

Identification of a Cytosolically Directed NADH Dehydrogenase in Mitochondria of *Saccharomyces cerevisiae*

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The reoxidation of NADH generated in reactions within the mitochondrial matrix of *Saccharomyces cerevisiae* is catalyzed by an NADH dehydrogenase designated Ndi1p (C. A. M. Marres, S. de Vries, and L. A. Grivell, Eur. J. Biochem. 195:857–862, 1991). Gene disruption analysis was used to examine possible metabolic functions of two proteins encoded by open reading frames having significant primary sequence similarity to Ndi1p. Disruption of the gene designated *NDH1* results in a threefold reduction in total mitochondrial NADH dehydrogenase activity in cells cultivated with glucose and in a fourfold reduction in the respiration of isolated mitochondria with NADH as the substrate. Thus, Ndh1p appears to be a mitochondrial dehydrogenase capable of using exogenous NADH. Disruption of a closely related gene designated *NDH2* has no effect on these properties. Growth phenotype analyses suggest that the external NADH dehydrogenase activity of Ndh1p is important for optimum cellular growth with a number of nonfermentable carbon sources, including ethanol. Codisruption of *NDH1* and genes encoding malate dehydrogenases essentially eliminates growth on nonfermentable carbon sources, suggesting that the external mitochondrial NADH dehydrogenase and the malate-aspartate shuttle may both contribute to reoxidation of cytosolic NADH under these growth conditions.

In eukaryotic cells, reoxidation of NADH generated by catabolic reactions in the cytosol requires fermentation reactions or delivery of reducing equivalents to the mitochondrial electron transport chain. Early studies indicated that isolated mitochondria from *Saccharomyces cerevisiae* are capable of oxidation of exogenous NADH (24, 25), as are mitochondria from plants and fungi. This suggests the existence of an externally directed NADH dehydrogenase that may catalyze the metabolic oxidation of cytosolic NADH. This direct oxidation could occur in lieu of indirect oxidation via the malate-aspartate shuttle cycle characteristic of mammalian cells. Additional studies have characterized at least two separate enzymatic activities in yeast mitochondria capable of delivery of reducing equivalents from NADH to the respiratory chain (5, 7, 20). These dehydrogenases differ from complex I of mammalian mitochondria in several ways, including the absence of coupling to site I phosphorylation, insensitivity to rotenone or piericidin, and the absence of an iron-sulfur redox center.

The best characterized of yeast NADH dehydrogenases is encoded by the *NDI1* gene (genome designation *YML120c*) isolated and characterized by de Vries et al. (10). The purified Ndi1 protein is composed of a single subunit (M_r , 53,000) and contains noncovalently linked flavin adenine dinucleotide (8). The protein has an amino-terminal mitochondrial targeting sequence which is removed upon import (10). Analysis of null mutants containing a disruption of the *NDI1* gene indicated that the enzyme is primarily involved in oxidation of NADH generated within the mitochondrial matrix (20). Gene disruption was found to have no effect on growth with fermentable carbon sources or ethanol but to reduce or eliminate growth on lactate, pyruvate, or acetate.

In contrast to the internal NADH dehydrogenase represented by Ndi1p, the identity of the external dehydrogenase(s)

has not been established. An activity oriented toward the intermembrane space has been described (5, 7, 13), but the protein has not been isolated. The *S. cerevisiae* genome sequence reveals two open reading frames (ORFs), *YMR145c* and *YDL085w*, with significant homology to *NDI1*. The current study describes effects of disruption of the corresponding genes to establish if either or both encode an externally directed NADH dehydrogenase.

Interest in the external NADH dehydrogenase derives, in part, from aspects of phenotypes determined for yeast mutants constructed by disruption of genes for cytosolic and mitochondrial isozymes of malate dehydrogenase. For example, disruption of the *MDH1* gene encoding the mitochondrial tricarboxylic acid cycle enzyme produces an inability to utilize acetate as a carbon source (21). Since this disruption does not eliminate growth with ethanol, it was speculated that sufficient energy for growth might be provided by delivery of reducing equivalents from reactions catalyzed by alcohol and aldehyde dehydrogenases and that this delivery could involve the external NADH dehydrogenase. Also, codisruption of *MDH1* and *MDH2*, the gene encoding the cytosolic enzyme, does not significantly affect growth on glucose or eliminate growth with other nonfermentable carbon sources like ethanol or glycerol-lactate (23), suggesting a metabolic alternative to the malate-aspartate shuttle. Thus, in addition to examining the metabolic effects of disruption of genes encoding putative external NADH dehydrogenases, we have also examined phenotypes resulting from codisruption of these genes with *MDH1* and *MDH2*. Our results suggest that shuttle cycle functions and external NADH oxidation may, in part, be interchangeable.

