View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

ELSEVIER
Biochimica et Biophysica Acta 1459 (2000) 274–283
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.elsevier.com/locate/bba

Diversity and origin of alternative NADH:ubiquinone oxidoreductases

Stefan J. Kerscher *

Universitätsklinkum Frankfurt, Institut für Biochemie I, ZBC, Theodor-Stern-Kai 7, Haus 25B, D-60590 Frankfurt am Main, Germany

Received 16 May 2000; received in revised form 15 June 2000; accepted 15 June 2000

Abstract

Mitochondria from various organisms, especially plants, fungi and many bacteria contain so-called alternative NADH:ubiquinone oxidoreductases that catalyse the same redox reaction as respiratory chain complex I, but do not contribute to the generation of transmembrane proton gradients. In eucaryotes, these enzymes are associated with the mitochondrial inner membrane, with their NADH reaction site facing either the mitochondrial matrix (internal alternative NADH:ubiquinone oxidoreductases) or the cytoplasm (external alternative NADH:ubiquinone oxidoreductases). Some of these enzymes also accept NADPH as substrate, some require calcium for activity. In the past few years, the characterisation of several alternative NADH:ubiquinone oxidoreductases on the DNA and on the protein level, of substrate specificities, mitochondrial import and targeting to the mitochondrial inner membrane has greatly improved our understanding of these enzymes. The present review will, with an emphasis on yeast model systems, illuminate various aspects of the biochemistry of alternative NADH:ubiquinone oxidoreductases, address recent developments and discuss some of the questions still open in the field. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Alternative NADH:ubiquinone oxidoreductase; Alternative NADH dehydrogenase; Respiratory chain; Electron transport

1. Introduction

The phenomenon of alternative NADH:ubiquinone oxidoreductase activity was first observed in 1961, when Bonner and Voss [1] found that plant mitochondria, in contrast to mammalian mitochondria, were capable of oxidising externally added NAD(P)H. A similar activity was seen with mitochondria prepared from the yeast *Saccharomyces carlsbergensis* [2]. Since NADH oxidation was found to be completely insensitive to amytal and rotenone, it was concluded that complex I is absent in this species. This was confirmed by EPR studies demonstrating that mitochondria from *S. carlsbergensis*, in contrast to mitochondria from *Torulopsis* (formerly: *Candida*) *utilis*, lack the iron-sulfur clusters characteristic of respiratory chain complex I [3]. A careful analysis revealed that respiratory chain NADH:ubiquinone oxidoreductase activity of *S. carlsbergensis* is due to at least two different alternative enzymes, directed towards the outer and the inner face of the mitochondrial inner membrane, respectively [4]. In contrast, mitochondria from *Neurospora crassa* were found to possess external and internal alternative NADH:ubiquinone oxidoreductase activities and the rotenone sensitive NADH:ubiquinone oxidoreductase activity of respiratory chain complex I [5].

* Fax: +49 (69) 63016970;
E-mail: kerscher@zbc.klinik.uni-frankfurt.de

0005-2728/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0005-2728(00)00162-6

2. The diversity of alternative NADH:ubiquinone oxidoreductases

In recent years, DNA sequences for several alternative NADH:ubiquinone oxidoreductases genes have become available and several enzyme proteins have been purified and analysed in vitro. As already mentioned, alternative NADH:ubiquinone oxidoreductases have adopted an important metabolic function in yeasts of the genus *Saccharomyces*, which stands out among ascomycetous fungi by its adaptation to ethanolic fermentation. In species like *Saccharomyces cerevisiae*, *S. carlsbergensis* and *Kluyveromyces marxianus*, complex I is absent [6] and internal alternative NADH:ubiquinone oxidoreductase is the only enzyme capable of feeding NADH generated in the mitochondrial matrix into the respiratory chain. Considering its vital importance in this well studied model organism, it is not surprising that much of our knowledge concerning alternative NADH:ubiquinone oxidoreductase has been gathered in *S. cerevisiae* and in other ascomycetous fungi.

The internal alternative NADH:ubiquinone oxidoreductase from *S. cerevisiae* has been purified and characterised in vitro [7]. It consists of a single polypeptide with an apparent molecular mass of 53 kDa for the mature protein, which lacks the N-terminal 26 amino acids of the mitochondrial targeting sequence. It contains a single molecule of non-covalently bound FAD as redox prosthetic group. A deletion mutant in the *SCNDI1* gene encoding the internal alternative NADH:ubiquinone oxidoreductase from *S. cerevisiae* [8] was unable to grow on highly oxidised carbon sources like acetate or pyruvate but, surprisingly, did not display a petite phenotype, demonstrating that glucose metabolism was respiratory.

In addition to this internal alternative enzyme, two external alternative enzymes, SCNDE1 and SCNDE2, are present in *S. cerevisiae* [9]. In a genome-wide study of gene expression using DNA microarrays [10] it was found that expression of all three alternative NADH:ubiquinone oxidoreductase genes of *S. cerevisiae* is linked to the activation of mitochondrial respiration. *scnde1*Δ or *scnde1*Δ, *scnde2*Δ strains displayed reduced specific growth rates with ethanol as the sole carbon source [9].

The obligate aerobic yeast *Yarrowia lipolytica* has only one single alternative enzyme, encoded by the *YLNDH2* gene, and oriented towards the external face of the mitochondrial inner membrane [11]. *ylndh2*Δ strains failed to exhibit appreciable growth defects on various media.

