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The *Saccharomyces cerevisiae NDE1* and *NDE2* Genes Encode Separate Mitochondrial NADH Dehydrogenases Catalyzing the Oxidation of Cytosolic NADH*

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In *Saccharomyces cerevisiae*, the *NDI1* gene encodes a mitochondrial NADH dehydrogenase, the catalytic side of which projects to the matrix side of the inner mitochondrial membrane. In addition to this NADH dehydrogenase, *S. cerevisiae* exhibits another mitochondrial NADH-dehydrogenase activity, which oxidizes NADH at the cytosolic side of the inner membrane. To investigate whether open reading frames *YMR145c/NDE1* and *YDL 085w/NDE2*, which exhibit sequence similarity with *NDI1*, encode the latter enzyme, NADH-dependent mitochondrial respiration was assayed in wild-type *S. cerevisiae* and *nde* deletion mutants. Mitochondria were isolated from aerobic, glucose-limited chemostat cultures grown at a dilution rate (D) of 0.10 h^{-1}, in which reoxidation of cytosolic NADH by wild-type cells occurred exclusively by respiration. Compared with the wild type, rates of mitochondrial NADH oxidation were about 3-fold reduced in an *nde1*Δ mutant and unaffected in an *nde2*Δ mutant. NADH-dependent mitochondrial respiration was completely abolished in an *nde1*Δ *nde2*Δ double mutant. Mitochondrial respiration of substrates other than NADH was not affected in *nde* mutants. In shake flasks, an *nde1*Δ *nde2*Δ mutant exhibited reduced specific growth rates on ethanol and galactose but not on glucose. Glucose metabolism in aerobic, glucose-limited chemostat cultures (D = 0.10 h^{-1}) of an *nde1*Δ *nde2*Δ mutant was essentially respiratory. Apparently, under these conditions alternative systems for reoxidation of cytosolic NADH could replace the role of Nde1p and Nde2p in *S. cerevisiae*.

During dissimilation of sugars via respiration by eukaryotic cells, glycolysis leads to NAD^+ reduction in the cytosol, whereas mitochondrial oxidation of pyruvate via the pyruvate-dehydrogenase complex and the trichloroacetic acid cycle yields NADH in the mitochondrial matrix. As the mitochondrial inner membrane is impermeable to NADH (1, 2), respiratory growth requires continuous reoxidation of this cofactor in the cytosol as well as in the mitochondrial matrix.

The mitochondrial inner membrane of the yeast *Saccharomyces cerevisiae* contains at least two NADH:ubiquinone-6 oxidoreductases ('NADH dehydrogenases') that may couple the oxidation of NADH to the mitochondrial respiratory chain (2 - 5). The catalytic site of one of these, commonly referred to as the 'internal' NADH dehydrogenase, faces the mitochondrial matrix. Thus, it can oxidize the intramitochondrial NADH generated by the pyruvate-dehydrogenase complex and the TCA cycle (2). In contrast to the classical 'complex I' NADH dehydrogenases of higher eukaryotes, the 'internal' NADH dehydrogenase of growing *S. cerevisiae* cells is not proton translocating (5, 6). The enzyme consists of a single subunit encoded by the nuclear *NDI1* gene (7). Mutants in which *NDI1* is inactivated do not oxidize intramitochondrial NADH (8).

In addition to the *NDI1*-encoded 'internal' NADH dehydrogenase, *S. cerevisiae* is able to synthesize another inner membrane NADH dehydrogenase, commonly referred to as external NADH dehydrogenase, the catalytic site of which faces the intermembrane space (Refs. 2, 3, and 5; Fig. 1).

In contrast to the mitochondria of fungi and plants (9, 10), mammalian mitochondria do not harbor external NADH dehydrogenases and therefore depend on redox shuttle mechanisms to couple the oxidation of cytosolic NADH to internal NADH dehydrogenases (11). The presence of an external NADH dehydrogenase in yeast mitochondria correlates with the absence of a functional malate-aspartate shuttle (5, 12), one of the major redox shuttles in mammalian mitochondria (11). However, the key enzymes for two alternative systems, the glycerol-3-phosphate dehydrogenase system and the ethanol-acetaldehyde shuttle (Fig. 1), have both been demonstrated in *S. cerevisiae* (2, 13, 14). A recent study indicated that the glycerol-3-phosphate dehydrogenase system contributes to the oxidation of cytosolic NADH under certain conditions but that it is not essential for respiratory growth of *S. cerevisiae* (15). The relative importance of the various proposed systems for respiratory oxidation of cytosolic NADH by *S. cerevisiae* mitochondria is at present unclear.