MATERIALS AND METHODS

Yeast strains and growth conditions. The *S. cerevisiae* strains used in this study were the parental haploid strain S173-6B (*MATa leu2-3,112 his3-1 ura3-52 trp1-289*) (2) and derivatives of this strain containing specific gene disruptions. These include strains containing a deletion and a *HIS3* insertion in the chromosomal locus for *MDH1* or *MDH2*, constructed as previously reported (21, 23), and strains containing disruptions of the *NDH1* and *NDH2* loci, constructed as described below.

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Yeast strains were cultivated in rich YP medium (1% yeast extract, 2% Bacto Peptone) or in minimal YNB medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, pH 6.0) containing supplements (20 µg/ml) to satisfy auxotrophic requirements for growth. Carbon sources used at a concentration of 3% were glucose, ethanol, and glycerol plus lactate. Potassium acetate (1%) was also used as a carbon source. With cultivation in liquid medium, 50 mM potassium phosphate (pH 6.8) was added as a buffer to prevent cell clumping. Culture growth rates were monitored by turbidity measurements using a Klett photometer (Manostat Corp., New York, N.Y.) with a KS-59 filter. For growth curves, cultures were inoculated from fresh logarithmic-phase starter cultures to an initial density of 40 Klett units.

Disruption of *NDH1* and *NDH2* loci. The *S. cerevisiae NDH1* gene (ORF *YMR145c*) was amplified from a yeast genomic DNA library by PCR. Oligonucleotides (5'-GGGGGATCCATGATTAGACAATCATTAAATGAAAAACAGTG-3' and 5'-CCCCGAATTCCTAGATAGATGAATCTCTACCCAAGAAATAAAC-3'), used as primers, were derived from sequences from the 5' and 3' termini of the coding region. The resulting PCR product was digested with *Bam*HI and *Eco*RI and ligated into the yeast shuttle vector pRS426 (28). The identity of the subcloned PCR product was confirmed by partial nucleotide sequence analysis (32). For disruption, the yeast *TRP1* gene was subcloned by blunt-end ligation into a unique *Ehe*I restriction site in the *NDH1* coding region. The *Bam*HI/*Eco*RI DNA fragment containing the interrupted coding region was used for transformation of strain S173-6B and one-step gene disruption (27).

The *NDH2* gene (ORF *YDL085w*) was disrupted by the heterologous cassette method of Wach et al. (33). The *kanMX4* selection gene was amplified by PCR using plasmid pFA6-*kanMX4* as the template. The oligonucleotides used as primers contained terminal 5' and 3' sequences derived from the *NDH2* coding region. The PCR product was used for direct transformation of various yeast strains and transformants identified as colonies resistant to geneticin (200 µg/ml). Yeast transformations were conducted by using lithium acetate (12, 15). Chromosomal gene disruptions were confirmed by Southern blot analysis (31) of genomic DNA isolated from Trp⁺ or geneticin-resistant colonies.

Northern blot analysis of *NDH1* expression was conducted by using RNA samples isolated as described in Rose et al. (26) from cultures of the parental strain harvested during logarithmic growth on YP medium with various carbon sources. DNA probes were prepared by the random primer labeling procedure (14) by using the *Bam*HI/*Eco*RI fragment from the cloned *NDH1* gene and a 1.1-kbp fragment from the yeast actin gene. Densitometry was used for quantitative comparison of autoradiographic results.

Cellular fractionation and protein analyses. For cellular fractionation, 200-ml cultures were grown with YP medium containing glucose or glycerol-lactate as the carbon source to an optical density at 600 nm of 1.0 to 2.0. Growth was terminated by addition of 0.2-mg/ml cycloheximide and incubation on ice for 15 min. Cell pellets were used for subcellular fractionation as described by Daum et al. (6), producing an organellar pellet containing mitochondria and peroxisomes and a soluble cytosolic fraction. For enzymatic assays, the organellar pellet was resuspended in 0.5 to 2.0 ml of 10 mM Tris-HCl (pH 7.4)-0.05% (wt/vol) phenylmethylsulfonyl fluoride and lysed by vortexing with glass beads.

