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A single external enzyme confers alternative NADH:ubiquinone oxidoreductase activity in *Yarrowia lipolytica*

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SUMMARY

NADH:ubiquinone oxidoreductases catalyse the first step within the diverse pathways of mitochondrial NADH oxidation. In addition to the energy-conserving form commonly called complex I, fungi and plants contain much simpler alternative NADH:ubiquinone oxido-reductases that catalyze the same reaction but do not translocate protons accross the inner mitochondrial membrane. Little is known about the distribution and function of these enzymes. We have identified *YLNDH2* as the only gene encoding an alternative NADH:ubiquinone oxidoreductase (NDH2) in the obligate aerobic yeast *Yarrowia lipolytica*. Cells carrying a deletion of *YLNDH2* were fully viable; full inhibition by piericidin A indicated that complex I activity was the sole NADH:ubiquinone oxidoreductase activity left in the deletion strains. Studies with intact mitochondria revealed that NDH2 in *Y. lipolytica* is oriented towards the external face of the mitochondrial inner membrane. This is in contrast to the situation seen in *Saccharomyces cerevisiae*, *Neurospora crassa* and in green plants, where internal alternative NADH:ubiquinone oxidoreductases have been reported. Phylogenetic analysis of known NADH:ubiquinone oxidoreductases suggests that during evolution conversion of an ancestral external alternative NADH:ubiquinone oxidoreductase to an internal enzyme may have paved the way for the loss of complex I in fermenting yeasts like *S. cerevisiae*.

Key words: *Yarrowia lipolytica*, Mitochondria, NADH:ubiquinone oxidoreductase, NADH dehydrogenase

INTRODUCTION

Despite of their central role in energy metabolism, rather little is known about the NADH:ubiquinone oxidoreductases, the enzymes that form the first step within the diverse pathways of mitochondrial NADH oxidation. In mammals, the first step in NADH oxidation via the mitochondrial respiratory chain is carried out by complex I, a multisubunit enzyme which couples electron transfer from NADH to ubiquinone to vectorial proton translocation across the inner mitochondrial membrane (reviewed in Brandt, 1997). In contrast, mitochondria from fungi typically contain alternative NADH:ubiquinone oxidoreductases in addition to complex I. These do not pump protons, are insensitive to classical inhibitors of complex I like rotenone and piericidin A and may compete with complex I for the substrates NADH and ubiquinone in the mitochondrial matrix (for an overview, see de Vries and Marres, 1987; Yagi, 1991). The discovery of alternative NADH:ubiquinone oxidoreductase activity in *Saccharomyces carlsbergensis* (von Jagow and Klingenberg, 1970) and *Neurospora crassa* (Weiss et al., 1970) dates back to 1970. Several reports indicate that this activity is under metabolic control in *Torulopsis utilis* (Katz et al., 1971) and in *N. crassa* (Schwitzguebel and Palmer, 1982): activity is highest during early log phase, but when cells enter the stationary phase due to depletion of the carbon source, it is downregulated and complex I becomes the predominant NADH dehydrogenase of the respiratory chain. However, the mechanisms underlying this metabolic regulation are far from being understood.

Among fungi, the genus *Saccharomyces* stands out by its adaptation to alcoholic fermentation and the absence of complex I. Sequences homologous to the highly conserved genes encoding the 14 'minimal' subunits could not be detected in the nuclear or mitochondrial genomes, respectively. NADH oxidation in the mitochondrial matrix must therefore proceed by default via an alternative NADH:ubiquinone oxidoreductase. Such an enzyme has been purified from mitochondrial membranes of *S. cerevisiae* (de Vries and Grivell, 1988). It consists of a single polypeptide chain and contains one molecule of noncovalently bound FAD. Deletion of SC*NDI1*, the gene encoding this alternative NADH:ubiquinone oxidoreductase (de Vries et al., 1992) makes the mutant unable to grow on highly oxidized carbon sources like acetate and pyruvate (Marres et al., 1991), consistent with the notion that the enzyme is located on the matrix side of the mitochondrial inner membrane.

The *S. cerevisiae* genome as deposited in the *Saccharomyces* Genome Database (SGD) at Stanford University contains two gene loci (*YMR145c*, bp 556474-554792 on chromosome 13 and *YDL085w*, bp 303210-304847 on chromosome 6) whose

open reading frames exhibit a high degree of sequence homology to the SC*NDI1* protein (locus name *YML120c*, bp 29807-28266 on chromosome 13). By searching a database established in a genome-wide study of gene expression using DNA microarrays (de Risi et al., 1997) it was found that expression of all three NADH:ubiquinone oxidoreductase genes of *S. cerevisiae* is linked to the activation of mitochondrial respiration.

In plant mitochondria, complex I and up to four different alternative NAD(P)H:ubiquinone oxidoreductase activities have been detected. They differ in terms of subcellular localization, Ca^{2+} requirements and induction kinetics. Some, but not all, plant mitochondria also have the capacity for respiration-linked oxidation of cytosolic NADPH (Soole and Menz, 1995). The purification of a 43 kDa rotenone-insensitive NADH dehydrogenase, located on the internal side of the inner mitochondrial membrane, has been reported (Menz and Day, 1996). Since sequence data are not available, the relationship of this protein to the SCNDI1 gene product is unclear, however.