Even under aerobic conditions, alcoholic fermentation rather than respiration is the predominant mode of sugar metabolism in *S. cerevisiae* (16). Fully respiratory growth on sugars is only possible during sugar-limited cultivation below the so called critical specific growth rate (μ_{crit}). Above μ_{crit}, respiration and aerobic alcoholic fermentation occur simultaneously, even in sugar-limited cultures (17–19). Aerobic fermentation negatively affects the biomass yield on sugars (20). Therefore, biomass-directed industrial applications of *S. cerevisiae*, such as the production of bakers' yeast and heterologous proteins, have to be performed at submaximal growth rates in aerobic, sugar-limited fed-batch cultures (21, 22). Competition between mitochondria and alcohol dehydrogenase for cytosolic NADH

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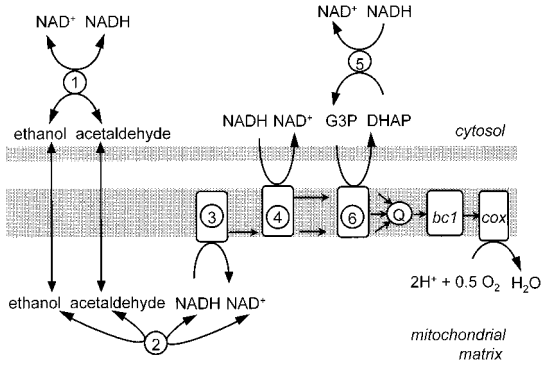

FIG. 1. **Proposed mechanisms for respiratory oxidation of cytosolic NADH in *S. cerevisiae* (2, 5): schematic representation of compartmentation and electron flow.** *Numbers* indicate the following enzymes: *1*, cytosolic NAD^+-dependent alcohol dehydrogenase; *2*, mitochondrial NAD^+-dependent alcohol dehydrogenase; *3*, mitochondrial internal NADH dehydrogenase; *4*, mitochondrial external NADH dehydrogenase; *5*, cytosolic NAD^+-dependent glycerol-3-phosphate dehydrogenase; *6*, mitochondrial flavoprotein glycerol-3-phosphate dehydrogenase.

formed in glycolysis may be a relevant factor in the occurrence of aerobic fermentation. Therefore, insight into the mechanisms of respiratory NADH oxidation by *S. cerevisiae* mitochondria is not only of fundamental interest but also relevant for the industrial application of this yeast.

Physiological characterization of mutants lacking one or more of the possible NADH-oxidizing mechanisms (Fig. 1) seems a powerful tool to investigate their physiological significance. A major complication for such studies is that, so far, the gene(s) encoding external NADH dehydrogenase(s) in *S. cerevisiae* have not been identified. The *S. cerevisiae* genome harbors two open reading frames (*YMR145c* and *YDL085w*), which exhibit high similarity with *NDI1*, the structural gene encoding the internal mitochondrial NADH dehydrogenase. The aim of this study was to investigate whether these open reading frames are structural genes encoding mitochondrial external NADH dehydrogenase and to determine whether their products are essential for respiratory growth on sugars.

EXPERIMENTAL PROCEDURES

Yeast Strains and Maintenance—The *S. cerevisiae* strains used in this study are listed in Table I. They were grown to stationary phase in shake-flask cultures on a mineral medium with vitamins (23), which was set at pH 6.0 and contained 20 g·liter^{-1} glucose. After adding sterile glycerol (30% v/v), 2-ml aliquots were stored in sterile vials at −80 °C. These frozen stock cultures were used to inoculate precultures for batch and chemostat experiments.

Construction of Null Mutants in Open Reading Frames YMR145c and YDL085w—Standard techniques and media for genetic modification of *S. cerevisiae* were used (24). Deletions in *YMR145c* and *YDL085w* were obtained by the short flanking homology (SFH)[1] method (25). SFH deletion cassettes were made with primers homologous to both the kanamycin resistance gene (the *kanMX* module) and the gene of interest. For each open reading frame, a pair of oligonucleotides (Table II) was designed to contain 40 nucleotides at the 5′-end homologous to the target yeast sequence and 19–21 nucleotides at the 3′-end homologous to the pUG6 multiple cloning site (26).

PCR was carried out as follows. Each 100-μl PCR reaction contained 10 μl of 10 × PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 200 μM dNTP mix, 30–60 ng of pUG6 plasmid as template DNA, 30 pmol of each primer, 1.5 mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase (27). Amplification parameters were 94 °C for 2 min (hot start) and then 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min. For quantification of the amount of PCR product, aliquots were loaded on an agarose gel. After ethanol precipitation of the SFH-PCR products, yeast cells were transformed using the improved lithium acetate method (26, 28). For selection of transformants, geneticin (G-418 sulfate, Life Technologies) was added at a final concentration of 200 μg·ml^{-1}. For the construction of single gene deletions in *YMR145c* and *YDL085w*, respectively, the SFH-PCR products were transformed to the prototrophic strains CEN.PK113–7D and/or CEN.PK122. To obtain a strain deleted for both *YMR145c* and *YDL085w*, strains CEN.PK152 (*nde1*Δ) and CEN.PK163 (*nde2*Δ) were crossed. After tetrad analysis, spores showing the nonparental ditype for the *kanMX* marker were subsequently analyzed by diagnostic PCR to confirm correct deletion of both genes.