A cDNA clone from *N. crassa*, encoding a 64 kDa alternative NADH:ubiquinone oxidoreductase, was found to contain an insertion related to Ca^{2+} binding EF-hand motifs [12].

Plant mitochondria contain complex I and up to four alternative NAD(P)H:ubiquinone oxidoreductases, which are associated with both faces of the mitochondrial inner membrane and display different induction kinetics and Ca^{2+} requirements [13]. Several authors have also speculated that alternative NADH:ubiquinone oxidoreductase activity of plant mitochondria could in part be due to assembly intermediates of complex I with rotenone-insensitive NADH oxidase activity [14]. The *Solanum tuberosum NDA* and *NDB* genes encode two alternative enzymes of potato tuber mitochondria, oriented towards the internal and external face of the mitochondrial inner membrane, respectively [15]. Like *N. crassa* p64, *S. tuberosum* NDB contains an insertion related to Ca^{2+} binding EF-hand motifs.

Dual pathways for respiratory chain NADH oxidation are also found in many bacteria, like *Escherichia coli*, which possesses a minimal form of complex I (called NDH1) encoded by the *nuo* operon [16] and an alternative NADH dehydrogenase (called NDH2) encoded by the *ndh* gene [17]. The *E. coli* NDH2 protein has been purified in native form [18,19] and, recently, also as an N-terminally His-tagged protein with much higher NADH:ubiquinone oxidoreductase activity [20].

Heterologous expression of alternative NADH: ubiquinone oxidoreductase proteins has been reported in two cases. SCNDI1 of *S. cerevisiae*, when expressed in *E. coli* with an N-terminally attached T7 tag was shown to be incorporated into the respiratory chain of the host cell [21]. SCNDI1 expressed in complex I deficient mammalian cells (Chinese hamster CCL16-B2 cells) was shown to restore respiratory chain NADH oxidation, allowing survival of the mutant cells [22]. These results lead the authors to conclude that 'the NDI1 gene provides a potentially useful tool for gene therapy of mitochondrial diseases caused by complex I deficiencies'.

3. Evolution of alternative NADH:ubiquinone oxidoreductases

Sequence similarity between alternative NADH: ubiquinone oxidoreductases and lipoamide dehydrogenases from various bacterial sources suggests common ancestry [20]. Both enzyme classes catalyse similar redox reactions, i.e. electron transfer from NADH to ubiquinone and from dihydrolipoamide to NADH, respectively, and contain one molecule of non-covalently bound FAD as redox prosthetic group. The major difference is the absence of a reactive cysteine pair, which is one of the hallmarks of the FAD dependent NAD(P)H (disulphide) oxidoreductase protein family. X-Ray structures are available for two bacterial lipoamide dehydrogenases from *Azotobacter vinelandii* [23] and from *Pseudomonas putida* [24] and may provide useful structural models.

Interestingly, the genome of the intracellular parasite *Rickettsia prowazekii* [25], which is believed to represent the closest eubacterial relative of mitochondria, does not contain a gene for an alternative NADH:ubiquinone oxidoreductase. This suggests that alternative NADH:ubiquinone oxidoreductases were most likely contributed by the nuclear genome and not by the endosymbiont.

It may be speculated that the eucaryotic alternative NADH:ubiquinone oxidoreductase initially had an external orientation. Species like *Y. lipolytica*, which has only one single external but no internal alternative NADH:ubiquinone oxidoreductase [11], may have conserved this original setup. Since different scenarios, including the loss of an internal enzyme in *Y. lipolytica* cannot be ruled out, this speculation clearly needs to be tested by the analysis of the alternative NADH:ubiquinone oxidoreductase complement of additional fungal species.

Subsequently, gene duplication and acquisition of a mitochondrial targeting sequence led to the recruitment of internal alternative NADH:ubiquinone oxidoreductase, as found in all other fungi and plants studied so far. In *S. cerevisiae*, there is good evidence for two consecutive gene duplication events, since the two external enzymes are much more closely related to each other than to the internal enzyme. The first event, leading to the separation of external and internal enzymes, may even have preceded speciation of the ascomycetous fungi. By phylogenetic analysis using the PAUP programme [26], the external enzymes from *S. cerevisiae* have been grouped together with the external enzyme from *Y. lipolytica* and a putatively external enzyme from *Schizosaccharomyces pombe* [11].