Among procaryotes, multiple respiratory chain NADH dehydrogenases are also known to exist, e.g. in *E. coli*, which has a 'minimal' form of complex I encoded by the *nuo* operon (Weidner et al., 1993) and an alternative NADH:ubiquinone oxidoreductase encoded by the *ndh* gene (Young et al., 1981).

Genetic dissection of NADH:ubiquinone oxidoreductase activity has only been performed in *S. cerevisiae* so far. Since complex I is absent in this yeast, it is not possible to address the question of how complex I and alternative NADH:ubiquinone oxidoreductase are regulated in terms of expression and activity under different growth conditions. We therefore chose the obligate aerobic yeast *Yarrowia lipolytica* as our model system. Here we report the identification of *YLNDH2*, the gene encoding alternative NADH:ubiquinone oxidoreductase. Analysis of a *YLNDH2* deletion mutant revealed a novel and unexpected design of the mitochondrial respiratory chain in this fungus.

MATERIALS AND METHODS

Strains

Yarrowia lipolytica strains E129 (*Mat*A, *lys11*-23, *ura3*-302, *leu2*-270, *xpr2*-322) and E150 (*Mat*B, *his*-1, *ura3*-302, *leu2*-270, *xpr2*-322) were a kind gift from Prof. C. Gaillardin, INRA, Paris, France. The diploid strain GB1 was produced by mating strains E129 and E150. *Y. lipolytica* genetic techniques were carried out according to Barth and Gaillardin (1996).

Cloning procedures

Degenerate primers YLNDH-1 5′-GTIGTIGGIGGIGGNCCNACN-GG-3′ (23mer, coding strand) and YLNDH-2 5′-GCYTCYTGRTG-IGCIACYTGNGC-3′ (23mer, noncoding strand) were deduced from the SCNDI1 protein sequence. PCR resulted in the amplification of one single 0.5 kb product in 3 out of 15 pools from our *Y. lipolytica* strain E150 genomic DNA library (see below), which was subcloned into pCR2.1 (Invitrogen) yielding clone pCRNDH2, and verified by sequencing.

A genomic library from *Y. lipolytica* was made by partially digesting DNA from strain E150 with *Sau*3AI and cloning of fragments in the size range 5-10 kb into *Bam*HI-cut pBluescriptSK^{-} (Stratagene). 15 primary pools with a complexity of 600-800 recombinant colonies were obtained, which corresponds to 4-8 genome equivalents. The fraction of insert-containing clones, as judged by plating on IPTG/X-Gal, was in the range of 80-90% for individual pools. Pools were screened by successive rounds of Southern hybridisation with the ^{32}P-labeled insert from clone pCRNDH2, using the Prime-It II Kit (Stratagene) for random prime labelling and QuickHyb (Stratagene) hybridisation solution. Screening resulted in the isolation of independent plasmid clones E8, F13 and H12, all of which were shown to belong to the *YLNDH2* gene locus by DNA sequencing. Clone E8, which contained a 5 kb insert, was used for further experiments. A 3.5 kb *Sac*I/*Pst*I fragment was sequenced by a combination of nested deletion (Henikoff, 1987) and primer walking strategies. The sequence of *YLNDH2* has been submitted to the EMBL database under accession number AJ006852.

Deletion of *YLNDH2*

The *YLNDH2* gene was deleted by homologous recombination with a *URA3* marked deletion allele. The complete insert from clone E8 was excised with *Eco*RI/*Not*I and subcloned into a pBluescriptSK^{-} (Stratagene) derivative from which part of the polylinker region had been deleted by digestion with *Kpn*I and *Hin*dIII, blunt ending using Klenow DNA polymerase and religation. A region of roughly 1800 bp, encompassing the complete *YLNDH2* ORF and about 70 bp of 5′ flanking region, were removed by digestion with *Xho*I and replaced with a 1.6 kb *Sal*I fragment from plasmid pINA311 (Barth and Gaillardin, 1996), carrying the complete *URA3* gene from *Y. lipolytica*. A clone in which the orientation of the *URA3* marker was opposite to the original *YLNDH2* gene was selected and a 4 kb *Xmn*I fragment was used for transformation of diploid *Y. lipolytica* cells. Screening of ten *URA*$^{+}$ colonies for homologous recombination with the wild-type *YLNDH2* locus by PCR on isolated genomic DNA, using combinations of inward primers derived from sequences outside of the 4 kb *Xmn*I fragment (H12up and E8dn) and outward primers derived from the *URA3* sequence (ura3up and ura3dn) and by Southern blotting, led to the identification of strain GB4. Haploid deletion strains were obtained through sporulation by random spore analysis or by the dissection of four-spored asci using a Singer MSM 200 instrument, after digestion of the ascus wall using zymolyase (ICN).