Verification of Gene Deletion—Correct gene deletion was verified by analytical PCR with whole yeast cells directly taken from a YPD plate. Two pairs of primers (A1/K1 and A4/K2, Table II) were used to check the two junctions corresponding to the replacement. For this, oligonucleotides were designed to bind outside the deleted gene 900–1000 nucleotides upstream of the ATG (A1) and 500 nucleotides downstream of the stop codon (A4). Primers K1 and K2 bind within the *kanMX* marker module. Amplification parameters were 94 °C for 2 min (hot start) and then 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1.5 min. About 5 μl of a 50-μl PCR reaction were loaded on an agarose gel.

Determination of the Mating Type—The mating types of the prototrophic CEN.PK strains used in this study were determined by PCR with whole yeast cells according to Huxley *et al*. (29).

Chemostat Cultivation—Aerobic chemostat cultivation was performed at 30 °C in 2-liter laboratory fermenters (Applikon, Schiedam, the Netherlands) at a stirrer speed of 800 rpm. The working volume was kept at 1.0 liter by a peristaltic effluent pump coupled to an electrical level sensor. Biomass concentrations in samples taken directly from the cultures differed by less than 1% from those in samples taken from the effluent line (30). The dilution rate (which, in steady-state cultures is equal to the specific growth rate) was set at 0.10 h^{-1}. A steady state was defined as the situation in which at least five volume changes had passed since the last change in dilution rate and in which the biomass concentration, as well as the specific rates of carbon dioxide production and oxygen consumption, had remained constant (<2% variation) for at least two volume changes. Steady-state cultures did not exhibit detectable metabolic oscillations. The pH was kept at 5.0 by an Applikon ADI 1030 biocontroller, via the automatic addition of 2 M KOH. The fermenter was sparged with air at a flow rate of 0.5 liter·min^{-1} using a Brooks 5876 mass-flow controller. The dissolved oxygen concentration was continuously monitored with an oxygen electrode (Ingold, model 34 100 3002) and remained above 50% of air saturation. The defined mineral medium with vitamins was prepared as described by Verduyn *et al*. (23) and contained 7.5 g·liter^{-1} glucose as the carbon source. Chemostat cultures were routinely checked for purity by phase-contrast microscopy and by plating on YPD agar plates.

Determination of Culture Dry Weight—Culture samples (10 ml) were filtered over preweighed nitrocellulose filters (pore size 0.45 μm, Gelman Sciences). After removal of medium, the filters were washed with demineralized water, dried in a Sharp type R-4700 microwave oven for 20 min at 360 W output, and weighed. Duplicate determinations varied by less than 1%.

Substrate and Metabolite Analyses—Glucose in reservoir media and supernatants of chemostat cultures was determined enzymatically with a hexokinase/glucose-6-phosphate dehydrogenase kit (Boehringer Mannheim). Concentrations of ethanol, glycerol, and acetate were determined by high pressure liquid chromatography (31). High pressure liquid chromatography analyses were confirmed by enzymic analysis of these metabolites (32).

Gas Analysis—The exhaust gas of chemostat cultures was cooled in a condenser (2 °C) and dried with a Perma Pure dryer (type PD-625-12P). Oxygen and carbon dioxide concentrations were determined with a Servomex type 1100A analyzer and a Beckman model 864 infrared detector, respectively. The exhaust gas flow rate was measured as described previously (33). Specific rates of carbon dioxide production and oxygen consumption were calculated as described by van Urk *et al*. (34).

Isolation of Mitochondria—Mitochondria were isolated from glucose-limited, aerobic chemostat cultures by a procedure based on that described for *Candida utilis* by Bruinenberg *et al*. (35). Biomass (approximately 1.5 g dry weight) was harvested by centrifugation at 1600 × *g* for 4 min. The pellet was then resuspended in 30 ml of Tris-HCl buffer (0.1 M, pH 9.3) containing 10 mM dithiothreitol and incubated at 30 °C for 10 min. After centrifugation (4 min, 1250 × *g*), the pellet was washed with 40 ml of buffer A (25 mM potassium phosphate, 1 mM $MgCl_2$, 1 mM EDTA, pH 7.5), containing 2 M sorbitol and resuspended in 35 ml of buffer A containing 2 M sorbitol. 10.2 mg of zymolyase (from *Arthobacter luteus*, 20.000 units·g^{-1}, ICN Biochemicals) was dissolved

[1] The abbreviations used are: SFH, short flanking homology; PCR, polymerase chain reaction.

TABLE I
Yeast strains used in this study
The numbers in parentheses indicate the deleted nucleotides (ATG = 1) of the corresponding genes.