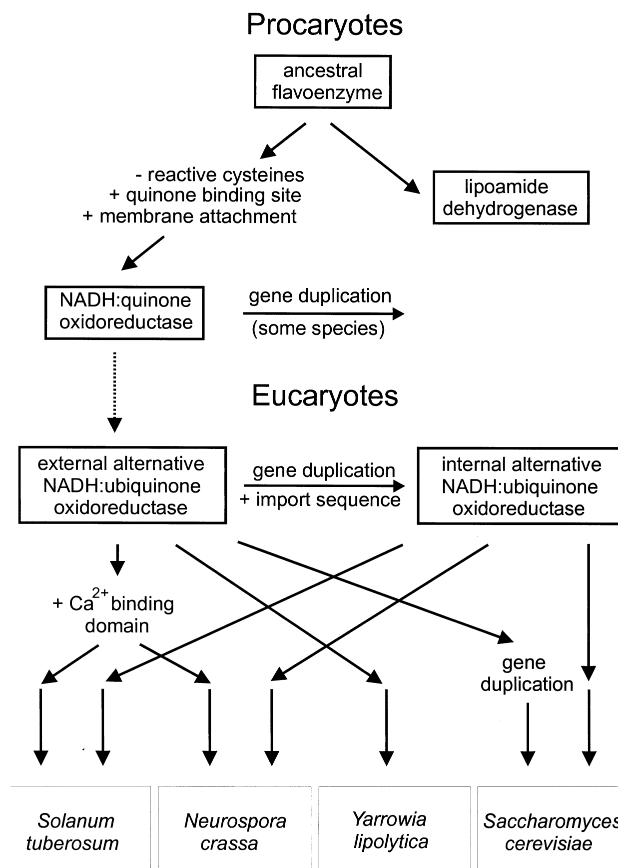

Fig. 1. Hypothetical scenario for the evolution of alternative NADH:ubiquinone oxidoreductases. See text for details.

Two alternative NADH:ubiquinone oxidoreductases, NDB from *S. tuberosum* [15], which was described as an external enzyme and p64 from *N. crassa* [12], which was described as an internal enzyme, were found to contain an insertion homologous to Ca^{2+} binding EF-hand motifs [27]. Under the assumption that acquisition of a mitochondrial import sequence and insertion of a Ca^{2+} binding EF-hand motif each occurred only once during evolution, the view that *N. crassa* p64 is an internal enzyme should be challenged (also see below). This hypothetical scenario for the evolution of alternative

NADH:ubiquinone oxidoreductases is summarised in Fig. 1.

Even some procaryotes, like the cyanobacterium *Synechocystis*, possess two gene copies for alternative NADH:ubiquinone oxidoreductases. One of these is more closely related to *Mycobacterium* homologues than to the other *Synechocystis* gene, suggesting that gene duplication and possibly functional specification occurred very early, probably even before the separation of the two species [15].

Recently, it was found that alternative NADH:ubiquinone oxidoreductase activity is also present in some archeae (Teixeira et al., personal communication). The thermoacidophilic archaeon *Acidianus ambivalens* which grows optimally at 82°C and pH 2.0 contains a very simplified respiratory chain. Its only terminal oxidase is an aa_3-type quinone oxidase which receives electrons from caldariella quinone. The quinone pool is reduced by succinate dehydrogenase and a 42 kDa NADH:quinone oxidoreductase which lacks EPR detectable iron-sulfur clusters. This is the first example of an alternative NADH:quinone oxidoreductase which has the flavin cofactor covalenty attached, presumably as an adaptation to the thermophilic environment. Evaluation of the question of common ancestry with eubacterial and eucaryotic enzymes will have to await the determination of its complete sequence.

4. Conserved sequence motifs

All alternative NADH:ubiquinone oxidoreductase proteins examined so far possess two regions that meet most of the criteria for a dinucleotide binding βαβ fold domain [28]. One of these lies very close to the N-terminus, the second one lies about 130–145 amino acids further downstream. Both these regions may form the binding site for the non-covalently attached FAD cofactor or the substrate NADH. Their identity is at present unclear, but based on the finding that an N-terminal attachment of an oligo-histidine stretch to the *E. coli* NDH2 protein stabilised the binding of the FAD cofactor, it was speculated that the first βαβ fold domain represents the FAD binding site [20]. This assumption is also supported by the homology of alternative NADH:ubiquinone oxidoreductases with lipoamide dehydrogenases. The structure of the latter enzyme with FAD bound to the first dinucleotide binding βαβ fold domain has been solved by X-ray crystallography [23,24].

Characteristic deviations from the βαβ fold consensus are found for both regions in some of the alternative NADH:ubiquinone oxidoreductase homologues (see Fig. 2). In the first region, the last of three highly conserved glycine residues is replaced by an alanine or serine residue in all the proteins from the ascomycetous fungi *S. cerevisiae*, *Y. lipolytica* and *N. crassa*, but not in the proteins from the plants *S. tuberosum* and *Arabidopsis thaliana*. A striking feature of this region of the *E. coli* NDH2 protein is the insertion of a highly basic stretch of amino acids (RKKKAK). A somewhat similar patch of basic residues is found immediately upstream from the first βαβ fold domain in all proteins from fungi and plants. Although the function of these basic amino acids is unclear, it is tempting to speculate that they might be necessary to stabilise the binding of the cofactor FAD. The second βαβ fold domain includes an unusually large loop region. Again, the significance of this feature is unclear.

Two alternative NADH:ubiquinone oxidoreductase proteins, NDB from *S. tuberosum* [15] and p64 from *N. crassa* [12], contain insertions with homology to Ca^{2+} binding EF-hand motifs [27]. The possible function of this domain in membrane association is discussed in the next section. The fact that both insertions reside in similar positions within the proteins makes it most likely that they originated from one single evolutionary event, although a direct alignment of these two insertions fails to reveal significant sequence identity. Apparently, the original insertion comprised two consecutive Ca^{2+} binding EF-hand motifs, of which only the first is well conserved in *S. tuberosum* NDB and only the second is well conserved in *N. crassa* p64 (see Fig. 2).