Preparation of unsealed mitochondrial membranes

Unsealed mitochondrial membranes were prepared from haploid parental strain E150 and haploid mutant strain GB5.2 (*ylndh2*::*URA3*, *ura3*, *leu2*, *his*, *Mat*B). Cells were grown in 2 l of YPD medium until mid-logarithmic phase, harvested by centrifugation for 10 minutes at 5000 ***g*** and 4°C and resuspended in 150 ml of 400 mM sucrose, 1 mM EDTA, 20 mM Na^{+}/Mops, pH 7.2. Cells were broken while chilled on ice in 5×1 minute intervals using a bead beater (Model 1107900, Biospec Products Inc) and 150 ml of 0.25-0.50 mm glass beads. A centrifugation step (30 minutes, 5000 ***g***, 4°C) to pellet glass beads, cell fragments and unbroken cells, was followed by a second centrifugation (40 minutes, 25000 ***g***, 4°C) to pellet mitochondrial membranes. Supernatants were discarded and pellets were gently homogenized in the same buffer to a concentration of 15-20 mg/ml total protein. Electron flow from NADH to NBQ (5-nonylubiquinone) and from NBQ to cytochrome *c*, assayed in the absence and presence of 0.4% Chaps (3-[(3-cholamidopropyl)-dimethylammonio]-1-propansulfonate), indicated that the preparation consisted of membrane fragments in which both faces of the inner mitochondrial membrane were fully accessible (data not shown).

Preparation of intact mitochondria

400 ml of *Y. lipolytica* culture in complete medium, containing approx. 1×10^{8} cells/ml, were harvested by centrifugation (10 minutes, 3000 ***g***, room temperature), washed once with distilled water, transferred into 50 ml polypropylene tubes and sedimented by centrifugation (10 minutes, 3000 ***g***, room temperature). Cells (approx. 4 g wet mass) were resuspended in 30 ml 0.1 M Tris/HCl, pH 9.3, dithioerythritol was added to a final concentration of 10 mM and the

suspension was incubated at 30°C for 10 minutes. Cells were then pelleted by centrifugation (4 minutes, 3000 ***g***, room temperature), resuspended in 40 ml of buffer A (25 mM potassium phosphate, 1 mM $MgCl_2$, 1 mM EDTA, 2 M sorbitol, pH 7.5) centrifuged as before and finally resuspended in 35 ml of buffer A. Enzymatic digestion of cell walls was carried out by adding 400 U of zymolyase (ICN) and incubation for 1 hour at 30°C with gentle agitation. Cells were then pelleted by centrifugation (10 minutes, 3500 ***g***, room temperature), washed with 40 ml of buffer A, centrifuged (7 minutes, 3500 ***g***, 4°C) and finally resuspended in 10 ml of buffer A. 30 ml of buffer B (like buffer A, but with 0.2 M sorbitol) was added and cells were homogenized in a glass homogenizer with a Teflon pistil (Heidolph) using ten strokes at 2000 rpm. Unbroken cells and cell fragments were pelleted by centrifugation (10 minutes, 3500 ***g***, 4°C) and the supernatant was centrifuged to pellet mitochondria (10 minutes, 5000 ***g***, 4°C). Pellets were carefully suspended in 0.5 ml of buffer C (25 mM potassium phosphate, 1 mM $MgCl_2$, 1 mM EDTA, 0.65 M sorbitol, 1 mg/ml bovine serum albumine, pH 7.5).

NADH:NBQ oxidoreductase assay conditions

NADH:NBQ oxidoreductase activity was assayed as NADH oxidation with a Shimadzu UV-300 spectrophotometer, dual wavelength mode, at 340-400 nm ($\varepsilon_{340\text{-}400}$=6200 $M^{-1}cm^{-1}$), using a stirred, thermostatted cuvette (30°C). Unsealed mitochondrial membranes equivalent to 20 µg/ml of total protein were suspended in 100 mM NaCl, 20 mM Na^+/Mops, pH 7.4, 0.4% Chaps and 2 mM KCN. The reaction was started by the addition of 60 µM NBQ. Michaelis-Menten parameters were determined by varying the concentration of NADH (0-200 µM). Data were analyzed using the ENZFITTER program (Elsevier). Catalytic activity of intact mitochondria (equivalent to 15 µg/ml of total protein) was determined with 200 µM NADH or deamino-NADH, 60 µM NBQ in buffer C plus 2 mM KCN. CCCP (carbonyl-cyanide-m-chlorophenylhydrazone) and valinomycin were added from stock solutions in DMSO to final concentrations of 20 µM and 2 µM, respectively. Mitochondria were made permeable for NADH by adding 30 µM alamethicin or 0.4% Chaps.

RESULTS

Sequence analysis of the *YLNDH2* gene

Genomic clone E8 contained a 582-amino-acid long, intronless open reading frame, encoding a protein with a predicted molecular mass of 65.8 kDa with high homology to known alternative NADH:ubiquinone oxidoreductases. Insights into the structure and function of the *YLNDH2* gene product NDH2 were obtained by comparison with homologous proteins. Four prominent blocks of excellent sequence conservation that became apparent from the alignment of the NDH2 with the three enzymes from *S. cerevisiae* (encoded by gene loci YML120c, YMR145c and YDL085w) were also retained when two more distantly related NADH:ubiquinone oxidoreductases from *Schizosaccharomyces pombe* (contained in chromosome 1 cosmid clone c3A11, accession number 299260), and *Arabidopsis thaliana* (contained in chromosome 4 BAC clone F18E5, accession number A2022603) that we identified by an exhaustive TFASTA search of the EMBL sequence database, and the *E. coli* NDH protein were included in the alignment (Fig. 1). The possible functional significance of these motifs is discussed below.