Strain	Relevant genotype
CEN.PK113–7D	MATa URA3 HIS3 LEU2 TRP1 MAL2–8^c SUC2
CEN.PK122	*MATa/MATα URA3/URA3 HIS3/HIS3 LEU2/LEU2 TRP1/TRP1 MAL2–8^c/MAL2–8^c SUC2/SUC2*
CEN.PK152	*MATa URA3 HIS3 LEU2 TRP1 MAL2–8^c SUC2 ymr145c*(41–1659)::*loxP-kanMX4-loxP*
CEN.PK162	*MATa URA3 HIS3 LEU2 TRP1 MAL2–8^c SUC2 ydl085w*(51–100)::*loxP-kanMX4-loxP*
CEN.PK163	*MATα URA3 HIS3 LEU2 TRP1 MAL2–8^c SUC2 ydl085w*(51–100)::*loxP-kanMX4-loxP*
CEN.PK167–2B	*MATa URA3 HIS3 LEU2 TRP1 MAL2–8^c SUC2 ymr145c*(41–1659)::*loxP-kanMX4-loxP ydl1085w*(51–100)::*loxP-kanMX4-loxP*

TABLE II
Oligonucleotides used for construction of disruption cassettes (S1 and S2) and as primers for analytical PCR of disruptants (A1/K1) and (A4/K2)
Construction of disruption cassettes and disruption of genes and analytical PCR were carried out as described under "Experimental Procedures." The sequences complementary to the multiple cloning site of pUG6 are underlined.

Open reading frame	Oligonucleotides
YMR145c/NDE1	S1: 5′-ATGATTAGACAATCATTAATGAAAACAGTGTGGGCTAACTCAGCTGAAGCTTCGTACGC-3′
YMR145c/NDE1	S2: 5′-TCTTGTATCTATTTCTACTAGATAGATGAATCTCTACCCAGCATAGGCCACTAGTGGATCTG-3′
YMR145c/NDE1	A1: 5′-CCTCTGGTACCCAATGCCG-3′
YMR145c/NDE1	A4: 5′-GCAACTGGCTGCAGGGAG-3′
YDL085w/NDE2	S1: 5′-GACTTGGTTTTGCGAGGACTGCTAGGTCCATACACCGTTTCAGCTGAAGCTTCGTACGC-3′
YDL085w/NDE2	S2: 5′-GCAGTGTAAGATTTCGATAGCTTCTGCTGTGGTCCGGGCTGCATAGGCCACTAGTGGATCTG-3′
YDL085w/NDE2	A1: 5′-GCCTCTCTTCTAGCATCTG-3′
YDL085w/NDE2	A4: 5′-GGTCCTCGGATACTTGATC-3′
kanMX	*K1: 5′-GGATGTATGGGCTAAATGTACG-3′*
kanMX	K2: 5′-GTTTCATTTGATGCTCGATGAG-3′

in 0.1 ml of buffer A containing 2 M sorbitol and added to the cell suspension, which was subsequently incubated at 30 °C for 1 h. This incubation period was chosen based on control experiments in which lysis of spheroplasts was periodically assayed by dilution of samples in demineralized water (35). All subsequent steps were carried out on ice or in a cooled (4 °C) centrifuge. Spheroplasts were harvested by centrifugation (7 min, 2800 × *g*). The pellet was washed with 40 ml of buffer A containing 2 M sorbitol and resuspended in 10 ml of the same buffer. Subsequently, 30 ml of buffer A containing 0.2 M sorbitol was added dropwise while the suspension was slowly stirred with a magnetic stirrer bar. The spheroplast suspension was subjected to 10 strokes in a cooled Potter-Elvehjem homogenizer (100 rpm, clearance 28 μm). After centrifugation (10 min, 2000 × *g*), the homogenate (supernatant) was separated from unbroken cells and debris and spun again (10 min, 7800 × *g*). The resulting pellet, containing the mitochondria, was resuspended in 5 ml of buffer A with 0.65 M sorbitol and 1 mg·ml^{-1} bovine serum albumin (fatty acid free, Sigma) and kept on ice. In this protocol, relative centrifugal forces represent average values at the applied rotational speeds, for completely filled centrifuge tubes in the Sorvall GSA and SS34 rotors, as reported by the manufacturer.

Oxygen Uptake Studies with Mitochondrial Preparations—Substrate-dependent oxygen consumption rates of mitochondria were determined polarographically at 30 °C with a Clark-type oxygen electrode. The assay mixture (3 ml) contained 25 mM potassium phosphate buffer (pH 7.0), 5 mM $MgCl_2$, and 0.65 M sorbitol. Reactions were started with ethanol (5 mM), succinate (5 mM), L-glycerol-3-phosphate (5 mM), or L-malate + pyruvate (5 mM). Commercial preparations of NADH are contaminated with ethanol (36). Therefore, NADH was generated in the oxygen uptake assays by addition of 5 mM glucose, 0.2 mM NAD^+, and 1.5 units·ml^{-1} of *Bacillus megaterium* glucose dehydrogenase (Sigma). Oxygen uptake rates were calculated based on a dissolved oxygen concentration of 236 μM in air-saturated water at 30 °C. Respiratory control values were determined by adding 0.25 mM ADP (37).

Protein Determination—The protein content of whole cells was estimated by a modified biuret method (38). Protein concentrations of mitochondrial preparations were estimated by the Lowry method. Dried bovine serum albumin (fatty acid free, Sigma) was used as a standard. Where necessary, protein determinations were corrected for bovine serum albumin present in the mitochondrial preparations.

