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New Insights into Type II NAD(P)H:Quinone Oxidoreductases

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INTRODUCTION

The electron transport chains of cytoplasmic membranes from bacteria and archaea, or of inner mitochondrial membranes, are the energetic factories of respiratory organisms. The respiratory chains catalyze the electron transfer from reduced substrates, such as NADH or succinate, to oxygen or other oxidizing compounds, such as fumarate or nitrate, in aerobic and anaerobic (or facultative) organisms, respectively. Coupled with the downhill electron transfer, there is the uphill proton or, in some cases, sodium translocation across the membranes, with the concomitant generation of a membrane potential that enables ATP synthase to synthesize ATP from ADP and P_i (25, 69).

In the respiratory chain of mammalian mitochondria, the paradigm of aerobic respiratory chains, there are five main inner membrane complexes engaged in energy production. The NADH:quinone oxidoreductase, also called rotenone-sensitive NADH dehydrogenase (complex I) (NDH-1), is the largest complex of the respiratory chain and is responsible for the transfer of electrons from NADH to quinones, coupled with proton or sodium translocation across the membrane. Succinate:quinone oxidoreductase, or succinate dehydrogenase (complex II), is an enzyme of the tricarboxylic acid cycle, which oxidizes succinate and reduces quinones, without proton translocation. Cytochrome bc_1, or quinol:cytochrome *c* oxidoreductase (complex III), transfers electrons from quinols to cytochrome *c* (or other electron transfer metalloproteins), and cytochrome *c*:oxygen oxidoreductase, an aa_3-type enzyme (complex IV), receives these electrons and transfers them to oxygen. Complexes III and IV translocate protons across the membrane, thus contributing to the electrochemical potential. Complex V is ATP synthase, which uses the ion motive force for the synthesis of ATP. Complex V is a functionally reversible enzyme, which, in the presence of high concentrations of ATP, also promotes its hydrolyses, producing ADP (69). In addition to the inner membrane complexes, there is an NADH-cytochrome b_5 reductase in the outer membrane of mammalian mitochondria that does not pump protons but that transfers electrons directly to cytochrome *c*, thus contributing to the formation of a membrane potential solely through complex IV (5).

Beyond the five main segments of the mammalian respiratory chains, which are generally present in all respiratory chains, "extra" enzymes are observed in organisms from other groups. An alternative way to drive electrons from quinol to oxygen, which is not involved in ion translocation (85), is present in the respiratory chains of plants (45) and fungi (38), the alternative oxidase. Five types of terminal oxygen reductases are known in prokaryotes: three types of heme-copper reductases (64), the cytochrome *bd* (quinol:oxygen oxidoreductase) (13), and the so-called alternative oxidase (quinol:oxygen oxidoreductase), which harbors a di-iron center (78).

Besides the canonical rotenone-sensitive NADH dehydrogenase activity, attributed to complex I, other enzymes perform this task in the aerobic respiratory chains (26, 53, 93, 94). Type II NADH:quinone oxidoreductases, also called rotenone-insensitive NADH dehydrogenases (NDH-2), are usually present in the electron transfer chains of bacteria (e.g., *Escherichia coli* [8]) and archaea (e.g., *Acidianus ambivalens* [23]), as well as in eukaryotic organisms from the fungal (16, 89) and plant (55) kingdoms. It is noteworthy that, as observed for *Saccharomyces cerevisiae* (16), these are the only enzymes known to be responsible for the oxidation of NADH in the respiratory chains of some aerobic organisms (see Table 1). Type III NADH dehydrogenases (Nqr), the so-called Na^+-translocating NADH:quinone oxidoreductases, are also present in the respiratory chains of several bacteria (e.g., *Vibrio cholerae* [4]).

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TABLE 1. Distribution of NADH:quinone oxidoreductases among fully sequenced aerobic (or facultative) prokaryotes genomes[a]

Organism	NDH-1	NDH-2	Nqr	Oragnism	NDH-1	NDH-2	Nqr
Aeropyrum pernix	Yes[b]	C	No	*Oceanobacillus iheyensis*[c]	No	A	No
Agrobacterium tumefaciens	Yes	A	No	*Pasteurella multocida*	No	No	Yes
Aquifex aeolicus	Yes	C	No	*Pirellula* spp.	No	A	Yes
Bacillus subtilis	No	A	Yes	*Porphyromonas gingivalis*	No	No	Yes
Bacteroides thetaiotaomicron	Yes	A	No	*Prevotella intermedia*	Yes[b]	No	Yes
Bradyrhizobium japonicum	Yes	A	No	*Pseudomonas aeruginosa*	Yes	A	Yes
Brucella melitensis	Yes	A	No	*Pyrobaculum aerophilum*	Yes[b]	Yes	No
Buchnera aphidicola	Yes	No	No	*Ralstonia solanacearum*	Yes	A	No
Campylobacter jejuni	Yes	No	No	*Rickettsia prowazekii*	Yes	A	No
Caulobacter crescentus	Yes	No	No	*Salmonella enterica*	Yes	A	No
Chlamydia pneumoniae	No	No	Yes	*Shewanella oneidensis*	Yes	A	Yes
Chlamydophila caviae	No	No	Yes	*Shigella flexneri*	Yes	A	No
Corynebacterium efficiens[c]	No	A	No	*Sinorhizobium meliloti*	Yes	A	No
Coxiella burnetii	Yes	No	No	*Staphylococcus aureus*[c]	No	A	No
Deinococcus radiodurans	Yes	A	No	*Streptococcus agalactiae*[c]	No	A	No
Enterococcus faecalis[c]	No	A	No	*Streptomyces coelicolor*	Yes	A	No
Escherichia coli	Yes	A	No	*Sulfolobus solfataricus*	Yes[b]	A,C	No
Geobacter sulfurreducens	Yes	A	No	*Sulfolobus tokodaii*	Yes[b]	A,C	No
Haemophilus influenzae	No	A	Yes	*Synechocystis* spp.	Yes[b]	A	No
Halobacterium spp.	Yes[b]	A	No	*Thermoplasma acidophilum*	Yes[b]	C	No
Helicobacter pylori	Yes	No	No	*Thermoplasma volcanium*	Yes[b]	C	No
Lactococcus lactis[c]	No	A	No	*Thermosynechococcus elongatus*	Yes	A,C	No
Leptospira interrogans	Yes	A	No	*Tropheryma whipplei*[c]	No	A	No
Listeria innocua[c]	No	A	No	*Vibrio cholerae*	No	A	Yes
Magnetococcus spp.	Yes	No	No	*Wigglesworthia glossinidia*[c]	No	A	No
Mesorhizobium loti	Yes	A	No	*Wolbachia pipientis*	Yes	No	No
Methanosarcina acetivorans	Yes[b]	A	Yes	*Wolinella succinogenes*	Yes	No	No
Mycobacterium tuberculosis	Yes	A	No	*Xanthomonas axonopodis*	Yes	A	No
Neisseria meningitidis	Yes	No	Yes	*Xylella fastidiosa*	Yes	No	No
Nitrosomonas europaea	Yes	No	Yes	*Yersinia pestis*	Yes	A	Yes
Nostoc spp.	Yes[b]	A	No				

[a] Data obtained from the TIGR database.
[b] Contains genes to encode an NDH I-like protein, whose electron donor and/or catalytic subunits are unknown, i.e., lack one or more of the genes that code for the flavoprotein subunits (*nqo*$_1$, *nqo*$_2$ and/or *nqo*$_3$).
[c] Organisms contain only NDH-2.

