respectively) from the cytosol to the mitochondrial matrix.

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Identification of the Mitochondrial NAD^+ Transporter in *Saccharomyces cerevisiae*

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