RESULTS

Generation of Ethanol-free NADH in Oxygen Uptake Studies—According to the literature, commercial preparations of NADH are contaminated with ethanol (36). high pressure liquid chromatography analysis of NADH obtained from Boehringer Mannheim confirmed that fresh solutions prepared in distilled water contained 0.48 ± 0.04 mol ethanol (mol NADH)$^{-1}$. Since ethanol is readily oxidized by *S. cerevisiae* mitochondria (Ref. 2, Table III), this precluded the direct use of commercial NADH in oxygen uptake studies with mitochondrial isolates. Attempts to remove ethanol by incubation with acetic acid bacteria resulted in the partial degradation of NADH (data not shown). As NAD^+ was found to be free from ethanol contamination, the possibility was investigated to generate NADH in the oxygen uptake assays by addition of glucose and NAD^+, together with NAD^+-dependent glucose dehydrogenase from *Bacillus megaterium* (see "Experimental Procedures"). Oxygen uptake by wild-type mitochondria could be observed only when all three components of this system for NADH regeneration were added (data not shown). Using this system, the respiratory control ratio for oxidation of NADH by wild-type mitochondria was about 3 (Table III), indicating that NADH oxidation was functionally coupled to oxidative phosphorylation. The glucose dehydrogenase system was used in all further experiments on NADH oxidation by mitochondrial preparations.

YMR145c and YDL085w Are Candidate Structural Genes for External NADH Dehydrogenase— To identify candidate structural genes encoding the mitochondrial external NADH dehydrogenase of *S. cerevisiae*, the deduced amino acid sequence encoded by the unique gene encoding the internal NADH dehydrogenase (7) was compared with the entire *S. cerevisiae* genome sequence (39). Data base searches using services offered by MIPS (BLAST, 40) yielded two open reading frames with unknown function, *YMR145c* and *YDL085w*. Sequence alignment of Ndi1 to the predicted peptide sequences encoded by *YMR145c* and *YDL085w* revealed identities of 48% and 46%, respectively. An even higher identity of 63% was found when the predicted peptide sequence encoded by *YMR145c* and *YDL085w* were compared. The observed sequence identity was found along the whole length of the three predicted peptide sequences. Interestingly in contrast to Ndi1p, the putative peptides encoded by *YMR145c* and *YDL085w* showed N-terminal extensions of 30 and 45 amino acids, respectively. These extensions are of interest as they might theoretically be involved in targeting of the proteins to the appropriate subcellu-

TABLE III

Substrate-dependent rates of oxygen consumption by mitochondria from wild-type S. cerevisiae CEN.PK113–7D and null mutants in open reading frames YMR145c (NDE1) and YDL085w (NDE2)

Cells were pregrown in aerobic, glucose-limited chemostat cultures at a dilution rate of 0.10 h^{-1}. The oxygen uptake rates given were measured in the presence of 0.25 mM ADP; respiratory control (RC) values represent the ratio of respiration rates in the presence and absence of ADP. Experimental results are the average ± S.D. (σ_n) of measurements with at least two independent mitochondrial isolations for each strain.

Substrate	*S. cerevisiae* CEN.PK113–7D (*NDE1 NDE2*)		*S. cerevisiae* CEN.PK152 (*nde1ΔNDE2*)		*S. cerevisiae* CEN.PK162 (*NDE1 nde2Δ*)		*S. cerevisiae* CEN.PK167–2B (*nde1Δnde2Δ*)	
	O_2 uptake rate	RC	O_2 uptake rate	RC	O_2 uptake rate	RC	O_2 uptake rate	RC
	μmol · (mg protein)$^{-1}$ · min^{-1}							
NADH	0.22 ± 0.06	3.0 ± 0.3	0.08 ± 0.04	1.8 ± 0.2	0.28 ± 0.01	2.6 ± 0.1	0.0 ± 0.0	
Malate + pyruvate	0.11 ± 0.01	1.7 ± 0.1	0.16 ± 0.01	2.0 ± 0.3	0.15 ± 0.01	2.0 ± 0.2	0.15 ± 0.02	2.0 ± 0.1
Ethanol	0.10 ± 0.02	1.4 ± 0.1	0.11 ± 0.02	1.4 ± 0.1	0.16 ± 0.03	1.7 ± 0.1	0.15 ± 0.03	1.6 ± 0.1
L-glycerol-3-phosphate	0.18 ± 0.04	2.3 ± 0.1	0.22 ± 0.06	2.5 ± 0.3	0.25 ± 0.04	2.2 ± 0.3	0.26 ± 0.03	2.6 ± 0.3
Succinate	0.10 ± 0.02	1.6 ± 0.1	0.13 ± 0.01	2.4 ± 0.4	0.13 ± 0.02	1.9 ± 0.2	0.17 ± 0.04	3.0 ± 0.3

lar locations. *YMR145c* exhibits a codon adaptation index of 0.26, which is indicative of a moderately expressed gene, similar to *NDI1* (codon adaptation index 0.19). *YDL085w* exhibited a lower codon adaptation index of 0.14. Based on their similarity with *NDI1*, *YMR145c* and *YDL085w* were tentatively named *NDE1* and *NDE2* (NADH dehydrogenase, external), respectively.