Dinucleotide fold I

This sequence conforms to most of the criteria for a dinucleotide binding βαβ fold (Wierenga et al., 1985), which

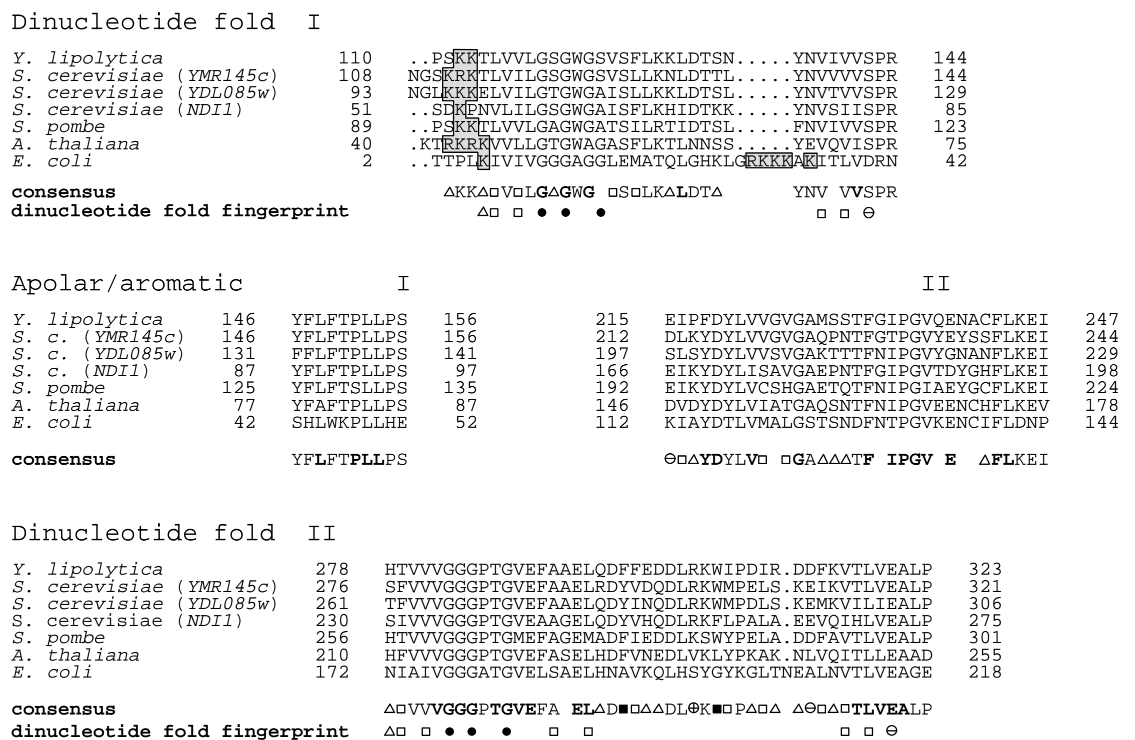


Fig. 1. Sequence motifs conserved between alternative NADH:ubiquinone oxidoreductases from *Y. lipolytica, S. cerevisiae, S. pombe, A. thaliana* and *E. coli.* The consensus sequence of the dinucleotide fold motifs is compared with the dinucleotide fold fingerprint as defined in Wierenga et al. (1985). In the consensus sequences, residues identical in at least five of seven sequences are indicated in one letter code and are printed in boldface, if this residue is present in the *E. coli* sequence. Conserved glycines are marked with filled circles in the fingerprint. Functionally similar residues, if present in five of the seven sequences, are indicated by the following symbols: hydrophobic residues by open squares, aromatic residues by filled squares, hydrophilic residues (irrespective of charge) by open triangles and acidic and basic residues by – and + symbols. Shaded in gray are stretches of basic residues clustered at the N terminus of the first dinucleotide fold in the eucaryotic proteins and forming an insertion into this motif in the *E. coli* protein.

include a set of three glycines, spaced GXGXXG, at the beginning, six regularly spaced hydrophobic residues, a conserved hydrophilic residue at the N terminus and a conserved acidic residue at the C terminus. This latter residue is aspartate in the *E. coli* protein, but is replaced by serine in all other cases. Also, the third, highly conserved but not invariant glycine residue of the GXGXXG motif in all five yeast proteins is replaced by other amino acids with small side chains, namely serine or alanine. In *E. coli*, this dinucleotide fold I domain lies close to the N terminus of the mature protein, in which the initiating N-formylmethionine is removed. Another peculiar feature of the *E. coli* protein is the insertion of the highly basic sequence RKKKAK at position 30-35. While this insertion is not found in any of the other protein sequences, there is a somewhat similar basic stretch at the beginning of dinucleotide fold I. It is tempting to speculate that these basic amino acids may serve a similar function, for example in stabilizing the binding of FAD or NADH.

Dinucleotide fold II

This sequence also conforms to the criteria for a dinucleotide binding βαβ fold (Wierenga et al., 1985), but includes an unusually large loop region. The corresponding part of the SCNDI1 protein has already been suggested to form the binding site for the non-covalently attached FAD cofactor or the substrate NADH (de Vries et al., 1992).