The mode of interaction with the hydrophobic substrate ubiquinone and the nature and localisation of the quinone binding site are unknown. Consensus patterns for quinone binding sites proposed by Rich and Fisher [29] cannot be found in alternative NADH:ubiquinone oxidoreductases. A tryptophan (W337 in the SCNDI1 precursor) that is conserved between all known alternative NADH:ubiquinone

Dinucleotide fold I

```
                                  βαβ
Y. lipolytica  (NDH2)                     KKTLVVLGSGWGSVSFLKKLDTSN.....YNVIVVSPR
S. cerevisiae  (NDE1)                    KRKTLVILGSGWGSVSLLKNLDTTL.....YNVVVVSPR
S. cerevisiae  (NDE2)                    KKKELVILGTGWGAISLLKKLDTSL.....YNVTVVSPR
S. cerevisiae  (NDI1)                     KPNVLILGSGWGAISFLKHIDTKK.....YNVSIISPR
S. tuberosum   (NDA)                      KPRIVVLGSGWAGCRLMKDIDTNI.....YDVVCVSPR
S. tuberosum   (NDB)                     KKKKVVVLGTGWGGTSFLKDVDISS.....YDVQVVSPR
N. crassa      (p64)                 KKKHKEKPRLVILGGGWGSVALLKELNPDD.....YHVTVVSPA
E. coli        (NDH2)                     TKKIVIVGGGAGGLEMATQLGHKLGRKKKAKITLVDRN

consensus                                 KK△□V□LG△GWG □S□LK△LDT△       Y△V VVSPR
dinucleotide fold fingerprint              △□ □ G G  G   □  □           □ □ ⊖
```

Dinucleotide fold II

```
                                  βαβ
Y. lipolytica  (NDH2)              HTVVVGGGPTGVEFAAELQDFFEDDLRKWIPDIR.DDFKVTLVEA
S. cerevisiae  (NDE1)              SFVVVGGGPTGVEFAAELRDYVDQDLRKWMPELS.KEIKVTLVEA
S. cerevisiae  (NDE2)              TFVVVGGGPTGVEFAAELQDYINQDLRKWMPDLS.KEMKVILIEA
S. cerevisiae  (NDI1)              SIVVVGGGPTGVEAAGELQDYVHQDLRKFLPALA.EEVQIHLVEA
S. tuberosum   (NDA)               HCVVVGGGPTGVEFSGELSDFILKDVHQRYAHVK.DYIHVTLIEA
S. tuberosum   (NDB)               HFVIVGGGPTGVEFAAELHDYVYEDLVKIYPSVK.DFVKITVIQS
N. crassa      (p64)               SFVVCGGGPTGVEFAAELFDLLNEDLTLHFPRLLRNEISVHLIQS
E. coli        (NDH2)              NIAIVGGGATGVELSAELHNAVKQLHSYGYKGLTNEALNVTLVEA

consensus                          △□VVVGGGPTGVEFAAEL△D■□△△DL△K□□P△□△ △⊖□△□TL□EA
dinucleotide fold fingerprint      △□ □ G G  G   □  □                    □ □ ⊖
```

Insertions with homology to Ca^{2+} binding EF-hand motifs

```
                  *  * **  **   **
S. tuber.   MEDISTIFEAADKDDSGTLSVEEFR-----DVLEDIIIRYPQVDLYLKNK--HLLEAKDLFRDSEGNEREEVDIEGFKLALSHVDSQMK
N. crassa   IGDCSTIQNNVADHIITFLRNLAWKHGKDPESLELHFSDWRDVAQQIKKRFPQATHLKRLDKLFEEYDKDQNGTLDFGELRELLKQIDS
                                                                                 *  * **  **   **
```

Fig. 2. Sequence motifs conserved between alternative NADH:ubiquinone oxidoreductases from various organisms. The consensus sequence of the two dinucleotide fold motifs is compared to the fingerprint defined in [28]. Residues identical in at least five out of eight sequences are indicated in one letter code, functionally similar residues are marked with the following symbols: open squares, hydrophobic; filled squares, aromatic; triangles, hydrophilic; +, basic; −, acidic. Shaded in grey 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. In the aligned insertions found in *S. tuberosum* NDB and *N. crassa* p64, residues conforming to the Ca^{2+} binding EF-hand pattern D-x-[DNS]-{ILVFYW}-[DENSTG]-[DNQGHRK]-{GP}-[LIVMC]-[DENQSTAGC]-x(2)-[DE]-[LIVMFYW] as defined in the PROSITE database, are indicated by asterisks. Conserved residues are shaded in grey.

oxidoreductases has, by analogy with the bacterial photoreaction centre [30,31], been proposed to be involved in ubiquinone binding [32].

Transmembrane helices have not been detected in the alternative NADH:ubiquinone oxidoreductases known so far. A segment of the *E. coli NDH2* protein was found to show a certain degree of homology to a segment of (*S*)-mandelate dehydrogenase from *P. putida* [33] which, based on mutagenesis studies, had been suggested to form the membrane attachment domain of the latter enzyme [34]. However, since this segment is not well conserved among various alternative NADH:ubiquinone oxidoreductases, this suggestion was in the following deemed less likely by the same authors [20]. It is unclear, therefore, how these proteins interact with biomembranes (also see below).