The complete genomes of aerobic and facultative anaerobic prokaryotes were searched for the presence of genes encoding NADH:quinone oxidoreductases by using both the available annotations and amino acid sequence comparisons. Since a clear distinction between aerobes and anaerobes is becoming difficult, it was decided to consider as an aerobe (or facultative) the organism that contains genes coding for canonical oxygen reductases. Table 1 lists the predictions for the presence of all types of NADH:quinone oxidoreductases in the fully sequenced genomes of prokaryotes matching the proposed criterion. NADH oxidation strategies are very diverse, as inferred from the existence of organisms with (i) a single type of NADH dehydrogenase, (ii) two types of NADH dehydrogenases, and (iii) all three types of NADH dehydrogenases. An interesting question is if these enzymes are expressed and active under the same conditions (see below). Particularly relevant and highlighting the key role of NDH-2 is the observation that in several organisms, only this type of NADH:quinone oxidoreductase is present. It is worth mentioning that some organisms present complex I-like enzymes, containing 11 (in archaea) or 12 (in cyanobacteria) of the 14 subunits usually present in the prokaryotic enzyme. The missing subunits belong to the flavoprotein fraction of NDH-1, the electron input modules; therefore, either they use an as yet unidentified electron donor or they have a quite distinct catalytic module. Moreover, from the analysis in Table 1, it can be concluded that all the "aerobes" included in this study contain at least one type of NADH dehydrogenase, in agreement with the main role of the [NADH]/[NAD^+] balance in their physiology.

The main reducing equivalent synthesized by the cell central metabolism is NADH, making it the principal electron donor to respiratory chains. In prokaryotes, NADH is produced in the cytosol, mainly by the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase, pyruvate dehydrogenase, and three enzymes in the tricarboxylic acid cycle: isocitrate, α-ketoglutarate, and malate dehydrogenases; in eukaryotes the corresponding Krebs cycle reactions take place at the mitochondrial matrix, thus yielding mitochondrial NADH (79). The reduced dinucleotide is oxidized by the respiratory chain NADH dehydrogenases, described above, with its energy ultimately conserved in the transmembrane electrochemical potential difference. In addition to its prominent role in energy production, NADH is a potential source of NAD^+, the main cellular oxidant. Since the amount of NAD^+ is small in comparison with the amount of substrates being oxidized, the NADH resulting from the oxidative reaction must be rapidly reoxidized, restoring the NAD^+ levels, to ensure that the oxidation is not limited by the lack of NAD^+ (14). Moreover, the [NADH]/[NAD^+] ratio is responsible for the regulation of some cell pathways leading to the synthesis of ATP. The cell requirements of NAD^+ make NADH turnover a top priority over ATP synthesis. Although the oxidation of one molecule of

NADH by NDH-2 yields smaller amounts of ATP compared with the same oxidation performed by complex I, their activity can be essential when the [NADH]/[NAD^+] ratio is very high, precisely because they are able to reoxidize more NADH and synthesize less ATP, therefore increasing the flux of substrate oxidation through the respiratory chain.

In this article, an overview of the historical, functional, and structural aspects of the type II NADH:quinone oxidoreductases is presented and the recent developments in the field are discussed. A preliminary study carried out for hyperthermophilic type II NADH:quinone oxidoreductases (63) has been extended to examples of NDH-2 from organisms belonging to all the three domains of life and living in different habitats, thus leading to further insights in this family of enzymes.

TYPE II NAD(P)H:QUINONE OXIDOREDUCTASES

NDH-2 are usually single polypeptides with molecular masses around 50 kDa and without any energy-transducing site (92), that catalyze a two-step transfer of electrons from NAD(P)H to quinones. These enzymes are resistant to the complex I-specific inhibitors piericidin A, capsaicin, and rotenone, and although a few compounds can prevent their activity, no general specific inhibitor was reported (94). The primary structures of these proteins generally contain two GXGXXG motifs within β-sheet–α-helix–β-sheet structures for binding NAD(P)H and flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) (90). In most cases, NADH-oxidizing enzymes can also oxidize NADPH, which is structurally similar to NADH with the exception of a phosphate group replacing the hydroxyl from position 2 in the ribose of AMP (20). At alkaline pH, the oxidation of NADPH can be prevented by electrostatic repulsion between the negative charges of the phosphate group of NADPH and the phospholipids of the membrane (57).

Until recently, all the rotenone-insensitive NA(D)PH:quinone oxidoreductases were described as lacking FMN and iron-sulfur clusters as cofactors but containing a noncovalently bound FAD instead. However, a few examples of NDH-2 with covalently bound FMN have recently been reported (2, 3), as well as a NDH-2 that contains a noncovalently bound FMN in place of FAD (19). There are also a few examples of NDH-2 that, beyond the typical feature of the two ADP-binding sites, also display an EF-hand motif that binds calcium (48, 67).

To address this diversity, it was suggested that these enzymes could be classified into three groups according to the conserved motives present in their primary and secondary structures (63) (Fig. 1). This classification is now firmly established, through a comprehensive analysis of the amino acid sequences of NDH-2. Hence, group A comprises the NDH-2 with two adenine dinucleotide-binding motives, involved in noncovalently binding of NAD(P)H and flavins. Enzymes of this kind are found in archaea, bacteria, and eukaryotes. Group B contains the NDH-2 that possess two ADP-binding motives plus a conserved EF-hand fold. To date, the reported enzymes of this group belong exclusively to eukaryotes, namely, fungi (48) and plants (67). There is also a third group of NDH-2, group C, that comprises the enzymes with a single conserved GXGXXG consensus motif in a βαβ fold and with a covalently bound flavin. In the primary structures of some of these proteins, the absence of the second dinucleotide-binding region is coincident with the presence of a conserved histidine residue (Fig. 2), suggested to be involved in the covalent binding of the flavin. However, this observation cannot be extended to all NDH-2C (Fig. 2), and it remains unclear which residue is involved in the covalent binding of the flavin. So far, biochemical data for group C enzymes are restricted to hyperthermophilic archaea (3, 23).