Apolar/aromatic

Two regions characterized by conserved apolar and aromatic residues can be identified. The first lies immediately after dinucleotide fold I and consists almost exclusively of apolar and aromatic residues. It is less well conserved in the *E. coli* sequence. The second is characterized by several conserved charged residues flanking stretches of apolar and aromatic residues. Both regions may form a pocket for the interaction of ubiquinone with the reactive moieties of FAD and NADH.

YLNDH2 is the only alternative NADH:ubiquinone oxidoreductase gene in *Y. lipolytica*

Both primers used to clone *YLNDH2* correspond to regions highly conserved among alternative NADH:ubiquinone oxidoreductases and can thus be expected to anneal to all members of this gene family. Three independently obtained positive plasmid clones were shown to belong to the *YLNDH2* gene locus by DNA sequencing. We were also unable to detect homologues of the *YLNDH2* gene by Southern hybridization under low stringency conditions (data not shown). These data strongly suggest that there is only one alternative NADH:ubiquinone oxidoreductase gene present in the genome of *Y. lipolytica.*

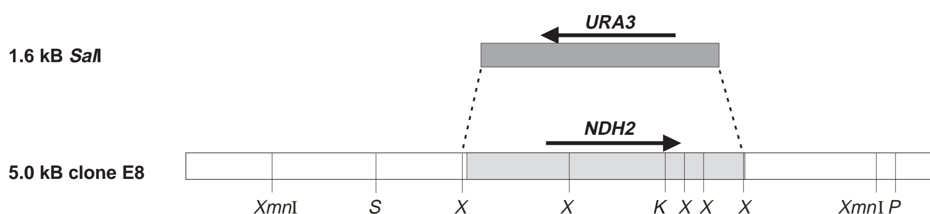
Deletion of *YLNDH2*

The *YLNDH2* gene was deleted by homologous recombination with a *URA3* marked deletion allele (Fig. 2). Deletion and cosegregation of the *URA3* marker with the *ylndh2*::*URA3* deletion allele was verified by Southern blot analysis (Fig. 3). Sporulation of the diploid GB4 strain revealed close to Mendelian segregation of all markers, with one exception: the *YLNDH2* locus seems to be closely linked to the mating type locus, since eight out of nine *ylndh2*::*URA3* spores tested were *Mat*B. Strains carrying the deletion of *YLNDH2* were found to be completely viable, since haploid parental and mutant strains showed comparable growth rates and yields on minimal and complete liquid media containing 10 g/l glucose or 4 g/l sodium acetate as carbon sources (data not shown).

Characterization of NADH:ubiquinone oxidoreductase activities in *Y. lipolytica*

Under substrate saturation conditions (Fig. 4), specific NADH:NBQ oxidoreductase activity in mutant membranes was about 30% of wild-type activity. In the parental strain NADH:NBQ oxidoreductase activity was reduced by 40% upon the addition of 2 μM piericidin A. In sharp contrast, the activity of mutant membranes could be completely inhibited

Construction of the *ylndh2*::*URA3* deletion allele



Deletion by homologous recombination

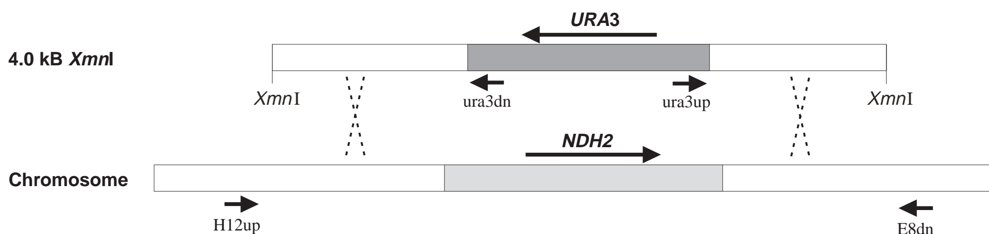


Fig. 2. Construction of the *ylndh2::URA3* deletion allele and gene deletion by homologous recombination. The *YLNDH2* ORF is shaded in gray. PCR primer pairs (H12up, ura3dn) and (ura3up, E8dn) were used to screen URA3+ colonies for marker insertion by homologous recombination. Restriction sites: K, *Kpn*I; P, *Pst*I; S, *Sac*I; X, *Xho*I; *Xmn*I, *Xmn*I.

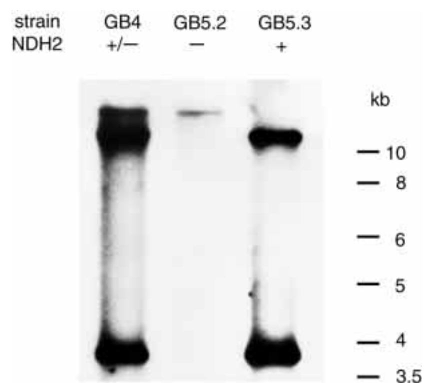


Fig. 3. Southern blot analysis of *YLNDH2* parental and deletion strains. Southern blot analysis of diploid strain GB4 (*ylndh2::URA3/YLNDH2*) and haploid strains GB5.2 (*ylndh2::URA3*) and GB5.3 (*YLNDH2*), derived from GB4 by sporulation. 1 μg of genomic DNA was loaded in each lane. The complete insert from clone E8 was used as a probe. Replacement by homologous recombination of the *YLNDH2* wild-type gene with the *URA3* marked deletion allele results in the loss of a *Kpn*I site within the *YLNDH2* ORF and the fusion of 12 and 3.75 kb *Kpn*I fragments into a novel 15.75 kb *Kpn*I fragment.