5. Substrates and inhibitors

The substrate specificities of alternative NADH:ubiquinone oxidoreductases have been studied for *S. cerevisiae* NDI1 [7], *E. coli* NDH2 [20] and *Y. lipolytica* NDH2 [11]. Their specificity for NADH contrasts with the much broader range of substrates accepted by respiratory chain complex I. In particular, the deamino analogue of NADH is not oxidised [35] and is therefore useful for specifically assaying complex I activity in the presence of alternative enzymes [11]. Also, NADPH is not accepted as a substrate by any of the three enzymes. No in vitro activity tests have been published for the proteins that contain Ca^{2+} binding EF-hand domains, namely NDB from *S. tuberosum* and p64 from *N. crassa*. It is tempting to speculate that these Ca^{2+} sensitive

enzymes confer NADPH dehydrogenase activity, although this speculation was discounted by Rasmusson et al. [15], based on the observation that acidic residues are present in the C-terminal part of the NDA and NDB motifs, downstream from the variable loop region, indicating that these proteins are unable to bind NADPH. Another possibility that cannot be ruled out at present would be that the oxidation of NADPH, which was historically the first alternative NAD(P)H activity to be discovered [1], is carried out by enzymes that are not homologous to the alternative NADH:ubiquinone oxidoreductases known so far.

Alternative NADH:ubiquinone oxidoreductases accept a broad range of quinone substrates. The SCNDI1 protein can use the natural substrate ubiquinone-6 (UQ-6), but ubiquinone analogues with shorter isoprenoid side chains, such as UQ-2, give much higher catalytic rates. No substrate inhibition can be observed with UQ-2, in contrast to bovine complex I [36]. EPR studies performed between 6 and 100 K failed to detect reaction intermediates like a semiquinone or a FADH radical [7]. The *E. coli* NDH2 protein, when overexpressed with an N-terminally attached His tag, is highly reactive with hydrophobic quinones such as *n*-decyl-ubiquinone (DBQ), hydroxydecyl-ubiquinone (idebenone), UQ-1 and UQ-2. The turnover rate with the more hydrophilic quinone analogue duroquinone and the artificial electron acceptors DCIP and ferrycyanide is very low. These studies demonstrate that the quinone reduction site of *E. coli* NDH2 is not easily accessible to water-soluble acceptors [20].

Inhibitors for alternative NADH:ubiquinone oxidoreductases are rare and mostly unspecific. It is a well established fact that classical inhibitors of complex I do not inhibit the alternative enzyme. Flavone, which inhibits SCNDI1 with an I_{50} of 95 μM [7], is known to inhibit other NADH dehydrogenases as well [37]. A study on the inhibitory effect of hydroxyflavones on the external alternative NADH:ubiquinone oxidoreductase of plants [38] revealed that platanetin (6-dimethylallyl-3,5,7,8-tetrahydroxyflavone) is a potent inhibitor of this enzyme ($I_{50} = 2$ μM). It remains unclear, however, whether specificity for the external alternative enzyme is caused by selective affinity or by the inability of platanetin to permeate across the inner membrane of the intact potato tuber mitochondria used in this study. Thus, additional specific inhibitors would certainly be valuable tools for further studies, especially on the quinone binding sites of alternative NADH:ubiquinone oxidoreductases.

6. Membrane association and import into mitochondria

Mitochondrial import of the SCNDI1 protein has been studied in detail [32]. It involves the cleavage of a 26 amino acid targeting sequence, probably by the matrix processing peptidase and is dependent on the membrane potential component of the proton-motive force. Processing also occurs during mitochondrial import of the internal NDA protein of *S. tuberosum*, although the cleavage site has not been determined [15]. These results indicate that the import pathway of this internal alternative NADH:ubiquinone oxidoreductase is very similar to the import of subunits of respiratory chain complexes [39]. How and when the FAD redox prosthetic group is inserted, is unknown.

Much less is known about the targeting of external alternative NADH:ubiquinone oxidoreductases to the outer face of the inner mitochondrial membrane. Targeting of the external *S. tuberosum* NDB protein does not seem to involve removal of a presequence, since no size difference was observed between the precursor and the mature protein [15]. On the other hand, preliminary data on the purification and N-terminal protein sequence of the SCNDE1 protein indicate that this external alternative NADH:ubiquinone oxidoreductase is processed by the removal of the first 41 amino acids [9]. This difference may be related to the fact that both external enzymes from *S. cerevisiae* possess N-terminal extension that exceed the length of the SCNDI1 presequence by 30 (SCNDE1) and 45 (SCNDE2) amino acid residues, respectively. No data are available regarding the targeting of external alternative NADH:ubiquinone oxidoreductases from other sources, such as *Y. lipolytica* and *N. crassa*.

Association of alternative NADH:ubiquinone oxidoreductases with the mitochondrial inner membrane may involve the C-terminal part of the protein, which interestingly is not conserved between these

enzymes and the water-soluble lipoamide dehydrogenases from bacteria. *S. tuberosum* NDA, expressed in *E. coli* with an N-terminally attached S tag, became unable to bind to biomembranes when the C-terminal part was lost by proteolytic degradation [15].

A different mode of membrane association may occur in the alternative NADH:ubiquinone oxidoreductases that contain Ca^{2+} binding EF-hand motifs. It has been proposed that Ca^{2+} binding facilitates association with the mitochondrial inner membrane, thereby regulating the activity of the enzyme [15]. Interestingly, of the two alternative NADH:ubiquinone oxidoreductases containing Ca^{2+} binding EF-hand motifs known so far, *S. tuberosum* NDB was described as an external and *N. crassa* p64 as an internal enzyme. This is in conflict with the assumption of evolutionary parsimony and requires further attention. The claim that *N. crassa* p64 is an internal enzyme is based on the following observations: p64 is resistant to protease K, both in intact mitochondria and in mitoplasts resulting from hypotonic swelling, which is assumed to destroy the integrity of the outer membrane only. During incubation with increasing concentrations of digitonin, resulting in sequential solubilisation of the outer and inner mitochondrial membranes, the pattern of release of the p64 protein paralleled that of the 20.8 and 30.4 kDa subunits of respiratory chain complex I [12].