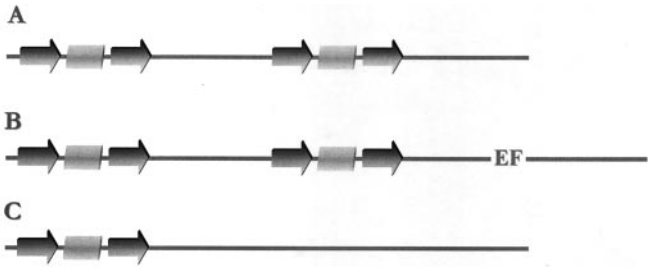

FIG. 1. Classification of type II NAD(P)H:quinone oxidoreductases according to their binding motifs. Cylinders represent α-helices, and arrows represent β-sheets; the EF-hand motif is represented by EF.

Biochemical and Genomic Aspects of NAD(P)H Dehydrogenases

A large number of microorganisms contain type II NADH:quinone oxidoreductases (Table 1), but only very few have been isolated and studied in detail. In the late 1960s, Bragg and Hou first reported the presence of two distinct NADH dehydrogenase activities in *E. coli* (10); these proved to correspond to NDH-1 and NDH-2 activities. This introduced a new field of biochemistry and bioenergetics, which is continuously increasing our knowledge of alternative strategies for respiratory chains. These authors have purified two menadione reductases from *E. coli*. One of them carried out the oxidation of NAD(P)H and was activated by FMN and FAD. It reduced 2,6-dichlorophenol-indophenol but, except in the presence of menadione, not cytochrome *c* or oxygen. This enzyme was strongly inhibited by dicumarol and *o*-phenantroline, and their effect suggested that it was the major NADH dehydrogenase of the *E. coli* respiratory chain. The second menadione reductase, which also catalyzed the oxidation of NAD(P)H, was slightly stimulated by the addition of flavins and showed resistance to the above-mentioned inhibitors (10). In the early 1980s, Young et al. cloned and sequenced the enzyme responsible for the second NADH:menadione reductase identified previously, providing clear evidence for its existence (95). Two ADP-binding motives are depicted in the primary structure of this protein; hence, it is an enzyme from group A. The *E. coli* NDH-2A was further characterized and showed to contain 1 mol of noncovalently bound FAD per mol of purified enzyme; moreover, it consists of a single polypeptide with M_r 47,200, which in the mature form has an apparent molecular mass of 45 kDa (30). The NDH-2A from the *E. coli* respiratory chain faces the cytosol (62).

The deamino-NADH:ubiquinone 1 reductase and the NADH:ubiquinone 1 reductase activities in *E. coli* membrane vesicles were compared, and it was observed that the former activity was more sensitive to piericidin A than the latter. Furthermore, the membranes exhibited two apparent K_ms for NADH but only one for deamino-NADH (43). These observations cor-

FIG. 2. Alignment of the amino acid sequences from selected NDH-2. When present, the GXGXXG consensus motifs are given a black background; the EF-hand motif is indicated by a dash over the sequence, whose strictly conserved and conserved amino acid residues are given a dark gray and gray background, respectively. Aam, *A. ambivalens* (AJ489504); Aaq, *A. aeolicus* (NP_214500); Aaq 1, *A. aeolicus* (AE000707); An, *Aspergillus nidulans* (XM_411637); At, *A. thaliana* NDI1 (T09038); Av, *A. vinelandii* (AF346487); Ec, *E. coli* (V00306); Hs, *Homo sapiens* AMID (NP_116186); Nc, *N. crassa* NDE1 (AJ236906); Sc, *S. cerevisiae* NDI1 (X61590); Sto, *S. tokodaii* C (AP000983); Stu, *S. tuberosum* NDA (AJ245861), NDB (AJ2455862); SyNdbA, *Synechocystis* (D90909); Tb, *T. brucei* (AY125472); Te, *T. elongatus* (AP005372); Te1, *T. elongatus* (AP005369); Tv, *T. volcanium* (AP000996).

FIG. 2—*Continued.*

roborated the previous indications for the existence of two types of NADH dehydrogenases in the respiratory chain of *E. coli*. The first enzyme is able to oxidize both deamino-NADH and NADH, and its turnover leads to the production of a proton-motive force at a site between the primary dehydrogenase and ubiquinone (NDH-1); the second enzyme oxidizes exclusively NADH and does not generate a proton-motive force before ubiquinone (NDH-2).

Since the identification of *E. coli* NDH-2, several homologous enzymes have been found in the most diverse organisms.

Prokaryotes. In the *Azotobacter vinelandii* respiratory chain there is a group A enzyme, since it contains two ADP-binding regions (Fig. 2); it is a capsaicin-resistant NADH dehydrogenase (7). The enzyme is particularly active at high concentrations of oxygen, in contrast to the low rates of NADH oxidation performed by the complex I of this organism under the same conditions. The disruption of the gene encoding the *A. vinelandii* NDH-2A resulted in a great decrease of the respiratory activity; the mutant could not grow diazotrophically at high levels of oxygen and was fully able to grow at low oxygen levels or in the presence of NH_4^+ (6).

In the genome of the cyanobacterium *Synechocistys* sp. strain PCC 6803, there are three open reading frames coding for group A NDH-2: *ndbA*, *ndbB*, and *ndbC*. The *ndb* genes have been cloned, and deletion mutants have been produced which led to small changes in the respiratory activity. In addition, an expression construct of *ndbB* complemented an *E. coli* strain lacking both NDH-1 and NDH-2 (28).

Sequences encoding putative type II NADH dehydrogenases from groups A and C were also identified in the genomes of hyperthermophilic bacteria such as *Aquifex aeolicus* (15), and *Thermosynechoccus elongatus* (58) (Fig. 2 and 3; Table 1).

The hyperthermophilic archaea *Sulfolobus tokodaii* (31), *Aeropyrum pernix* (32), and *Thermoplasma volcanium* (33) and the mesophilic archaeon *Halobacterium* spp. (59) have genes for putative type II NADH:quinone oxidoreductases. *Halobacterium* spp. contain one gene for a putative NDH-2, and *S. tokodaii*, *A. pernix*, and *T. volcanium* contain genes for putative type II NADH dehydrogenases from group C (Fig. 2 and 3; Table 1). There is a report on the electron transport chain of *H. salinarum*, where the presence of a type II NAD(P)H: quinone oxidoreductase is proposed (77). This NDH-2 is likely to belong to group A, since in the genome of *Halobacterium* spp. there is only one open reading frame that putatively codes for a NDH-2A, which could be the homologue of the *H. salinarum* NDH-2.