by this classical inhibitor of complex I. Inhibitor-insensitive NADH:NBQ oxidoreductase activity could be restored near to wild-type levels by introducing the *YLNDH2* gene on pINA240, a *LEU2* marked replicative plasmid carrying a chromosomal ARS/CEN and thus present in about one copy per cell (data not shown). These data demonstrate that the *YLNDH2* gene accounts for all alternative NADH:ubiquinone oxidoreductase activity of *Y. lipolytica*. It should be noted that some variation of the relative activities of complex I and alternative NADH:ubiquinone oxidoreductase was observed with different batches of membranes and may also reflect sensitive regulation of the expression of these enzymes in *Y. lipolytica*.

Since the presence of any additional NADH:ubiquinone oxidoreductase activity can be ruled out, a direct assessment of the K_M(NADH) values of complex I and NDH2 is possible (Fig. 5). Using unsealed membranes prepared from the mutant, a K_M(NADH) of 15±1 μM was determined for complex I. Conversely, after inhibition of complex I with 2 μM piericidin A, NDH2 is the only NADH:ubiquinone oxidoreductase active in unsealed membranes prepared from the parental strain. A very similar K_M(NADH) of 14±1 μM was found for NDH2. This similarity may be crucial for the maintenance of a balanced redox poise between the cytoplasm and the mitochondrial matrix.

We were unable to detect any NADPH:NBQ oxidoreductase activity, either in parental or mutant membranes (data not shown). No calcium-dependent NADH:ubiquinone oxidoreductase seems to be present: after addition of 5 mM Ca^{2+} to mutant membranes in which complex I had been inhibited by the addition of 5 μM piericidin A, no NADH:NBQ oxidoreductase activity was detectable (data not shown).

NDH2 is an external NADH:ubiquinone oxidoreductase

To test for the orientation of NADH:ubiquinone oxidoreductases, NADH:NBQ oxidoreductase activities were measured before and after permeabilization of intact mitochondria by the addition of 0.4% Chaps or 30 μM alamethicin, which form pores large enough to permit the rapid diffusion of NADH (Cafiso, 1994). To avoid the buildup of electrochemical gradients and allow the reaction to proceed at constant rate, CCCP and valinomycin were added as uncouplers. After permeabilization of membranes from the *YLNDH2* deficient mutant, a sevenfold increase of complex I activity was observed. As NADH has to enter the mitochondrial matrix to react with complex I, this indicates that more than 80% of the mitochondria were intact (Table 1). A very similar result was obtained for membranes prepared from the parental strain, in which complex I activity could be assayed selectively by using deamino-NADH as a substrate (Table 1) that is not oxidized by alternative NADH:ubiquinone oxidoreductases (Matsushita et al., 1987).

In stark contrast, NDH2 activity in membranes from the parental strain monitored as NADH:NBQ oxidoreductase activity in the presence of piericidin A was not affected by permeabilization of the mitochondrial membranes using two different methods (Table 1). These data clearly demonstrate that in *Y. lipolytica* the only alternative NADH:ubiquinone oxidoreductase NDH2 is oriented towards the external face of the mitochondrial inner membrane and that on the matrix side complex I is the only enzyme capable of directly passing electrons into the respiratory chain from NADH.

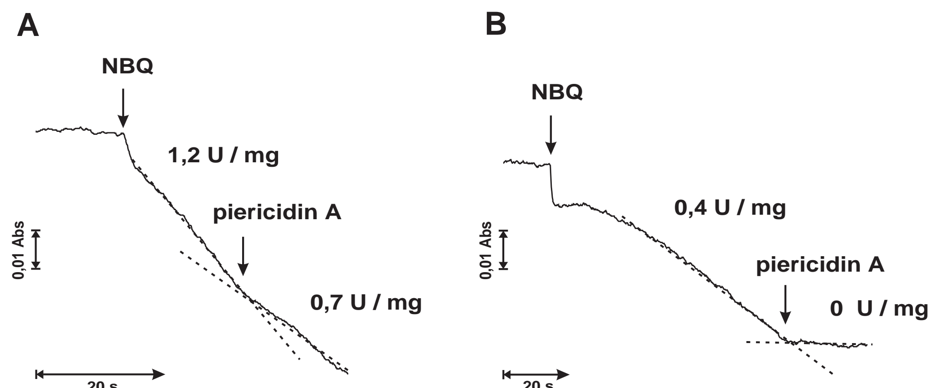


Fig. 4. NADH:ubiquinone oxidoreductase activities in *Y. lipolytica*. NADH:NBQ oxidoreductase activities of mitochondrial membranes (20 μg/ml total protein) were tested under conditions of substrate saturation (200 μM NADH, 60 μM NBQ). The reaction was started by the addition of 60 μM NBQ, piericidin A was added from a 1 mM solution in ethanol to a final concentration of 2 μM. In controls, addition of up to 10% ethanol had no effect on NADH:NBQ oxidoreductase activity (data not shown). (A) Parental strain E150. The reaction rate was inhibited by 40% upon addition of piericidin A; (B) *ylndh2::URA3* mutant GB5.2 Total NADH:NBQ oxidoreductase activity was only one third of the wild-type level. Addition of piericidin A led to complete inhibition.