Oxygen Uptake Studies with Mitochondria from Wild-type S. cerevisiae and Deletion Mutants—To investigate whether open reading frames *YMR145c*/*NDE1* and *YDL085w*/*NDE2* are indeed structural genes encoding mitochondrial external NADH dehydrogenases, NADH-dependent respiration was studied in isolated mitochondria from wild-type *S. cerevisiae* and from isogenic mutants in which either *NDE1*, *NDE2*, or both had been deleted.

Mitochondria were isolated from aerobic, glucose-limited chemostat cultures grown at a dilution rate of 0.10 h^{-1}. Under these conditions, wild-type *S. cerevisiae* did not exhibit alcoholic fermentation (see below), indicating that cytosolic NADH was efficiently reoxidized by the mitochondria. Such a situation cannot be accomplished in batch cultures on glucose where, because of glucose repression of respiratory enzymes, alcoholic fermentation is the predominant mode of NADH reoxidation (16, 41).

As discussed above, wild-type mitochondria readily oxidized exogenous NADH. The respiratory control ratio of 3 (Table III) strongly suggested that this NADH-oxidizing activity was due to the presence of an external NADH dehydrogenase rather than to exposure of the internal enzyme because of the presence of damaged mitochondria. Wild-type mitochondria were also capable of oxidizing pyruvate when this substrate was added in combination with malate. This indicates that the oxidation of intramitochondrial NADH, formed by the pyruvate-dehydrogenase complex and the trichloroacetic acid cycle, could be functionally coupled to the respiratory chain via internal NADH dehydrogenase. Similarly, the respiration rates observed with L-glycerol-3-phosphate, succinate, and ethanol (Table III) were indicative of functional coupling of mitochondrial glycerol-3-phosphate dehydrogenase, succinate dehydrogenase, and mitochondrial alcohol dehydrogenase to the respiratory chain.

Deletion of *NDE1* caused a 3–4-fold decrease in NADH-dependent oxygen uptake by mitochondria, whereas no decrease was observed for the other substrates tested (Table III, Fig. 2). The residual NADH-oxidizing activity in mitochondrial preparations of the mutant still exhibited respiratory control (Table III), indicating that this activity was not entirely due to contamination with nonrespiratory chain-linked oxidases (42).

Deletion of *NDE2* alone did not have a significant effect on the rate of NADH oxidation by isolated mitochondria, nor was a clear effect observed for any of the other substrates tested

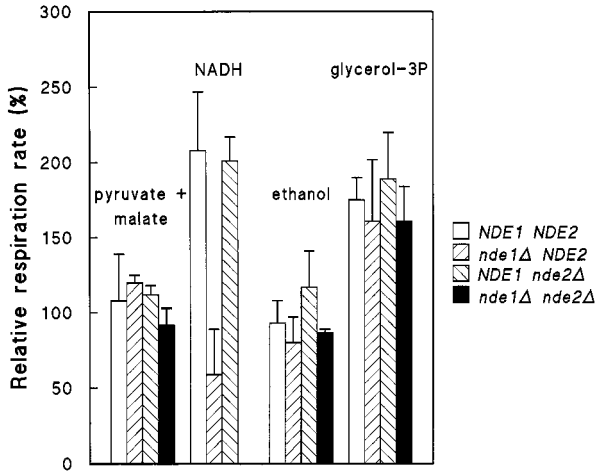

FIG. 2. **Substrate-dependent respiration rates of mitochondria isolated from wild-type *S. cerevisiae* CEN.PK113-7D and isogenic *nde* deletion mutants (for absolute values see Table III).** In view of the experimental variation in the overall respiratory activities of various batches of mitochondria, respiration rates for each batch were normalized for the succinate-dependent oxygen uptake, which was arbitrarily set at 100%. Data are the average ± S.D. (σ_n) of at least two independent mitochondrial isolations for each strain.

(Table III, Fig. 2). However, when both *NDE1* and *NDE2* were deleted, NADH-dependent oxygen uptake by isolated mitochondria was completely abolished (Table III, Fig. 2). In the *nde1Δ nde2Δ* mutant, oxidation rates with other substrates were not significantly lower than in the wild type (Table III, Fig. 2), indicating that the *nde* deletions did not affect coupling of other dehydrogenase systems to the respiratory chain.

Growth Characteristics of nde Null Mutants in Chemostat Cultures—In aerobic, glucose-limited chemostat cultures of the wild-type strain CEN.PK113-7D grown at a dilution rate of 0.10 h^{-1}, neither ethanol nor glycerol was found in culture supernatants, and all glucose carbon in the feed could be quantitatively recovered as biomass and carbon dioxide (Table IV). The biomass yield on glucose was 0.49 g of biomass·g $glucose^{-1}$, which is typical for respiratory growth of wild-type *S. cerevisiae* strains (20). A further confirmation that glucose metabolism in these cultures was fully respiratory was that the ratio between specific rates of carbon dioxide production and oxygen uptake was close to unity (Table IV).