Biochemical evidence of group C NDH-2 was obtained by studying the archaea *Sulfolobus metallicus* (3) and *Acidianus ambivalens* (2, 23). The purified enzymes from both organisms catalyze NADH:quinone oxidoreduction. These proteins are monomers with apparent molecular masses of 47 and 49 kDa, respectively, and have a covalently bound FMN molecule. The absence of the second dinucleotide-binding region leaves the first region as the only one capable of binding the substrate. This idea is strengthened by previous indications from the structure of the *Neurospora crassa* NDE1 (47). The authors proposed that, in NDE1, the first motif should bind the substrate, NADPH, on the basis of the observation that the third glycine amino acid residue of the first GXGXXG motif, in NADPH-binding proteins, is generally replaced by serine, ala-

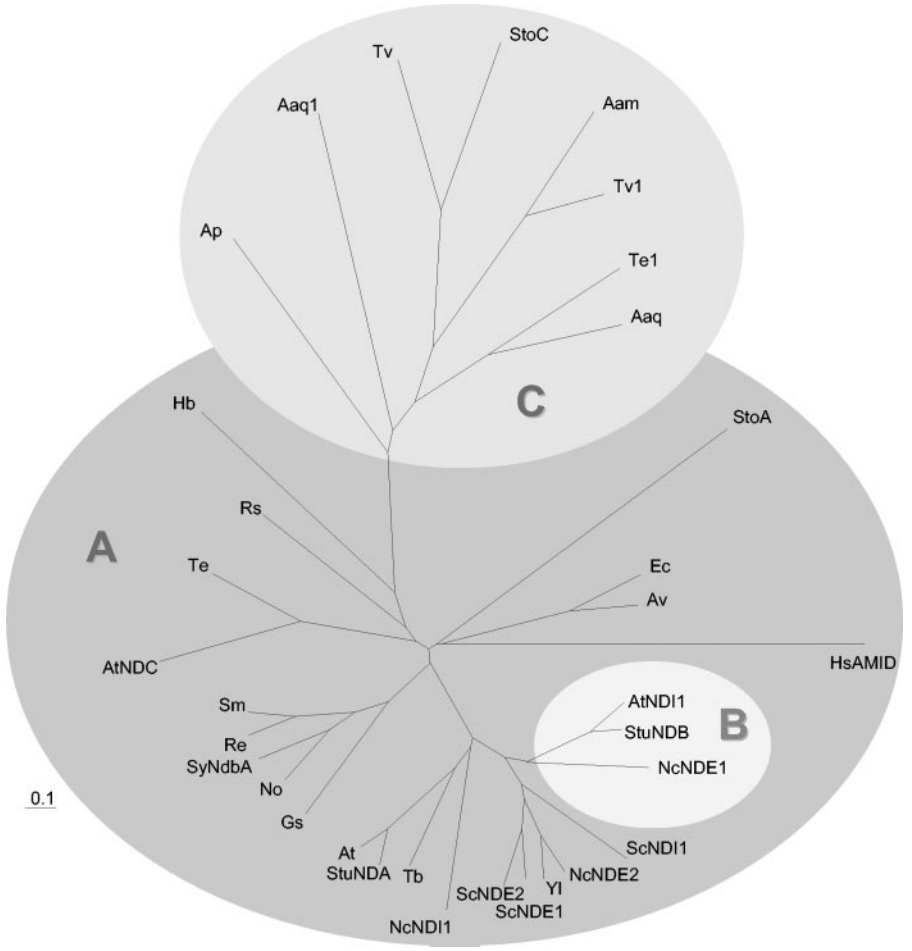

FIG. 3. Dendrogram of type II NAD(P)H:quinone oxidoreductases, based on an amino acid sequence alignment of NDH-2 from prokaryotic and eukaryotic organisms. The alignment was manually adjusted prior to dendrogram production by using Clustal X (82), excluding positions with gaps and correcting for multiple substitutions. Aam, *A. ambivalens* (AJ489504); Aaq, *A. aeolicus* (NP_214500); Aaq1, *A. aeolicus* (AE000707); Ap, *A. pernix* (AP000060); At, *A. thaliana* (AY084663), AtNDC, *A. thaliana*. (NM_120955), AtNDI1, *A. thaliana* (BX828330); Av, *A. vinelandii* (AF346487); Ec, *E. coli* (V00306); Gs, *Geobacter sulfurreducens* (AE017208); Hb, *Halobacterium* sp. (AE005028); Hs, *Homo sapiens* AMID (NP_116186); NcNDI1, *N. Crassa* (XM_322238); NcNDE1, *N. crassa* (AJ236906); NcNDE2, *N. crassa* (XM_331371); No, *Nostoc* (AP003584); Re, *Rhizobium etli* (U80928); ScNDI1, *S. cerevisiae* (X61590); ScNDE1, *S. cerevisiae* (Z47071); ScNDE2, *S. cerevisiae* (Z74133); Rs, *Rhodobacter sphaeroides* (ZP_00006015); Sm, *Sinorhizobium meliloti* (AL591789); StuNDA, *S. tuberosum* (AJ245861); StuNDB, *S. tuberosum* (AJ245862); StoA, *S. tokodaii* A (AP000990); StoC, *S. tokodaii* C (AP000983); SyNdbA, *Synechocystis* (D90909); Tb, *T. brucei* (AY125472); Te-*T. elongatus* (AP005372); Te1, *T. elongatus* (AP005369); Tv, *T. volcanium* (AP000996); Tv1, *T. volcanium* (AP000995); Yl, *Yarrowia lipolytica* (AJ006852).

nine, or proline amino acid residues and also that a conserved negatively charged amino acid residue at the end of the second β-sheet is missing (28). This residue could be replaced by an asparagine residue, avoiding the unfavorable interaction between the negatively charged residue with the negatively charged 2′-phosphate of NADPH (54). These features are observed in the first and absent in the second dinucleotide-binding motif of NDE1 (48). The average reduction potentials of the *A. ambivalens* and *S. metallicus* enzymes were determined to be 70 and 160 mV, respectively, which are very high compared to the −370 mV reduction potential of *S. cerevisiae* NDI1. Nevertheless, other respiratory enzymes, such as the succinate:quinone oxidoreductases (SQR), also exhibit a quite positive flavin reduction potential (for an example, see reference 37a). The covalent attachment between the protein backbone and the flavin, also present in SQRs, may be responsible for increasing the reduction potential of the *A. ambivalens* and *S. metallicus* enzymes (2, 3). It should be noted that caldariella quinone, the endogenous quinone of these archaea, has a high reduction potential of +100 mV. For the archaeal enzymes, no flavin-derived semiquinone radical was observed, suggesting that a two-electron transfer reaction is favored, as expected for enzymes having two-electron redox compounds as electron donor and acceptor. The fact that either genomic or biochemical evidence of NDH-2C was found only in hyperthermophilic organisms may suggest the association of these enzymes with the extreme conditions under which the host organisms live. However, the possibility that they also exist in mesophilic organisms cannot be excluded. It is also crucial to gain biochemical support for NADH:quinone oxidoreduction activity of more NDH-2C, in particular from hyperthermophilic bacterial enzymes, to confirm that these enzymes are not restricted to hyperthermophilic archaea and to establish which flavin type is assembled in the bacterial NDH-2C.