The external localisation of NDB was demonstrated by an experiment using the water-soluble cross-linker 3,3′-dithiobis-sulphosuccinimidylpropionate (DTSSP), which is unable to penetrate the inner mitochondrial membrane and can only react with proteins exposed to the internal medium or the intermembrane space. NDA and NDB were synthesised in vitro by transcription and translation in the presence of [^{35}S]methionine, followed by a mitochondrial import assay, reisolation of the mitochondria and cross-linking with DTSSP. NDB, in contrast to NDA, was accessible to DTSSP in these experiments. Imported NDB was, however, resistant to protease K treatment and digitonin extraction [15]. These findings suggest that such behaviour does prove tight association with the inner mitochondrial membrane, but not internal localisation.

7. Metabolic function

7.1. NAD(P)H metabolism in yeasts

Little is known about the metabolic function of external and internal alternative NADH:ubiquinone oxidoreductases, and the mechanisms underlying their metabolic regulation. It is anticipated that ascomycetous yeasts will provide the system of choice for the analysis of this question since NADH and NADPH in the cytoplasm and NADH in the mitochondrial matrix can be assumed to form separate metabolic pools for the following reasons: transhydrogenase is known to be absent in *S. cerevisiae* [40]. Recently, we found that loss of function mutations in complex I are lethal in *Y. lipolytica* which has only one external, but no internal alternative NADH:ubiquinone oxidoreductase (Kerscher et al., in preparation). This finding strongly suggests that NADH shuttle systems, such as an ethanol/acetaldehyde shuttle [4], that were proposed to substitute for the mammalian malate/aspartate shuttle which is missing in fungi [41], are either absent or inadequate in this yeast (also see below).

7.2. External alternative NADH:ubiquinone oxidoreductases

External enzymes are obviously involved in feeding electrons from NADH generated in the cytoplasm into the respiratory chain. It should be stressed that in *S. cerevisiae*, where ethanolic fermentation is the preferred mode of glucose metabolism [42], fully respiratory growth can only be observed when the cultures are kept below the so-called critical specific growth rate (μ_{crit}). However, from the finding that growth of *scnde1*Δ or *scnde1*Δ, *scnde2*Δ strains was fully respiratory in such glucose-limited chemostat cultures [9], it appears that external alternative NADH:ubiquinone oxidoreductase is only one out of several redundant pathways fulfilling this role in metabolism. This is also the reason why *scnde1*Δ, *scnde2*Δ and even *scndi1*Δ, *scnde1*Δ, *scnde2*Δ strains do not display a petite phenotype on glucose containing solid media (Kötter, personal communication).

Potential pathways include the hypothetical etha-

nol/acetaldehyde shuttle [4], lactate:cytochrome *c* oxidoreductase [43] and the glycerol-3-phosphate dehydrogenase system [44,45]. The hypothetical ethanol/acetaldehyde shuttle is the only one of these mechanisms that can be envisaged as a real shuttle, capable of exchanging reduction equivalents in the form of NADH in both directions between the cytoplasm and the mitochondrial matrix. However, the lethality of loss of function mutations in genes for nuclear coded subunits of complex I of *Y. lipolytica* (Kerscher et al., in preparation) which has only an external, but no internal alternative NADH:ubiquinone oxidoreductase [11], argues against its operation, at least in this organism. If a shuttle system was active, it should allow NADH transfer to the cytoplasm, where it could be fed into the respiratory chain by the action of external alternative NADH:ubiquinone oxidoreductase. In sharp contrast, loss of function mutations in complex I of *N. crassa* [46], generated by homologous recombination with genetically marked deletion alleles or by RIP mutagenesis [47], is viable due to the presence of internal alternative NADH:ubiquinone oxidoreductase activity.

The glycerol-3-phosphate dehydrogenase system of *S. cerevisiae* consists of a soluble NAD-dependent glycerol-3-phosphate dehydrogenase, encoded by the *GPD1* gene and a FAD-dependent glycerol-3-phosphate dehydrogenase located on the outer face of the inner mitochondrial membrane and encoded by the *GUT2* gene. Although the shuttle was used extensively in wild type cells grown in aerobic batch cultures with reduced substrates such as ethanol, growth rate and biomass yield of *gut2*Δ mutants were unaffected, even when grown on ethanol [44]. This finding demonstrates that external alternative NADH:ubiquinone oxidoreductase activity can fully substitute for the glycerol-3-phosphate dehydrogenase system. However, when grown in aerobic batch cultures with glucose or ethanol as carbon source, *gut2Δ* mutants did secrete glycerol into the culture medium, a phenomenon which is characteristic for anaerobic growth of wild type cells. In anaerobic cultures, the excretion of reduced metabolites, like ethanol and glycerol, is the only way to rid the cell of the reduction equivalents generated during glycolysis. Since the combination of anaerobic glycolysis and ethanol production is redox neutral, glycerol production by dephosphorylation of glycerol 3-phosphate is the only way to rid the cell of reduction equivalents. The analysis of glycerol-3-phosphate dehydrogenase mutants has clearly demonstrated the necessity of glycerol formation as a redox sink for *S. cerevisiae* under anaerobic conditions [45].