Malate dehydrogenase activity was measured as previously described (21). NADH dehydrogenase activity was measured as the NADH-dependent reduction of cytochrome *c* by a modification of the method of Sottocasa et al. (30). Assays were conducted in a volume of 1.0 ml containing 50 mM potassium phosphate (pH 7.4), 2 mM potassium cyanide, and 0.1 mM cytochrome *c*. Assays were initiated by addition of NADH (or NADPH) to a final concentration of 0.1 mM and monitored spectrophotometrically at 550 nm. Protein concentrations were measured by the method of Bradford (3) by using bovine serum albumin as the standard.

Organellar pellets were resuspended for measurements of oxygen consumption by using a Clark-type oxygen electrode (YSI Inc., Yellow Springs, Ohio) as described by Balcaevage et al. (1). Respiratory substrates were 5 mM succinate, 5 mM pyruvate plus 5 mM malate, and 0.1 mM NADH (or NADPH). Measurements were made in the absence and presence of 0.1 mM ADP, and all respiration was found to be sensitive to inhibition by cyanide or azide.

RESULTS

Disruption of putative *NDH* genes. A search of the *S. cerevisiae* genome sequence database revealed two ORFs, *YMR145c* and *YDL085w*, with extensive sequence similarity to the previously identified gene (*NDH1*, ORF *YML120C*) encoding the internal form of mitochondrial NADH dehydrogenase (20). For simplicity, the *YMR145c* and *YDL085w* ORFs potentially encoding NADH dehydrogenases are designated *NDH1* and *NDH2*, respectively. The aligned primary sequences of Ndh1p and Ndh2p share 62% residue identity and 48 and 46% residue sequence identity, respectively, with Ndi1p. The ORFs can also encode polypeptides of similar sizes: 513 residues for Ndi1p, 560 for Ndh1p, and 545 for Ndh2p. Among striking differences,

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NDI1:
NDH1: MIRQSLMKTVWANSRRFSLQSKSGLVKYAKNRSFHAARNLLEDKVKLQKVAP 53
      ..  ..:
NDH2:      MLPRLGFARTARSIRHFKMTQISKIPFFHSTEVGPKPGPQQLSK 43

[MLSKNLYSNKRLTSTNTLVRFSTR]STGVENSAGGPTSFKTKMVIDPQHS DK 53
      ..  ..:
TTGVVAKQSPFKRKTGKPTLKALLYLAGATAYVSYSLYREANPSTQVQPQSDTFPNQSKR 112
      ..  ..:
SYTAVFKKWFVR--G---LKLTFYTLAGTLYVSYELYKESNPPKQVQSTAFANGLKK 97

PNVLILGSGWGAISFLKHIDTKKYNVSIISPRSYFLFTPLLPSPAPVGTVDEKSIIEPIV 112 (513)
      ..  ..:
KTLVILGSGWGSVSLKLNLDLTLNVTVVSPRNYFLFTPLLPSTPVTGTEIELKSIVPEVR 171 (560)
      ..  ..:
KELVILGTGWGAISLLKLDLTLNVTVVSPRNYFLFTPLLPSTPVTGTEIEMKSIIVEPVR 156 (545)

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FIG. 1. Comparison of amino-terminal sequences of yeast NADH dehydrogenase homologs. Shown is a partial alignment of amino acid sequences for Ndi1p (20) (ORF *YML120c* on chromosome XIII), Ndh1p (ORF *YMR145c* on chromosome XIII), and Ndh2p (ORF *YDL085w* on chromosome IV). Residue identities are indicated by colons, and similarities are indicated by periods. Brackets enclose the mitochondrial targeting sequence of Ndi1p (10). The total number of residues in each ORF is shown in parentheses.

as illustrated in the partial sequence comparison in Fig. 1, are sequences near the amino termini. Ndh1p and Ndh2p have long amino-terminal extensions with significant homology beginning at respective residue positions 49 and 39. The amino terminus of Ndi1p is substantially shorter, but the sequence of the mature polypeptide commences with significant homology to the Ndh1p and Ndh2p sequences. The 26-residue mitochondrial targeting sequence of Ndi1p (10) is bracketed. The first 20 to 30 residues of the amino-terminal extensions of Ndh1p and Ndh2p also contain multiple residues with positively charged and hydroxylated side chains, characteristic of mitochondrial targeting sequences.