Eukaryotes. The *Trypanosoma brucei* NDH-2 is the first example of an eukaryotic NDH-2 containing FMN. Unlike the archaeal FMN-containing NDH-2, in the enzyme of *T. brucei* the FMN is noncovalently bound. It was described as a functional dimer of 33-kDa monomers, with the catalytic site facing the matrix (19). The analysis of *T. brucei* NDH-2 primary and secondary structures showed two conserved GXGXXG motifs in a βαβ structure; therefore, it is a group A enzyme (Fig. 2 and 3).

The obligate aerobic yeast *Yarrowia lipolytica* has a group A type II NAD(P)H dehydrogenase in the outer surface of the inner mitochondrial membrane. Deletion mutants of the enzyme were fully viable. Complete inhibition of NADH oxidation in mitochondria solubilized with 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) was achieved with piericidin A, indicating that complex I activity was the sole NADH:quinone oxidoreduction activity left in those strains. The orientation of the alternative NADH dehydrogenase was assessed by measuring NADH:5-nonylubiquinone oxidoreductase activity before and after solubilization of the inner mitochondrial membrane. In the presence of piericidin A, this activity was not affected by solubilization, indicating that the active site of the enzyme faced the intermembrane space (35).

The respiratory chain of the facultative aerobic yeast *S. cerevisiae* does not contain a complex I. The oxidation of cytosolic and matrix NAD(P)H is performed by two external (NDE1 and NDE2) (41, 73) and one internal (NDI1) (42) NAD(P)H dehydrogenases. The purification of an external NADH dehydrogenase from *S. cerevisiae* mitochondria was described. The protein consisted of a single subunit with a molecular mass of 53 kDa that contained FAD and was insensitive to rotenone and piericidin A (16). Later, the protein was isolated, its encoding gene was disrupted, and the oxidation of several substrates by mitochondria from wild-type and mutant strains was measured. While the oxidation of external NADH was not affected in mutant mitochondria, the oxidation of substrates generating internal NADH was severely decreased or missing, showing that the inactivated enzyme was, in fact, the internal NADH dehydrogenase of *S. cerevisiae* (42). In 1998, Small and McAlister-Henn (73) and Luttik et al. (41) identified two other genes coding for mitochondrial NADH dehydrogenases (NDE1 and NDE2) that oxidize NADH from the cytosol. Both genes were deleted, and the NADH oxidation was monitored in mitochondria from the wild type and from *nde* deletion mutants. Compared to wild-type mitochondria, exogenous NADH oxidation was drastically reduced in one of the mutants, although the other showed no difference. However, in mitochondria from the double mutant, oxidation of external NADH was completely absent, confirming the external location of NDE1 and NDE2. According to the features of their primary and secondary structures, all NDH-2 reported in *S. cerevisiae* are group A enzymes (Figs. 2 and 3). Recently, Fang and Beattie suggested that the *S. cerevisiae* external NADH dehydrogenases are a potential source of mitochondrial superoxide (18).

In the early 1970s, the presence of two rotenone-insensitive NAD(P)H dehydrogenases in *N. crassa* mitochondria was reported (89). Oxygen consumption was observed after addition of rotenone to mitochondria respiring pyruvate/malate, thus indicating the activity of a rotenone-resistant NADH dehydrogenase facing the matrix. In 1982, Moller et al. showed that the oxidation of NADH by *N. crassa* mitochondria was stimulated in the presence of cations (57). Later, Melo et al. (46) reported the existence of two enzymes oxidizing cytosolic NAD(P)H in *N. crassa* mitochondria. They sequenced and disrupted the gene encoding a putative NAD(P)H dehydrogenase, NDE1, from the inner membrane of *Neurospora* mitochondria. The NAD(P)H oxidation was monitored in wild-type and mutant mitochondria, and it was observed that the mutant lacked exogenous NADPH oxidation at physiological pH, while exogenous NADH oxidation was still carried out by these mitochondria, indicating the presence of a second NAD(P)H dehydrogenase, NDE2, in the *Neurospora* respiratory chain. Moreover, it was reported that the inactivated enzyme was calcium dependent, in accordance with what was expected from its amino acid sequence data (Fig. 2), which, beyond the two ADP-binding regions, included an EF-hand motif. This was the first time that a calcium-dependent NADPH:quinone oxidoreductase was reported, and a new group of type II NAD(P)H:quinone oxidoreductase, NDH-2B, was introduced (47, 48). With the release of the *N. crassa* genome (22), at least two other sequences encoding putative NDH-2 dehydrogenases were identified. These proteins have recently been characterized; one corresponds to NDE2 (11), and the other was identified as the internal rotenone-insensitive NADH dehydrogenase (NDI1) (17).

Type II NAD(P)H:quinone oxidoreductases have also been described in the electron transport chain of plant mitochondria. There are reports of the purification of 42-kDa (39), 26-kDa (66), and 43-kDa (50) NAD(P)H dehydrogenases from red beetroot mitochondria. In these mitochondria, the purification of a 58-kDa protein was associated with external NAD(P)H oxidation activity (40). Studies of NADH and NADPH oxidation by intact mitochondria from *Arum maculatum* and potato tubers (68) and by inside-out submitochondrial particles from potato tubers and Jerusalem artichoke (49, 56, 65) led to the conclusion that there are four distinct NDH-2, two on each side of the inner membrane. Rasmusson et al. (67) described two different cDNAs from potato, homologous to genes encoding rotenone-insensitive NADH dehydrogenases in yeast and bacteria. The encoded proteins have approximate molecular masses of 55 kDa (NDA1) and 65 kDa (NDB1) and are located in the inner and outer surfaces of the inner mitochondrial membrane, respectively. It is noteworthy that the structure of NDB1 also displays an EF-hand motif, like the one previously described for the *Neurospora* homologue; therefore, it constitutes another example of a NDH-2B. Concerning NDA1, it is a type II NADH dehydrogenase from group A. Further studies of potato leaves revealed that the expression of *nda1* is light dependent and that the expression levels of NDA1 are severely decreased on cold exposure, with a concomitant decrease in the rotenone-insensitive oxidation of matrix NADH (81).