Also in the *nde* deletion mutants, growth was essentially respiratory, as evident from the absence of ethanol in culture supernatants and a respiratory coefficient close to 1 (Table IV). Both in the *nde1Δ* mutant and in the *nde1Δ nde2Δ* double mutant, low concentrations of glycerol were detected in culture supernatants (Table IV). Glycerol formation is the major pathway for reoxidation of cytosolic NADH during anaerobic growth

TABLE IV

Growth parameters of wild-type S. cerevisiae CEN.PK113–7D and isogenic null mutants in open reading frames YMR145c (NDE1) and YDL085w (NDE2) in aerobic, glucose-limited chemostat cultures grown at a dilution rate of 0.10 h^{-1}

Other growth conditions: T = 30 °C, pH 5, glucose concentration in reservoir 7.5 g · $liter^{-1}$. Experimental results are the average ± S.D. (σ_n) of at least two independent steady-state measurements. Carbon recoveries were calculated based on a carbon content of dry biomass of 48%.

	S. cerevisiae CEN.PK113–7D (*NDE1 NDE2*)	*S. cerevisiae* CEN.PK152 (*nde1Δ NDE2*)	*S. cerevisiae* CEN.PK162 (*NDE1 nde2Δ*)	*S. cerevisiae* CEN.PK167–2B (*nde1Δ nde2Δ*)
Biomass yield (g biomass · g $glucose^{-1}$)	0.49 ± 0.00	0.48 ± 0.01	0.49 ± 0.01	0.45 ± 0.01
Residual glucose (mM)	<0.2	<0.2	<0.2	<0.2
Ethanol (mM)	<0.1	<0.1	<0.1	<0.1
Glycerol (mM)	<0.1	0.57 ± 0.03	<0.1	0.78 ± 0.03
qCO_2 (mmol · g^{-1} · h^{-1})	2.7 ± 0.1	2.9 ± 0.1	2.7 ± 0.1	3.0 ± 0.1
qO_2 (mmol · g^{-1} · h^{-1})	2.6 ± 0.1	2.9 ± 0.1	2.6 ± 0.1	3.0 ± 0.1
RQ	1.03 ± 0.02	0.99 ± 0.01	1.03 ± 0.02	1.00 ± 0.01
Carbon recovery (%)	98 ± 1	100 ± 1	99 ± 2	99 ± 3

of *S. cerevisiae* (14, 43). However, the amount of glycerol produced by the mutant strains corresponded to less than 1% of the glucose carbon fed to the cultures, indicating that glycerol production was not a major means of reoxidizing cytosolic NADH in these aerobic cultures (Table IV).

The biomass yield of the *nde1Δ nde2Δ* strain in the glucose-limited chemostat cultures was about 10% lower than that of the wild type. As this difference cannot be explained from the small amounts of glycerol produced by the cultures, it suggests that rerouting of the oxidation of cytosolic NADH via alternative pathways led to a lower energetic efficiency.

Growth of Wild-type S. cerevisiae and nde Mutants in Shake-flask Cultures—To further investigate the phenotype of *nde* mutants, specific growth rates were determined in shake-flask cultures. In glucose-grown cultures, deletion of *NDE1*, *NDE2*, or both had no significant effect on the specific growth rate (Table V). This is consistent with the notion that alcoholic fermentation rather than respiration is the key mode of glucose dissimilation in batch cultures (16).

Galactose represses respiratory enzymes to a lesser extent than glucose and is metabolized at a lower rate than glucose by wild-type *S. cerevisiae* strains. Consequently, during batch cultivation on galactose, the relative contribution of respiration is larger than during growth on glucose (16). The specific growth rates of the *nde1Δ* and *nde1Δ nde2Δ* mutants on galactose were about 30% lower than those of the isogenic wild type. Deletion of only *NDE2* did not have a significant effect on the specific growth rate on galactose (Table V). This indicates that Nde1p is involved in the dissimilation of galactose via respiration and that alternative systems for reoxidation of cytosolic NADH cannot sustain wild-type growth rates.

S. cerevisiae contains both mitochondrial and cytosolic isoenzymes of alcohol dehydrogenase and acetaldehyde dehydrogenase (2, 44). Exclusive involvement of the mitochondrial isoenzymes of these dehydrogenases might, at least in theory, prevent the generation of cytosolic NADH in the initial steps in ethanol metabolism. Nevertheless, deletion of both *NDE1* and *NDE2* caused a significant reduction of the specific growth rate on ethanol. The corresponding single mutants exhibited essentially the same specific growth rate on ethanol as the wild type (Table V).