Gene disruption analysis was conducted to test the enzymatic and metabolic functions of Ndh1p and Ndh2p. Cassettes for disruption were constructed by PCR as described in Materials and Methods. A haploid yeast strain containing a disruption of the *NDH1* gene was constructed by transformation with a DNA fragment containing the *NDH1* coding region interrupted by the yeast *TRP1* gene. The *NDH2* genes in this strain and the parental strain were subsequently disrupted by replacement with a *kanMX4* gene. Trp⁺ and geneticin-resistant transformants representing single- and double-mutant strains were isolated, and the gene disruptions were verified by Southern blot analyses.

Initial analyses of activity were conducted by using extracts from soluble cytosolic and organellar (mitochondrial) fractions following cultivation of mutant and parental strains on rich medium with various fermentable or nonfermentable carbon sources. Under all conditions, NADH dehydrogenase activity was found to be associated with the organellar pellet. These assays, which monitor oxidation of NADH as a function of cytochrome *c* reduction by the *bc*₁ complex, were conducted following mechanical disruption of organellar membranes and thus are a measure of total mitochondrial activity. As illustrated in Table 1, levels of total NADH dehydrogenase activity associated with mitochondria from the parental strain are elevated approximately threefold with growth on glycerol-lactate relative to growth on glucose as the carbon source. Similarly elevated levels are detected in mitochondrial fractions from the Δ *NDH* mutant strains following growth on glycerol-lactate, and differences among the various strains are not significant.

TABLE 1. NADH dehydrogenase activity in mitochondrial extracts from parental and *NDH* disruption strains

Relevant genotype	Mitochondrial NADH dehydrogenase activity ($\mu\text{mol}/\text{min}/\text{mg}$)	
	Glucose ^a	Glycerol-lactate
Parental	0.48 ± 0.12^b	1.49 ± 0.22
ΔNDH1	0.18 ± 0.07	1.59 ± 0.11
ΔNDH2	0.63 ± 0.24	1.24 ± 0.22
$\Delta\text{NDH1 } \Delta\text{NDH2}$	0.22 ± 0.01	1.48 ± 0.45

^a Yeast strains were cultivated on rich medium with the indicated carbon source prior to cellular fractionation.

^b Values represent averages and standard deviations of two or three independent measurements.

However, significant differences in activity are measurable following growth on glucose. An approximate threefold decrease in activity is measured for strains containing the *NDH1* gene disruption, whereas disruption of *NDH2* has no measurable effect on activity under these conditions. These results are consistent with previous reports that Ndh1p activity is depressed with growth on nonfermentable carbon sources (8). They also suggest that Ndh1p is the primary contributor to NADH dehydrogenase activity in cells grown on glucose. Northern blot analyses (data not shown) indicate similar levels of *NDH1* mRNA, relative to yeast actin mRNA levels, with growth on all of the carbon sources used in this study, suggesting that expression may be constitutive.

To distinguish relative contributions to oxidation of external NADH, rates of oxygen consumption with this and other respiratory substrates were compared by using mitochondria isolated from cells grown with glucose as the carbon source. As shown in Table 2, rates of oxygen consumption with succinate are similar for mitochondria from the parental strain and the ΔNDH1 , ΔNDH2 , and $\Delta\text{NDH1 } \Delta\text{NDH2}$ disruption strains, whereas rates with pyruvate plus malate as the substrate are slightly reduced for strains with the ΔNDH1 disruption. In contrast, the ΔNDH1 disruption is associated with a threefold reduction in respiratory rates with NADH as the substrate. For the parental and ΔNDH2 disruption strains, the approximate 4:1 ratio of rates with NADH versus succinate is similar to published values for mitochondria from glucose-grown cells (13, 29). However, this ratio for mitochondria from the ΔNDH1 and $\Delta\text{NDH1 } \Delta\text{NDH2}$ disruption strains is reduced approximately fourfold. Thus, these data suggest that Ndh1p is the dehydrogenase primarily responsible for delivery of reducing equivalents from external NADH to the respiratory chain. The residual oxidative capacity with NADH of mitochondria isolated from ΔNDH1 strains may be due to another external activity; however, the inherent fragility of isolated mitochondria could also account for some access to the internal NADH dehydrogenase.

