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 Δndt1 Δndt2 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 digotin-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, 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 Δndt1 and Δndt2 cells exhibited lower levels of NAD$^+$ and NADH in their mitochondria and decreased activity of the NAD$^+$-requiring mitochondrial enzymes PDH and ACDH. The Δndt1 Δndt2 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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Yeast Strains, Media, and Preparation of Mitochondria—BY4742 (wild-type), \( \Delta ndt1 \) and \( \Delta ndt2 \) yeast strains were provided by the EURO- FAN resource center EUROSCARF (Frankfurt, Germany). The \( NDT1 \) (\( YIL006W \)) or \( NDT2 \) (\( YEL006W \)) locus of the \( S. cerevisiae \) strain BY4742 (MAT\( a \)his3\( \Delta 1 \), leu2\( \Delta 0 \), lys2\( \Delta 0 \), ura3\( \Delta 0 \)) was replaced by kanMX4. The double deletion strain \( \Delta ndt1 \Delta ndt2 \) 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 \( \Delta ndt1 \) mutant (17). All deletions were verified by PCR. The wild-type and deletion strains were grown in rich medium (YP) containing 2% glucose, 2% ethanol, 3% acetate, 2% DL-lactate, or 2% pyruvate) and with synthetic minimal medium (SM) (18). All media were supplemented 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 \( E. coli \) DH5\( \alpha \) cells were selected on ampicillin (100 \( \mu \)g/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 \( \times \) 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 \( [3H] \)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 \( \mu \)M \([3H] \)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-\( \mu \)m 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 NAM/NMN adenylyl-transferase 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 \( \sim \) 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 \( \alpha \)-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
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residue in the signature motif of the second repeat (PIWYVVK). This characteristic feature is found in a mitochondrial carrier subfamily, which in S. cerevisiae comprises Flx1p (encoded by YLJ134W) 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 [3H]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 [3H]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 before induction of expression. Likewise, no such activity was detected 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.

To investigate the substrate specificity of Ndt1p in more detail, we measured the uptake of [3H]NAD$^+$ into proteoliposomes that had been preloaded with a variety of potential substrates (Fig. 1). The highest activity of [3H]NAD$^+$ uptake into proteoliposomes was with internal NAD$^+$. [3H]NAD$^+$ also exchanged substantially with internal NAAD, (d)AMP, and (d)GMP. Notably, internal α-NAD$^+$, NAADH, 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 [3H]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, [3H]NAD$^+$ also exchanged, to some extent, with internal FMN, FAD, and 3'-AMP. In contrast, the uptake of [3H]NAD$^+$ was negligible in the presence of internal cAMP, adenosine, coenzyme A, pyruvate, thiamine monophosphate, ornithine, NaCl (Fig. 1), and cGMP, thiamine, thiamine diphosphate, S-adenosylmethionine, succinate, maleate, 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 [3H]NAD$^+$ was observed in liposomes reconstituted with Ort1p, Sfc1p, Sam5p, Ggc1p, APC, GC1, or Rim2p containing their best substrate, i.e. ornithine, fumarate, S-adenosylmethionine, GTP, ATP-Mg, glutamate, or TTP, respectively. The [3H]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 bromocresol 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, carboxyatractysolide, and bongkrekic acid (inhibitors of other mitochondrial carriers).
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Kinetic Characteristics of Recombinant Ndt1p—The kinetics were compared for the uptake of 1 mM [3H]NAD$^+$ into proteoliposomes either as unidirectional uptake (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 unidirectional uptake of NAD$^+$ was very low. The unidirectional uptake of NAD$^+$ was further investigated by measuring the efflux of [3H]NAD$^+$ from prelabeled active proteoliposomes, because it provides a more convenient assay for unidirectional transport (23). In these experiments, little yet significant efflux of [3H]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 uptake rate at various external [3H]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$^+$ uptake were obtained at 25°C (Table 1). The $K_m$ value of Ndt1p for NAD$^+$ reported here is virtually the same as that found for the uptake of [3H]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 [3H]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 only about three times lower than those of TMP and UMP.