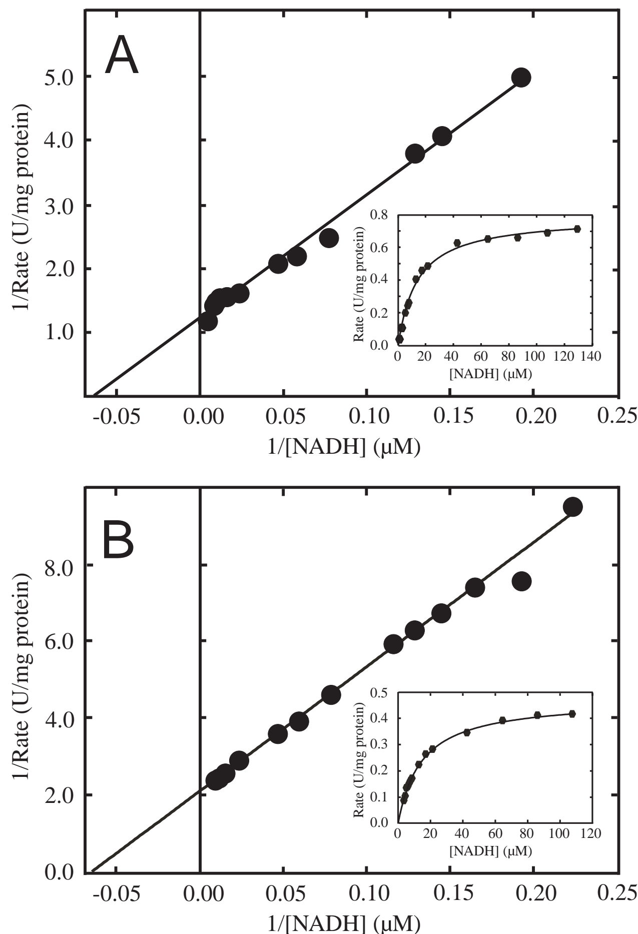


Fig. 5. Michaelis-Menten parameters for complex I and NDH2. Double reciprocal plots of Michaelis-Menten kinetics for NADH:NBQ oxidoreductase activities in unsealed mitochondrial membranes from *Y. lipolytica*. The inserts show a direct plot of the data. (A) NDH2, assayed using membranes from parental strain E150 in the presence of 2 µM piericidin A; K_M(NADH) = 15±1 µM, V_{max} = 0.79 U/mg; (B) Complex I, using membranes prepared from *ylndh2::URA3* mutant GB5.2; K_M(NADH) = 15±1 µM, V_{max} = 0.47 U/mg. For details of the assay, see Materials and methods.

DISCUSSION

We have established *Y. lipolytica* as a useful model to study the metabolic roles and structure/ function relationships of external NADH:ubiquinone oxidoreductase and complex I in a strictly aerobic fungus.

Our results provide conclusive evidence that NDH2 is the only alternative NADH:ubiquinone oxidoreductase in *Y. lipolytica*. The presence of homologous and non-homologous enzymes could be ruled out by the observation that mitochondrial membranes from a strain in which the *YLNDH2* gene was deleted were fully sensitive to piericidin A, an inhibitor specific for proton translocating NADH:ubiquinone oxidoreductase (complex I).

Since the *YLNDH2* deletion mutant was found to be fully viable when grown on glucose or acetate, NDH2 activity seems to be dispensable and yet other mechanisms for the oxidation of cytoplasmic NADH must also exist in *Y. lipolytica*. This situation is similar to *S. cerevisiae*, where, although a functional malate-aspartate shuttle, one of the major redox shuttles in mammalian mitochondria, is absent (Hollenberg et al., 1970), activities of the ethanol-acetaldehyde shuttle (von Jagow and Klingenberg, 1970) and of glycerol-3-phosphate dehydrogenase, a flavoprotein of the mitochondrial inner membrane (Ronnow and Kielland-Brandt, 1993; Bjorkqvist et al., 1997), have been demonstrated. Consequently, a *S. cerevisiae* strain carrying deletions of both external NADH:ubiquinone oxidoreductase genes was capable of respiratory growth on glucose at wild-type rate (Luttik et al., 1998). The finding that this double mutant had a slower growth rate on ethanol (Luttik et al., 1998) indicates that the turnover rate of such systems can be a limiting factor under certain growth conditions and may also constitute the basis for interspecific differences in metabolism. Further work is clearly needed to study the various systems which may feed cytoplasmic NADH into the mitochondrial respiratory chain in *Y. lipolytica* and clarify the identities and relative contributions of these seemingly redundant pathways.

Table 1. NADH:ubiquinone oxidoreductase activities in intact and permeabilized mitochondria from *Y. lipolytica*

Enzyme	Activity (U/mg) in mitochondria: As prepared	Activity (U/mg) in mitochondria: Permeabilized	Accessible activity (%)
Complex I[a]	0.19±0.07	1.15±0.16	17
Complex I[b]	0.13±0.07	0.90±0.12	14
NDH2[c]	0.55±0.02	0.51±0.01	approx. 100

[a]From parental strain E150; 200 µM deamino-NADH, 60 µM NBQ.
[b]From *ylndh2::URA3* mutant GB5.2; 200 µM NADH, 60 µM NBQ.
[c]From parental strain E150; 200 µM NADH, 60 µM NBQ, 2 µM piericidin A.
All tests were carried out in triplicate. For details of the assay, see Materials and methods.