There is at least one report that yeast mitochondria may be capable of oxidation of NADPH (11). We measured NADPH-dependent reduction of cytochrome *c* by mitochondrial extracts from the strains listed in Table 1. Levels of activity in extracts were approximately 10% of that measured for NADH in the parental extract, and no significant differences were detected among various strains. Also, we found no measurable oxidation of NADPH by respiring mitochondria isolated from any of the strains in this study. Thus, any mitochondrial oxidation of NADPH likely occurs internally and may be attributable to Ndh1p. This would be analogous to utilization of both NADH and NADPH by an internal dehydrogenase in plant mitochondria (22).

Growth phenotypes associated with *NDH* and *MDH* gene disruptions. The effects of *NDH1* and *NDH2* gene disruptions on growth rates with various carbon sources were examined by using logarithmically growing cultures in both rich and minimal media. As shown in Table 3, experiment A, disruption of either or both genes was found to have no effect on growth rates with glucose. Also, single disruption of *NDH2* does not significantly alter growth rates relative to the parental strain on any carbon source tested. However, disruption of *NDH1*, alone or in combination with disruption of *NDH2*, was found to reduce growth rates with nonfermentable carbon sources, including glycerol-lactate (data not shown) and ethanol. The most dramatic effect is an ~twofold lengthening of generation time with ethanol as the carbon source in minimal medium (Table 3, experiment A); this effect is also observed but is less pronounced with rich medium. These results are consistent with an important role for Ndh1p in reoxidation of NADH, which may accumulate to significant levels in the cytosol during ethanol utilization due to the reaction catalyzed by cytosolic alcohol dehydrogenase (34).

That ΔNDH1 strains grow on nonfermentable carbon sources, albeit at reduced rates, suggests that reoxidation of cytosolic NADH by this enzyme is not essential or that an alternative mechanism is available. To test if one alternative could be the malate-aspartate shuttle, the *NDH1* gene disruption was introduced into yeast strains containing disruptions of the genes encoding the mitochondrial (Mdh1p) or cytosolic (Mdh2p) isozyme of malate dehydrogenase, an enzymatic participant in the shuttle cycle. As shown in Table 4, disruption of *NDH1* in these strains results in an approximate threefold reduction in NADH dehydrogenase activity in mitochondria from cells grown with glucose analogous to that described for the parental strain disruption. Disruption of *MDH1* or *MDH2* reduces respective compartmental activity by 3- to 10-fold in subcellular fractions, as previously reported (21, 23).

As illustrated in Table 3, experiment B, and as previously reported (23), the most dramatic phenotype associated with disruption of *MDH2* is an inability to grow with ethanol as the carbon source on minimal medium. Since growth with ethanol on rich medium is less affected, this phenotype may indicate a function in gluconeogenesis for production of C4 metabolites.

TABLE 2. Rates of oxygen consumption of isolated mitochondria from parental and *NDH* disruption strains

Relevant genotype	Oxygen consumption ($\mu\text{mol}/\text{mg}$ of protein/min)			Ratio of:	
	NADH ^a	Succinate	Pyruvate + malate	NADH/succinate	NADH/pyruvate + malate
Parental	0.25 ± 0.08^b	0.06 ± 0.02	0.14 ± 0.03	4.2	2.5
ΔNDH1	0.08 ± 0.04	0.07 ± 0.03	0.10 ± 0.02	1.1	0.8
ΔNDH2	0.23 ± 0.05	0.06 ± 0.01	0.13 ± 0.03	3.8	2.2
$\Delta\text{NDH1 } \Delta\text{NDH2}$	0.07 ± 0.02	0.06 ± 0.01	0.08 ± 0.01	1.2	0.9

^a Polarographic measurements were conducted as described in Materials and Methods by using mitochondria isolated from cells grown in YP glucose medium.

^b Values represent averages and standard deviations of two or three independent measurements.