The genome of *Arabidopsis thaliana* contains several open reading frames encoding NDH-2 homologues. Recently, these were compared to the potato homologues, and their expression responses to light were analyzed. Three distinct types of NDH-2 were identified: (i) two *nda*-like genes, one of which, *nda1*, showed light-dependent expression, related to the circadian cycle; (ii) four genes closely related to *ndb1*; and (iii) a novel homologue, *ndc1*, which is associated with cyanobacterial

TABLE 2. Summary of NDH-2 biochemical features

Organism	Group	E_m	K_m (μM)	Mm (kDa)	Flavin	QBS
E. coli	A	ND[d]	50	45	FAD	IB
A. vinelandii	A	ND	13	ND	FAD	IB
Synechocystis	A	ND	ND	49[c]	FAD	IB
A. ambivalens	C	+70	6	47	FMN	IA
S. metallicus	C	+160	2	49	FMN	ND
T. brucei	A	ND	120	2 × 33	FMN	IC
Y. lipolytica	A	ND	15	66[c]	FAD	IB
S. cerevisiae						
NDI1	A	−370	31, 9[a]	53	FAD	IA
NDE1	A	ND	ND	62[c]	FAD	IB
NDE2	A	ND	ND	65[c]	FAD	IB
N. crassa						
NDI1	A	ND		57	FAD	IB
NDE1	B	ND	11[b]	64	FAD	IB
NDE2	A	ND	12	65[c]	FAD	IB
S. tuberosum						
NDA	A	ND	14[b]	55[c]	FAD	IA
NDin	ND	ND	14[b]	ND	FAD	ND
NDB	B	ND	ND	65	FAD	IC
NDex	ND	ND	ND	ND	FAD	ND

[a] Values obtained using Q_6 or DCPIP, respectively.
[b] The experiments were carried out with intact mitochondria (*N. crassa*) or inside-out submitochondrial particles (*S. tuberosum*); thus, the K_m values stand for external or internal NAD(P)H dehydrogenases, respectively.
[c] Estimated from the sequence of the precursor protein.
[d] ND, not determined. Fingerprints of type 1 Quinone-Binding Sites (QBS): type IA $L(X)_3H(X)_2T$, type IB $(A/L/I)(X)_3H(X)_2L$, and type IC $L(X)_3H(X)_3S$.

NDH-2 genes. *ndc1* is suggested to have entered the eukaryotic cell via the chloroplast progenitor; it is likely that it was transferred to the nucleus and then fused with a mitochondrion targeting signal (51). The *nda*-like genes encode NDH-2A enzymes, while the *ndb*-like genes encode proteins belonging to group B (Fig. 3).

A compilation of data from the type II NADH:quinone oxidoreductases described in the present review is summarized in Table 2. In spite of the presence of one or two ADP-binding domains, the prokaryotic NDH-2 have similar molecular masses, around 47 kDa. The molecular mass of the putative NDH-2, whose presence is based exclusively on the genomes of their organisms, was calculated according to their primary structures, and similar values were obtained. It is likely that the size of the protein is important for accomplishing function, namely, to assume a correct tertiary structure that ensures an efficient binding and catalysis of the substrate and interactions with both quinones and the membranes. The affinity of these proteins to NADH is a delicate point: there are insufficient data from enzymes to make a prudent generalization. The range of $K_{m(NADH)}$ presented is very broad, from 2 to 50 μM. The hyperthermophilic archaeal enzymes from group C have the lowest $K_{m(NADH)}$ values (e.g. *A. ambivalens* [23]; Table 2). The enzymes from mesophilic bacteria have K_ms ranging from 13 μM in *A. vinelandii* (6) to 50 μM in *E. coli* (43). With the exception of the *T. brucei* NDH-2A, the $K_{m(NADH)}$ values of the eukaryotic enzymes are within the same range, in spite of being determined for different electron acceptors (Table 2). The K_m determinations were carried out using different electron acceptors: potassium ferricyanide for the *S. metallicus* and *A. ambivalens* enzymes; quinones for the NDH-2 from *T. brucei* (19), *S. cerevisiae* NDI1 (16), and *Y. lipolytica* (35); and oxygen for the *A. vinelandii* (6), *N. crassa* NDE1 and NDE2 (46); and *S. tuberosum* (65) internal-type II NAD(P)H:quinone oxidoreductases. The $K_{m(NADH)}$ of the *E. coli* NDH-2 was determined using either quinones or oxygen as electron acceptors, and the same value was obtained (43). However, artificial electron donors may affect substrate affinity; for instance, the $K_{m(NADH)}$ for the *S. cerevisiae* NDH to dichlorophenolindophenol (DCPIP) was determined as 9.4 μM (86), which is a very different value from that obtained using Q_6 as the electron acceptor (Table 2). Therefore, these values must be carefully compared. It is also important to stress that the catalytic activity of NDH-2 from *E. coli* (8), *S. metallicus* (3), and *A. ambivalens* (23) is severely affected by the presence of lipids in the reaction mixture: a 3-fold and 300-fold increase in activity was determined for the *S. metallicus* and *E. coli* enzymes, respectively; furthermore, for the *A. ambivalens* enzyme, activity was detected only in the presence of lipids (see also "Membrane interaction of NDH-2" below). The mechanism through which the NADH:quinone oxidoreduction reaction takes place is not clear, since the only data available refer to the NDH-2 from *T. brucei* and the NDI1 from *S. cerevisiae*, for which the oxidation of NAD(P)H via a ping-pong mechanism was proposed (19, 86).

Analyses of NDH-2 Primary Structures

Sequence comparisons of NDH-2. The primary structures of NDH-2 from 17 representative organisms plus a homologue sequence of the human apoptosis-inducing factor Mitochondrion-associated Inducer of Death (AMID) (91) were aligned using Clustal X followed by manual adjustment (Fig. 2). The sequence alignment makes it clear that these proteins are not very highly conserved, when the full sequence of all types of bacterial, archaeal, and eukaryotic enzymes is considered; however, they present at least one strictly conserved region to bind ADP. This feature, together with their average size, is likely enough to ensure that all of them are able to catalyze the same reaction. The lowest percent identity and similarity between the different type II NADH dehydrogenases listed are found between group B and group C enzymes (Table 3). The highest similarity is observed between the *S. tokodaii* and *A. ambivalens* NDH-2C, which belong to two closely related organisms (Table 3). The human AMID, which contains two ADP-binding motifs, has significant similarity to all NDH-2 considered, ranging between 22% (e.g., Te1) and 33% (e.g. Te) (Table 3), suggesting that it should be able to carry out NADH oxidation activity. In fact, this ability is observed in the mouse mitochondrial apoptosis-inducing factor (52).

From the amino acid sequence analyses, a relationship between group C NDH-2 and hyperthermophilic organisms can be established, as was previously suggested (63) (Fig. 2). In contrast to the conservation of the first ADP-binding domain, the presence of the second GXGXXG motif is clear in only 12 of the 18 enzymes compared in Fig. 2. The other seven sequences are from hyperthermophilic archaea and bacteria. The results of the hyperthermophilic NDH-2C, with the exception of the *A. ambivalens* and *S. metallicus* enzymes, are based exclusively on genomic data. It is also important to mention that in the *S. tokodaii* and *T. elongatus* genomes, two of the seven hyperthermophilic organisms analyzed, there are also sequences that putatively code for group A NDH-2.