*scnde1*Δ or *scnde1*Δ, *scnde2*Δ mutant strains of *S. cerevisiae* showed reduced specific growth rates for respiratory growth on ethanol [9]. This demonstrates that external alternative NADH:ubiquinone oxidoreductase activity is the most important pathway for the reoxidation of cytoplasmic NADH under aerobic conditions and that SCNDE1 is the more important one of the two external alternative enzymes. Also, biomass yield of the deletion strains was lowered by about 10% compared to the parental strain. This indicates that rerouting of the oxidation of cytoplasmic NADH via other pathways results in lower energetic efficiency.

In contrast, the specific growth rate of a *ylndh2*Δ strain of *Y. lipolytica* was not markedly reduced (Kerscher et al., in preparation). This difference could mean that ethanol oxidation in *Y. lipolytica* occurs preferentially in the mitochondrial matrix, or that the relative contribution of external alternative NADH:ubiquinone oxidoreductase to the reoxidation of cytoplasmic NADH is lower in this species.

7.3. *Internal alternative NADH:ubiquinone oxidoreductases*

Internal alternative enzymes may compete with complex I for the substrates NADH and ubiquinone. Respiratory chain electron transport via an internal alternative enzyme versus via complex I will results in a lower P/O ratio, but the possibility to build a functional electron transport chain by the expression of a single polypeptide instead of the at least 35 subunits of complex I may be advantageous under conditions where the carbon source is abundant and rapid growth is essential. Consistent with this hypothesis, it was found that the activities of the two types of NADH:ubiquinone oxidoreductase are under metabolic control. In *T. utilis* [48] and in *N. crassa* [49], the alternative enzyme acts as the major respiratory chain NADH dehydrogenase during exponential phase and complex I during stationary phase, respectively.

In fermentative yeasts, alternative NADH:ubiquinone oxidoreductases have acquired a special function. Since glucose is preferentially metabolised via anaerobic glycolysis and ethanolic fermentation, the expression of respiratory chain enzymes is repressed by high levels of glucose in the culture medium. Reduced significance of oxidative phosphorylation for ATP generation led to the loss of respiratory complex I, made possible by the possession of internal alternative NADH:ubiquinone oxidoreductase. In *S. cerevisiae*, SCNDI1 is the only enzyme capable of feeding NADH generated in the mitochondrial matrix into the respiratory chain. Consistent with its role as the metabolic equivalent of complex I, expression of *SCNDI1* is subject to catabolite repression with glucose [7]. A deletion mutant of the *SCNDI1* gene [8] has essentially the same phenotype as a deletion mutant in the *MDH1* gene for mitochondrial malate dehydrogenase [50]. Inability to use the tricarboxylic acid cycle for ATP generation eliminates growth on acetate but not on glucose or ethanol. This finding demonstrates that enough ATP to permit growth can be generated by feeding NADH derived from the reactions catalysed by alcohol and aldehyde dehydrogenases into the respiratory chain, mainly via the external alternative NADH:ubiquinone oxidoreductases [7].

8. Conclusion

Although a large amount of new information concerning alternative NADH:ubiquinone oxidoreductases has been gathered in recent years, many questions are still open. These include the way how these largely hydrophilic proteins associate with biological membranes, the interaction with their hydrophobic quinone substrates and their function in metabolism. Since the genes encoding alternative NADH:ubiquinone oxidoreductases have become available in several genetic model systems, these questions are now amenable for experimentation. The small alternative NADH dehydrogenase proteins should also provide useful model systems to investigate the interaction of respiratory chain enzymes with ubiquinone in general.

Acknowledgements

I would like to thank Ulrich Brandt and Volker Zickermann for critically reading the manuscript and many helpful discussions.