TABLE 3. Growth rates of yeast strains with various carbon sources

Expt and relevant genotype	Doubling time (h) ^a			
	YP glucose ^b	YNB ethanol	YP ethanol	YP acetate
A				
Parental	2.2	9.6	7.7	10.6
$\Delta NDH1$	2.4	19.5	12.7	14.0
$\Delta NDH2$	2.2	10.6	7.7	10.1
$\Delta NDH1 \Delta NDH2$	2.3	18.4	12.6	16.6
B				
$\Delta MDH2$	2.2	NG ^c	14.2	13.9
$\Delta NDH1 \Delta MDH2$	2.2	NG	NG	NG
C				
$\Delta MDH1$	2.3	16.5	12.8	NG
$\Delta NDH1 \Delta MDH1$	2.2	NG	NG	NG

^a Culture growth rates were measured during logarithmic growth.

^b Rich YP medium and minimal YNB medium were used for cultivation with various carbon sources as described in Materials and Methods.

^c NG, no growth after 30 h of incubation.

Codisruption of *MDH2* and *NDH1* was found to essentially eliminate growth on other nonfermentable carbon sources with either rich or minimal medium. One interpretation of this "synthetic" phenotype is that Mdh2p and Ndh1p have complementary functions in reoxidation of cytosolic NADH during growth on nonfermentable carbon sources.

The most dramatic phenotype associated with disruption of *MDH1* is inability to grow with acetate as the carbon source on either rich or minimal medium (21), while growth with other nonfermentable carbon sources is reduced. The acetate growth phenotype, also observed for mutants with a disruption in the *CIT1* gene encoding mitochondrial citrate synthase (17), has been interpreted as an energetic defect. Since *MDH1* and *CIT1* mutants grow well on rich medium with ethanol, production of NADH during ethanol utilization and conversion to acetate was proposed to allow bypass of the energetic defect. Codisruption of *MDH1* and *NDH1* was found in this study (Table 3, experiment C) to significantly reduce growth with ethanol as the carbon source on both rich and minimal media, suggesting that Ndh1p is responsible for this bypass, allowing delivery of cytosolic reducing equivalents for energy production.

In contrast, codisruption of *NDH2* and *MDH1* or *MDH2* was found to have no effect on growth rates measured for the malate dehydrogenase mutants, suggesting no overlap of the physiological functions of these enzymes.

DISCUSSION

The functions of two proteins with extensive primary sequence homology with yeast Ndi1p, the matrix-oriented mitochondrial NADH dehydrogenase, were investigated by disruption of the corresponding genes in *S. cerevisiae*. Evidence is presented that the protein designated Ndh1p catalyzes the oxidation of cytosolic NADH or of exogenous NADH in the case of isolated mitochondria, but no physiological function has been determined for Ndh2p. This family of homologous proteins in yeast shares some similarity with single-subunit NADH dehydrogenase of *Escherichia coli* (20), but these proteins appear to be evolutionarily and structurally distinct from the multisubunit complex I ubiquinone oxidoreductases characteristic of mammalian cells. Independent function of yeast Ndi1p has been demonstrated by its purification as a single

polypeptide (8) and by demonstration of function in the bacterial respiratory chain when *NDH1* is expressed in *E. coli* (18).

Disruption of the *NDH1* gene results in an approximate threefold reduction in rates of oxygen consumption by isolated mitochondria with NADH but has little effect on respiration with pyruvate-malate as the respiratory substrate, suggesting that the Ndh1p catalytic function primarily involves external reducing equivalents. Essentially opposite effects were obtained with disruption of *NDH2* (20), i.e., loss of respiration with pyruvate-malate but not with NADH. Mitochondria from both types of mutants utilize succinate due to independent delivery of reducing equivalents from flavin adenine dinucleotide-H₂. In terms of contribution to total mitochondrial activity, disruption of *NDH1* (and *NDH2*) has little effect on elevated levels in extracts from cells grown with nonfermentable carbon sources. Differences in NADH dehydrogenase activity attributable to Ndh1p are measurable only with growth on glucose, a condition associated with repressed expression of Ndi1p (8).