TABLE 3. Percent identity and similarity between the NDH-2 aligned in Fig. 2[a]

Organism	Av	Sy	Te	Tb	Sc	Nc	StuA	Aam	Sto	Tv	Aaq1	Aaq	Te1	Hs	At	An	StuB
E. coli	54	23	20	21	20	15	20	12	12	12	14	13	12	17	16	14	16
	71	36	37	36	37	32	39	26	26	27	28	27	27	30	29	28	32
A. vinelandii		23	24	20	21	17	20	11	12	11	11	13	12	15	18	16	17
		40	39	37	40	31	38	26	28	26	27	25	25	31	29	28	31
Synechocystis			24	22	22	17	20	12	12	14	12	16	14	16	16	14	16
			41	38	38	30	40	26	26	26	26	28	29	30	28	29	30
T. elongatus				20	20	15	22	13	12	14	15	14	16	19	14	13	14
				35	35	28	37	27	27	27	29	27	30	33	27	27	28
T. brucei					23	23	38	11	11	11	11	9	10	15	20	20	23
					42	38	55	24	25	26	24	24	25	27	34	35	37
S. cerevisiae						26	28	12	12	13	12	11	12	15	25	24	23
						44	51	25	27	26	24	25	26	29	40	40	27
N. crassa							27	9	9	9	10	11	11	13	26	58	30
							44	23	22	21	23	22	23	25	41	71	44
S. tuberosum								13	13	11	12	11	12	12	24	23	29
								27	28	27	27	28	28	26	42	40	45
A. ambivalens									80	18	21	21	20	14	10	8	10
									89	35	40	38	38	28	23	21	23
S. tokodaii										20	22	20	22	14	12	8	12
										38	40	38	40	29	24	22	24
T. volcanium											17	19	20	10	10	9	10
											36	38	37	25	23	20	23
A. aeolicus 1												21	22	16	11	8	10
												37	36	31	23	21	23
A. aeolicus													36	10	11	11	11
													55	23	22	22	24
T. elongatus 1														9	10	9	11
														22	22	21	24
H. sapiens															12	12	11
															22	22	22
A. thaliana																28	64
																46	78
A. nidulans																	33
																	49

[a] Percent identity is given in the first row for each organism, and percent similarity is given in the second. Abbreviations in the column heads are spelled out in the left-hand column.

Envisaging the corroboration of the observations made from the alignment, a dendrogram was constructed, using Clustal X, based on the sequence alignment (Fig. 3). The obtained dendrogram supports the suggestion that the NDH-2 affiliate according to the motives present in their primary and secondary structures, thus sustaining the three-group classification for alternative NADH dehydrogenases. Enzymes from group A are present in organisms from the three domains of life, while NDH-2B were observed only in plants and fungi. From the analysis of the dendrogram, the idea that group C enzymes are typical of those from hyperthermophilic prokaryotes is strengthened. Both NDH-2 from the hyperthermophilic bacterium *A. aeolicus* and one NDH-2 from *T. elongatus* (which also contains a NDH-2A) are associated with NDH-2C from the hyperthermophilic archaea reported above (Fig. 3). Moreover, the archaeal NDH-2A from *Halobacterium* spp. and *S. tokodaii* are associated with the group A NDH-2. These results weaken the argument that the NDH-2C would cluster according to their phylogenetic origin and emphasize the fact that they group according to the conserved motives present in their primary and secondary structures (Fig. 1). The human AMID grouped together with the NDH-2A, as expected from the analysis of its primary and secondary structures.

A human (mammalian) homologue of NDH-2? As mentioned above, the mammalian respiratory chains are described as devoid of type II NAD(P)H:quinone oxidoreductases. However, Nohl et al. (60) and Oliveira et al. (61) reported an external NADH dehydrogenase from rat heart mitochondria.

The *E. coli* NDH-2A was used to search for a homologous enzyme in the human genome. The protein displaying more significant similarity to the *E. coli* enzyme (see above) is a mitochondrial AIF homologue, AMID (91), whose primary structure displays the curious feature of having the typical two ADP-binding motifs characteristic of the group A and B enzymes perfectly conserved, suggesting that this protein could have NAD(P)H dehydrogenase activity. The AIF is a mitochondrial FAD-containing protein that activates caspase-independent apoptosis (80). The mitochondrion-homologous AMID is suggested to trigger a caspase- and p53-independent apoptosis (91). AMID expression in several human tissues was analyzed by Northern blotting. AMID mRNA was not detected in the tested healthy tissues but was observed in colon cancer cell lines DLD and HCT116 and also in B lymphoma cell line RPMI8226 (91). Could AMID be the human homologue of the protein described by Nohl and Oliveira? This is an interesting question, deserving further investigation.

Membrane interaction of NDH-2. Membranes are composed of a lipid monolayer (archaea) or bilayer (bacteria and mitochondria), hydrophilic on the outer surfaces and hydro-

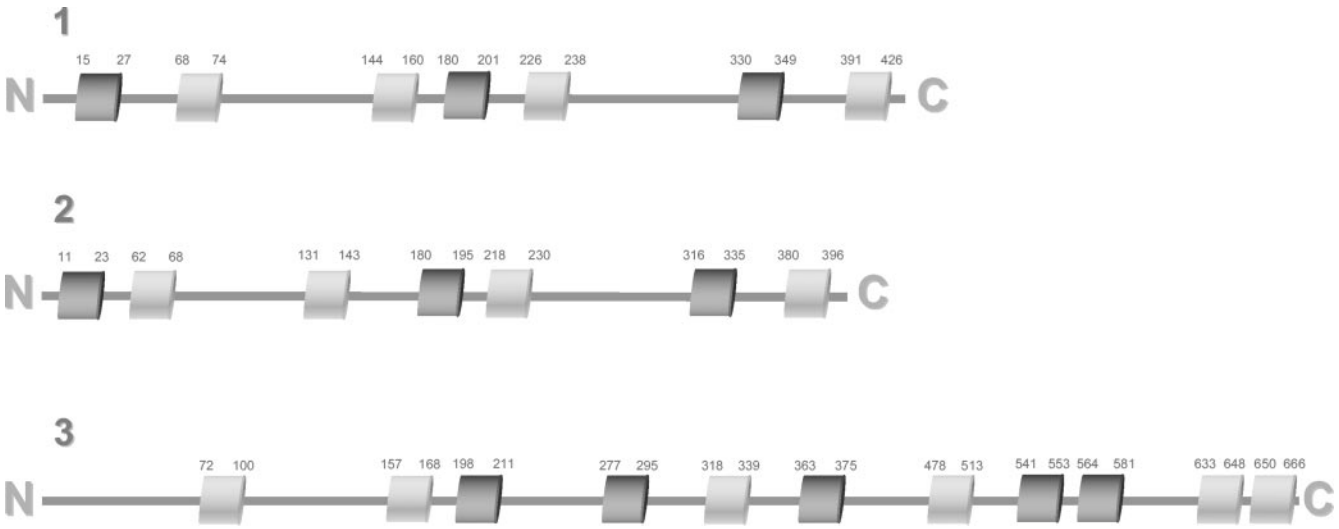

FIG. 4. Illustration of putative amphipathic helices present in NDH-2 from groups A, B, and C. Highlighting indicates putative α-helices in the secondary structures. Dark shading in α-helices indicates possible amphipathic nature. 1, *E. coli* NDH-2 (group A); 2, *A. ambivalens* NDH-2 (group C); 3, *N. crassa* NDE1 (group B). Numbers refer to helix position in the amino acid sequence.