References

[1] W.D. Bonner, D.O. Voss, Nature 191 (1961) 682–684.
[2] T. Ohnishi, K. Kawaguchi, B. Hagihara, J. Biol. Chem. 241 (1966) 1797–1806.
[3] T. Ohnishi, Biochim. Biophys. Acta 301 (1973) 105–128.
[4] G. von Jagow, M.E. Klingenberg, Eur. J. Biochem. 12 (1970) 583–592.
[5] H. Weiss, G. von Jagow, M.E. Klingenberg, T. Bücher, Eur. J. Biochem. 14 (1970) 75–82.
[6] R. Büschges, G. Bahrenberg, M. Zimmermann, K. Wolf, Yeast 10 (1994) 475–479.
[7] S. de Vries, L.A. Grivell, Eur. J. Biochem. 176 (1988) 377–384.
[8] C.A.M. Marres, S. de Vries, L.A. Grivell, Eur. J. Biochem. 195 (1991) 857–862.
[9] M.A.H. Luttik, K.M. Overkamp, P. Kötter, S. de Vries, P. van Dijken, J.T. Pronk, J. Biol. Chem. 273 (1998) 24529–24534.
[10] J.L. DeRisi, V.R. Iyer, P.O. Brown, Science 278 (1997) 680–686.
[11] S. Kerscher, J.G. Okun, U. Brandt, J. Cell Sci. 112 (1999) 2347–2354.
[12] A.M. Melo, M. Duarte, A. Videira, Biochim. Biophys. Acta 1412 (1999) 282–287.
[13] A.G. Rasmusson, V. Heiser, E. Zabaleta, A. Brennicke, L. Grohmann, Biochim. Biophys. Acta 1364 (1998) 101–111.
[14] K.L. Soole, R.I. Menz, J. Bioenerg. Biomembr. 27 (1995) 397–406.
[15] A.G. Rasmusson, A.S. Svensson, V. Knoop, L. Grohmann, A. Brennicke, Plant J. 20 (1999) 79–87.
[16] U. Weidner, S. Geier, A. Ptock, T. Friedrich, H. Leif, H. Weiss, J. Mol. Biol. 233 (1993) 109–122.
[17] I.G. Young, B.L. Rogers, H.D. Campbell, A. Jaworowski, D.C. Shaw, Eur. J. Biochem. 116 (1981) 165–170.
[18] A. Jaworowski, H.D. Campbell, M.I. Poulis, I.G. Young, Biochemistry 20 (1981) 2041–2047.
[19] A. Jaworowski, G. Mayo, D.C. Shaw, H.D. Campbell, I.G. Young, Biochemistry 20 (1981) 3621–3628.
[20] K. Björklöf, V. Zickermann, M. Finel, FEBS Lett. 467 (2000) 105–110.
[21] T. Kitajima-Ihara, T. Yagi, FEBS Lett. 421 (1998) 37–40.
[22] B.B. Seo, T. Kitajima-Ihara, E.K.L. Chan, I.E. Scheffler, A. Matsuno-Yagi, T. Yagi, Proc. Natl. Acad. Sci. USA 95 (1998) 9167–9171.
[23] A. Mattevi, A.J. Schierbeek, W.G.J. Hol, J. Mol. Biol. 220 (1991) 975–994.

[24] A. Mattevi, G. Obmolova, J.R. Sokatch, C. Betzel, W.G.J. Hol, Proteins Struct. Funct. Genet. 13 (1992) 336–351.
[25] S.G.E. Andersson, A. Zomorodipour, J.O. Andersson, T. Sicheritz-Pontén, U.C.M. Alsmark, R.M. Podowski, C.G. Kurland, A.-S. Eriksson, H.H. Winkler, C.G. Kurland, Nature 396 (1998) 133–140.
[26] D.L. Swofford, PAUP: Phylogenetic Analysis Using Parsimony 3.1.1, 1992.
[27] B.J. Marsden, G.S. Shaw, B.D. Sykes, Biochem. Cell Biol. 68 (1990) 587–601.
[28] R.K. Wierenga, M.C.H. de Maeyer, W.G.J. Hol, Biochemistry 24 (1985) 1346–1357.
[29] P. Rich, N. Fisher, Biochem. Soc. Trans. 27 (1999) 561–565.
[30] J. Deisenhofer, H. Michel, Science 245 (1989) 1463–1473.
[31] J. Deisenhofer, H. Michel, Annu. Rev. Cell Biol. 7 (1991) 1–23.
[32] S. de Vries, R. van Witzenburg, L.A. Grivell, C.A.M. Marres, Eur. J. Biochem. 203 (1992) 587–592.
[33] M. Finel, FEBS Lett. 393 (1996) 81–85.
[34] B. Mitra, J.A. Gerlt, P.C. Babbitt, C.W. Koo, G.L. Kenyon, D. Joseph, G.A. Petsko, Biochemistry 32 (1993) 12959–12967.
[35] K. Matsushita, T. Ohnishi, H.R. Kaback, Biochemistry 26 (1987) 7732–7737.
[36] G. Lenaz, Biochim. Biophys. Acta 1364 (1998) 207–221.
[37] T.P. Singer, Methods Enzymol. 55 (1979) 454–462.
[38] P. Ravanel, S. Creuzet, M. Tissut, Phytochemistry 29 (1990) 441–445.
[39] F.U. Hartl, N. Pfanner, D.W. Nicholson, W. Neupert, Biochim. Biophys. Acta 988 (1989) 1–45.
[40] R. Lagunas, J.M. Gancedo, Eur. J. Biochem. 37 (1973) 90–94.
[41] C.P. Hollenberg, W.F. Riks, P. Borst, Biochim. Biophys. Acta 201 (1970) 13–19.
[42] R. Lagunas, Yeast 2 (1986) 221–228.
[43] S. de Vries, C.A.M. Marres, Biochim. Biophys. Acta 895 (1987) 205–239.
[44] C. Larsson, I.L. Pahlman, R. Ansell, M. Rigoulet, L. Adler, L. Gustafsson, Yeast 14 (1998) 347–357.
[45] S. Bjorkqvist, R. Ansell, L. Adler, G. Liden, Appl. Environ. Microbiol. 63 (1997) 128–132.
[46] A. Videira, Biochim. Biophys. Acta 1364 (1998) 89–100.
[47] E.U. Selker, P.W. Garrett, Proc. Natl. Acad. Sci. USA 85 (1988) 6870–6874.
[48] R. Katz, L. Kilpatrick, B. Chance, Eur. J. Biochem. 21 (1971) 301–307.
[49] J.P. Schwitzguebel, J.M. Palmer, J. Bacteriol. 149 (1982) 612–619.
[50] L. McAlister-Henn, L.M. Thompson, J. Bacteriol. 169 (1987) 5157–5166.