TABLE V

Specific growth rates of S. cerevisiae CEN.PK113–7D and isogenic nde1 and nde2 null mutants on a defined mineral medium supplemented with glucose, galactose, or ethanol as the sole carbon source

Data are the average of two independent shake-flask experiments. The standard deviation (σ_n) was less than 5% of the measured value for all duplicates.

Strain and relevant genotype	Specific growth rate (h^{-1})		
	Glucose	Galactose	Ethanol
S. cerevisiae CEN.PK113–7D (*NDE1 NDE2*)	0.40	0.18	0.17
S. cerevisiae CEN.PK152 (*nde1Δ NDE2*)	0.38	0.12	0.15
S. cerevisiae CEN.PK162 (*NDE1 nde2Δ*)	0.39	0.17	0.17
S. cerevisiae CEN.PK167–2B (*nde1Δ nde2Δ*)	0.37	0.12	0.12

DISCUSSION

In this study, the *S. cerevisiae* open reading frames *YMR145c*/*NDE1* and *YDL085w*/*NDE2* have been unambiguously identified as two structural genes that each encode a mitochondrial external NADH dehydrogenase. An independent corroboration of the identity of *NDE1* was obtained by sequencing of the N-terminal amino acid sequence of a NADH dehydrogenase purified from an *S. cerevisiae* mutant lacking the *NDI1*-encoded internal NADH dehydrogenase.[2] The obtained sequence of the purified 54-kDa flavoprotein was *XXXX*-VILQKVAT (*i.e.* the first four amino acids could not be identified; the T was ambiguous). The residues VILQKVA correspond to amino acids 46–52 of the predicted amino acid sequence of Nde1p.

Identification of *NDE1* and *NDE2* required a special protocol for generation of ethanol-free NADH in the oxygen-uptake experiments with isolated mitochondria. The importance of this experimental detail became evident when commercial NADH was used in preliminary studies with mitochondria isolated from the *nde1Δ nde2Δ* mutant. In these studies, a significant residual respiration rate was observed (0.05 μmol O_2·min^{-1}·mg $protein^{-1}$). Control experiments in which ethanol was provided at the same concentration as was calculated to be present in the experiments with commercial NADH yielded the same (<10% difference) respiration rates as those observed with commercial NADH. The indirect method for generating NADH used in this study may also be applicable in other systems in which ethanol interferes with the quantitation of NADH-dependent reactions.

Although mitochondria from the *nde1Δ nde2Δ* mutant failed to oxidize external NADH (Table III), glucose-limited, aerobic chemostat cultures grown at a dilution rate of 0.10 h^{-1} did not exhibit alcoholic fermentation. Instead, glucose dissimilation occurred virtually completely via respiration (Table IV). This observation constitutes the first experimental evidence that systems other than the external NADH dehydrogenase can sustain mitochondrial reoxidation of cytosolic NADH in growing *S. cerevisiae*. As indicated in the literature (2, 5, 15), both the glycerol-3-phosphate system and an ethanol-acetaldehyde shuttle might theoretically fulfill this role. The small amounts

[2] S. de Vries, unpublished data.

of glycerol produced by glucose-limited cultures of the *nde1*Δ *nde2*Δ mutant (Table IV) may be indicative of an increased activity of the glycerol-3-phosphate dehydrogenase system compared with the wild type. Although glycerol itself is not an intermediate in this proposed system for NADH reoxidation (Fig. 1), it can be formed by dephosphorylation of glycerol-3-phosphate via the *GPP1* and *GPP2*-encoded glycerol-3-phosphatases (15, 45).

With the identification of the *NDE1* and *NDE2* genes, structural genes have now been identified for all three major mechanisms proposed to contribute to the reoxidation of cytosolic NADH by *S. cerevisiae* mitochondria (Fig. 1). Construction of mutants in which different combinations of these proposed systems have been eliminated will eventually show whether all three systems can function in growing *S. cerevisiae* and whether other redox shuttle systems are also operating.

Under the experimental conditions investigated in this study, the phenotype of null mutants seems to indicate that Nde1p is the more important of the two external NADH dehydrogenases; absence of Nde2p did not, by itself, result in a clear phenotype. Of course, the relative expression of *NDE1* and *NDE2* may strongly depend on growth conditions. A recent study on transcription of the yeast genome has demonstrated that both *YMR145c*/*NDE1* and *YDL 085w*/*NDE2* are transcribed during growth on glucose in batch cultures, with *NDE2* transcription being strongly induced when the cultures switched to ethanol utilization (46). Further studies, involving a broad range of growth conditions, are required to investigate the regulation of these two external NADH dehydrogenases.

Identification of the *NDE1* and *NDE2* genes makes *S. cerevisiae* the first eukaryote in which the genes encoding external NADH dehydrogenase have been identified. It will be of interest to investigate whether, and to what extent, Nde1p and Nde2p are similar in structure, function, and regulation to the external NADH dehydrogenases in other fungi and in plants. Functional complementation of *S. cerevisiae nde1*Δ *nde2*Δ mutants with plant homologues may prove to be an attractive model system for such studies.

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