**Subcellular Localization of Ndt1p**—Because some members of the MCF are localized in non-mitochondrial membranes (42, 43), the subcellular localization of Ndt1p was investigated using a GFPTagged 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-
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 adenyllytransferases, 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 adenyllytransferase, 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. 6A shows that PDH activity was lower in mitochondria (mitochondrial extracts) from the Δndt1 and Δndt2 single deletants, and even more so from the Δndt1 Δndt2 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. 6B). In contrast, the activity of the NAD⁺-requiring ADH, measured in the post-mitochondrial supernatant, was not significantly different in Δndt1 and Δndt2 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 Δndt1 and Δndt2 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 Δndt1 Δndt2 strain, the NAD⁺ content in mitochondria was significantly lower than that of the single deletants (p < 0.05). In the Δndt1 Δndt2 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 Δndt1 or Δndt2 single mutants, respectively.

The NAD⁺ levels assayed in the same strains showed changes similar to those observed for NAD⁺ (Fig. 7). Because NAD⁺ 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 Δndt1 Δndt2 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...
observed in the SM than in the YP medium, although maximal growth
was achieved in the SM using the same media supplemented
with ethanol (Fig. 8A) as well as with lactate, pyruvate, or acetate
(data not shown). In the SM medium, growth of the

\[
\text{ndt1} \quad \text{ndt2}
\]

mutant displayed a growth delay in the YP medium sup-
plemented 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.

**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 analy-
sis. Data represent means \( \pm \) S.E. of at least three independent experiments. The signifi-
cance of the difference between the intramitochondrial NAD\(^+\) level of \(\text{ndt1} \quad \text{ndt2}\) compared with that of \(\text{ndt1}\) is indicated (*, \( p < 0.05\), one-way analysis of variance). In the
right-hand panel, the mitochondria were incubated with N,N,N’-tetramethyl-p-
phenylenediamine (TMPD)/ascorbate for 1 min before being extracted.

**FIGURE 8.** Effect of \(\text{NDT1} \quad \text{NDT2}\) single and double deletion on the growth of
yeast cells on ethanol. Wild-type ( ), \(\text{ndt1}\) ( ), \(\text{ndt2}\) ( ), \(\text{ndt1} \quad \text{ndt2}\) pRS416-\(\text{NDT1}\) ( ), and
\(\text{ndt1} \quad \text{ndt2}\) pRS416-\(\text{NDT2}\) ( ) were inoculated in YP (A) or SM (B) medium supple-
mented 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.

**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 trans-
porter 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 reconstitut-
ing 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
isofoms (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 \(\text{ndt1}\) mutant exhibited no inhibitor-
sensitive transport of pyruvate (47). However, recombinant and reconsti-

tuted Ndt1p does not transport pyruvate either as \(\text{[14C]}\text{pyruvate} /
pyruvate or as \(\text{[3H]}\text{NAD}^+\) (pyruvate exchange. Furthermore, external
pyruvate (20 mM) does not enhance loss of \(\text{[3H]}\text{NAD}^+\) from preloaded
proteoliposomes and, when added together with \(\text{[3H]}\text{NAD}^+\), does not
inhibit the reconstituted NAD\(^+\)/NAD\(^+\) exchange. In addition, in a phy-
logenetic 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. How-
ever, several protein sequences available in databases are or may be
orthologs of these transporters in other organisms. These sequences
include: XP_4536881 from Kluyveromyces lactis (47% of identical

\(^3\) S. Todisco, G. Agrimi, and F. Palmieri, unpublished observations.
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amino acids with Ndt1p; EAK9561i.1 from Candida albicans (45%); XP_661991.1 from Aspergillus nidulans (34%); EAA31572.1 from Neurospora crassa (30%); AAH90770.1 from Danio rerio (34%); AAA68821.1 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 charac-
terized biochemically. However, NP_110407.2 (named hMFT), the
closest human relative of Ndt1p and Ndt2p (30% identical amino
carriers display some substrate overlap-
ning. For example, pyrimidine nucleotides are transported poorly by
Dip1p, Sfc1p, and Tpc1p, respectively, and quite well by Rim2p. Similarly, sulfate, citrate, and ATP are transported poorly by Dip1p, Scf1p, and Tpc1p, respectively, and quite well by Oac1p, Ctp1p, and Acp2p, respectively. The marked decrease in the mitochondrial NAD\(^+\) and NADH content in the Δndt1 Δndt2 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 met-
abolite re-instates folate in the mitochondria of transfected glyB cells (49).

The findings of the present report add two new members, Ndt1p and
Ndt2p, to the group of already identified mitochondrial transporters
(Fix1p (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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