We could demonstrate in intact mitochondria that NDH2 is oriented towards the external face of the mitochondrial inner membrane and that complex I is the only internal NADH:ubiquinone oxidoreductase in the strictly aerobic yeast *Y. lipolytica*. These findings are in contrast to both the situation found in *S. cerevisiae* and to current concepts about respiration-linked NADH oxidation in strictly aerobic fungi, and green plants.

In *S. cerevisiae*, which is adapted to alcoholic fermentation, complex I is absent and three NADH:ubiquinone oxidoreductase isogenes, *SCNDI1*, *YMR145c* and *YDL085w*, are found. Recently, it has been demonstrated by deletion analysis that the latter two enzymes are oriented towards the external face of the inner mitochondrial membrane (Luttik et al., 1998; Small and McAlister-Henn, 1998). In other fungi, e.g. *Neurospora crassa* and in plants, multiple internal and external NADH:ubiquinone oxidoreductase enzymes have been postulated (Weiss et al., 1970; von Jagow and Klingenberg, 1970; Soole and Menz, 1995; Rasmusson et al., 1998).

Analysis of the phylogenetic relationship of the alternative NADH:ubiquinone oxidoreductase proteins from *Y. lipolytica*, *S. cerevisiae*, *A. thaliana* and *E. coli* revealed that the SCNDI1 protein is evolutionarily more distant from the other three fungal enzymes (Fig. 6). It is tempting to speculate that the

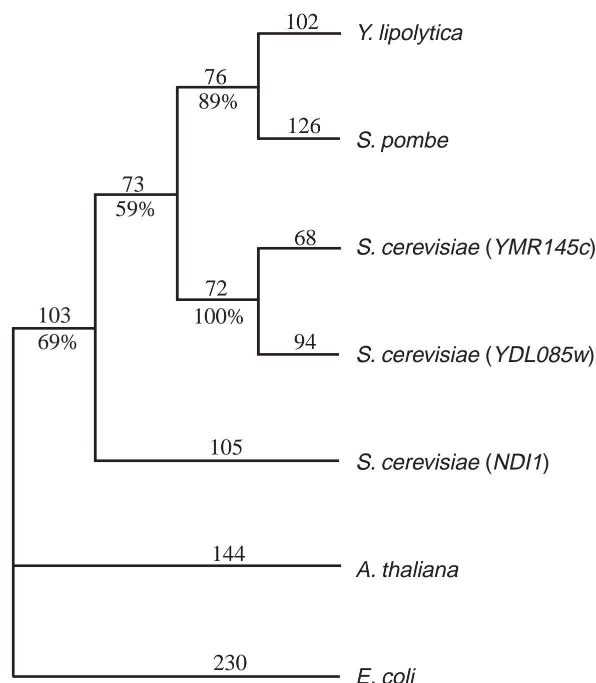


Fig. 6. Phylogenetic relationship of the alternative NADH:ubiquinone oxidoreductases from *Y. lipolytica, S. cerevisiae, A. thaliana* and *E. coli*. N-terminally truncated protein sequences, starting at the first dinucleotide fold, were aligned using the CLUSTAL program of the HUSAR 4.0 package, DKFZ Heidelberg, Germany. A 50% majority rule consensus tree was calculated using the PAUP 3.1.1 program (Swofford, 1992). The tree was rooted with basal polytomy using the *E. coli* sequence as outgroup. Bootstrap values (given as % below branches) indicate the percentage of trees in which a given subset of sequences was grouped together in 100 replicates; branch lengths are proportional to the number of substitutions (given above branches) from the hypothetical common ancestor sequence at the respective branch point.

common ancestor of fungal alternative NADH:ubiquinone oxidoreductases originally had an external orientation and that the internal form in *S. cerevisiae* emerged from divergent evolution following an early gene duplication event. This development had to involve the acquisition of a targeting signal for the mitochondrial matrix and may have paved the way to the loss of complex I in *S. cerevisiae*. It should be noted that targeting of different isoforms to opposite sides of the inner mitochondrial membrane is in itself a unique feature of the family of alternative NADH:ubiquinone oxidoreductases.

The similarity between the two external enzymes of *S. cerevisiae* is strikingly high and may point to a second, more recent gene duplication event. It remains to be determined whether multiple NADH:ubiquinone oxidoreductase gene copies are present in other ascomycetous yeasts, especially in fermentative species like *Schizosaccharomyces pombe* and *Kluyveromyces marxianus.* The *S. pombe* enzyme that was included in this analysis may, because of its close relatedness to the NDH2 protein and the two external enzymes from *S. cerevisiae*, be predicted to be an external enzyme as well.

The data presented here point to an unexpected diversity in the design of mitochondrial electron transport chains among these taxa and provide some interesting clues regarding the evolutionary origin of alternative NADH:ubiquinone oxidoreductase enzymes.

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