Despite lower levels of Ndh1p activity relative to Ndi1p with growth on nonfermentable carbon sources, the phenotypes exhibited by haploid yeast strains containing a disruption of the *NDH1* gene suggest that direct oxidation of cytosolic NADH is crucial for optimum growth under these conditions. Ndi1p is also important for optimum growth on most nonfermentable carbon sources (20). In fact, the major difference between strains with corresponding gene disruptions appears to be the reduction in growth with ethanol associated with *NDH1* disruption; this phenotype and its absence with *NDH2* disruption suggest the importance of the external activity when net oxidation is required for assimilation of a carbon source. Growth with glucose as a carbon source is not affected by disruption of any of the genes encoding putative NADH dehydrogenases (Table 3) (20). Also, as shown in this study, concomitant disruption of *NDH1* and *NDH2* and repressed expression of Ndi1p do not reduce glucose growth rates, suggesting the sufficiency of fermentation reactions for reoxidation of NADH.

It has been proposed that the malate-aspartate shuttle cycle may not be functional in yeast due to the capacity for direct oxidation of cytosolic NADH (9, 16). We have attempted to address this question by analysis of mutants lacking both Ndh1p and either the cytosolic or mitochondrial isozyme of malate dehydrogenase. One aspect of growth phenotypes exhibited by strains lacking cytosolic Mdh2p is reduced growth on rich medium with ethanol as the carbon source (Table 3). This is distinct from the auxotrophic phenotype exhibited on minimal medium. Since codisruption of *MDH2* and *NDH1* eliminates

TABLE 4. Enzyme activities in extracts from parental and disruption mutant strains

Relevant genotype	Activity ($\mu\text{mol}/\text{min}/\text{mg}$) of:		
	NADH dehydrogenase in mitochondria ^a	Malate dehydrogenase in:	
		Mitochondria ^b	Cytosol
Parental	0.48 \pm 0.12 ^c	1.85 \pm 0.12	0.27 \pm 0.09
$\Delta NDH1$	0.18 \pm 0.07	2.16 \pm 0.04	0.22 \pm 0.04
$\Delta MDH1$	0.39 \pm 0.06	0.38 \pm 0.09	0.28 \pm 0.15
$\Delta NDH1 \Delta MDH1$	0.19 \pm 0.06	0.45 \pm 0.01	0.21 \pm 0.01
$\Delta MDH2$	0.51 \pm 0.02	1.93 \pm 0.22	0.05 \pm 0.01
$\Delta NDH1 \Delta MDH2$	0.17 \pm 0.01	1.45 \pm 0.06	0.03 \pm 0.01

^a Subcellular fractionation was conducted as described in Materials and Methods by using cells cultivated on YP glucose medium.

^b Subcellular fractionation was conducted by using cells cultivated on YP glycerol-lactate.

^c Values represent averages and standard deviations of two or three independent measurements.

the residual capacity of the single-disruption mutant strains for growth on ethanol with rich medium, it is possible that the two gene products provide complementary or additive physiological functions, and it seems reasonable to assume that the common function is oxidation of cytosolic NADH. Similarly, disruption of *MDH1* only slightly reduces growth with ethanol on rich or minimal medium, indicating that an intact tricarboxylic acid cycle is not essential under these conditions. Codisruption with *NDH1*, however, eliminates ethanol growth, indicating that redox balance may require either an intact cycle-shuttle function or the external dehydrogenase activity. Collectively, these results suggest that both direct and indirect paths for oxidation of NADH may be operative. An alternative possibility is that the malate dehydrogenases are important substrate sources for the external NADH dehydrogenase.

A potential alternative for oxidation of cytosolic NADH is the glycerol phosphate shuttle. However, a recent study by Larsson et al. (19) indicates that although this shuttle is very active in yeast cells grown with ethanol, disruption of genes encoding enzymatic components of the shuttle has no effect on ethanol growth rates. Thus, this shuttle is not physiologically essential, at least under aerobic conditions. Of interest for future studies are correlations between the functions of the different shuttle cycles. Also, although no deleterious consequences for growth have been found to correlate with disruption of the *NDH2* gene, further examination of expression and other growth conditions is required to elucidate or eliminate a possible metabolic function. Along these lines, it is of interest to determine the identity of a high-molecular-weight complex with NADH dehydrogenase activity which is reportedly induced by starvation (4). Finally, the yeast NADH dehydrogenases provide an interesting system for examination of mechanisms for differential compartmental-membrane localization.

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