phobic in the middle. The simplest method of membrane anchoring involves one transmembrane α-helix traversing the membrane once, in contrast to the case where several α-helices or β-strands pass through the bilayer several times. An alternative strategy for membrane anchoring of proteins uses α-helices parallel to the plane of the membrane, which are sometimes amphipathic, with, in this case, predominantly polar residues along one side of the helix and nonpolar side chains on the remainder opposite the helical structure, as observed in several enzymes such as prostaglandin synthase (9, 74) and proposed for type E succinate:quinone oxidoreductases (37). The anchoring of NDH-2 to the membrane remains controversial, since transmembrane helices are not commonly present among NDH-2 proteins. The secondary structures of NDH-2 were studied using PSIPRED (44), and the transmembrane topology was then predicted using SOSUI (27). Sequences from integral membrane proteins with known structures were also analyzed and used to confirm the correct prediction of transmembrane α-helices. In addition, the amino acid sequence of human AIF (1M6IA) was analyzed and the obtained predictions were cross-checked with the structural data available for this enzyme. All these controls gave correct predictions, thus supporting the predictions of NDH-2 secondary structures.

Among all the enzymes used in the dendrogram (Fig. 3), only two are predicted to contain transmembrane helices: the NDH-2A from *Geobacter sulfurreducens*, which has three putative transmembrane helices, and *N. crassa* NDE1, a NDH-2B, with only one. The latter prediction is consistent with the fact that, on alkaline treatment of *N. crassa* mitochondria, the enzyme remains in the membrane fraction (48). The absence of transmembrane helices in the remaining enzymes and the observation that hydrophobic and hydrophilic amino acids are located on opposite sides in some of the predicted α-helices suggest a membrane-protein interaction through the hydrophobic face of these amphipathic α-helices (2). The analyses of *E. coli* (group A) and *A. ambivalens* (group C) NDH-2 primary structures revealed an example where the most probable α-helices are conserved (Fig. 4), sharing a high probability of having an amphipathic nature. This conservation is extended to other examples of NDH-2A (e.g., *S. cerevisiae* NDH) and NDH-2C (e.g., *S. tokodaii*), but other enzymes exist where no conservation of the position of the amphipathic α-helices was observed (Fig. 4). Nevertheless, the analyses of the secondary structures of all NDH-2 used in this study predicted the presence of amphipathic α-helices, reinforcing our previous hypothesis that this strategy is used for membrane attachment.

The absence of transmembrane domains in most of the NDH-2 considered corroborates the observation that these enzymes do not contribute directly to the formation of a membrane potential (93). In fact, the reaction that they catalyze can take place only on one side of the membrane, making it impossible to translocate protons across the membrane. However, it is clear that they are strongly attached to the membranes: the fact that the activity of NDH-2 increases significantly on incubation with lipids clearly reveals that a hydrophobic environment is needed for their activity (3, 8, 23). Further structural studies are in progress and will help clarify this issue.

Quinone interactions of NDH-2. Quinones, which ensure electron transfer between several membrane-bound electron transport complexes, are essentially of three types: naphthoquinones, benzoquinones, and benzothiophenoquinones. Due to this variability and to the very small number of three-dimensional structures of quinone-containing proteins, it is very difficult to identify, solely on the basis of amino acid sequences, the quinone-binding sites. These structural motifs are often the site of action of several inhibitors. Fisher and Rich proposed the existence of at least two types of quinone-binding sites (21). The type I site is characterized by one conserved central histidine residue flanked downstream by an aliphatic residue, usually a conserved leucine residue, and upstream by a threonine (type IA), leucine (type IB), or serine (type IC) residue. The type II motif contains a conserved tyrosine and a phenyl-

alanine in a central core, flanked upstream by a leucine residue and downstream by an isoleucine and a proline residues. The analysis of NDH-2 sequences from different species and groups identified type I quinone-binding motifs as the predominant ones present in this family of enzymes (Table 2). Moreover, most of the predicted binding sites are located between the two dinucleotide-binding regions, although their relative position is not strictly conserved. An attempt to establish a relationship between the position of the quinone-binding site and the secondary structure of the NDH-2 was also performed, but no evidence was found. In addition, a correlation between quinone-binding types and NDH-2 groups could not be established. However, the presence of different quinone-binding motifs within the NDH-2 family may suggest that the type of quinone-binding site can change according to the nature of the quinone molecule present in each organism, in this way allowing a more effective protein-quinone interaction.

Physiological Role of NDH-2

In organisms where type II NADH:quinone oxidoreductases are the sole NADH oxidizing enzymes, their main function is respiratory chain-linked NADH turnover, with the concomitant production of ATP. Where these proteins coexist with the other ion-gradient generating NADH:quinone oxidoreductases, they are likely to play a more important role in keeping the cell [NADH]/[NAD^+] balance. There are also reports associating the highest levels of NDH-2 expression with specific phases of growth, meaning that they are the main NADH dehydrogenases during these periods.

In *E. coli*, complex I is more strongly expressed during the early and late exponential phases and also during stationary phase (87), while the highest levels of rotenone-insensitive NAD(P)H dehydrogenase expression are found during the exponential phase (24). In addition, under aerobic or nitrate respiratory conditions, NDH-2 is the preferred NADH dehydrogenase, while NDH-1 is preferred during fumarate respiration (83). It was also reported that the *E. coli ndh-2* is repressed under anaerobic conditions (75, 76), due to the direct action of the oxygen-responsive transcription factor of the fumarate and nitrate reduction (FNR), which binds to the *ndh-2* promoter sites FNR I and FNR II (24). Expression of *ndh-2* from *E. coli* is also regulated by the growth phase-responsive transcription factor Fis, which binds to three distinct sites of the *ndh-2* promoter. The binding to Fis I and Fis II promoter sites mediates the activation of protein expression, while binding to the Fis III promoter site leads to repression (29). The expression of the *E. coli* complex I is positively regulated by Fis (87), whose levels are high in the transition of the lag phase of growth to the exponential phase and fall during the exponential phase, in accordance with the decrease in the expression levels of NDH-1 and enhancement of NDH-2 expression, which is the main NADH dehydrogenase, in this phase. The amounts of Fis increase during the stationary phase, leading to a concomitant increase in NDH-1 expression.

At high oxygen concentrations, the *A. vinelandii* NDH-2A is particularly active, while complex I rates of NADH oxidation are significantly lower under the same conditions, suggesting that NDH-2A may play an important role in the respiratory protection of nitrogenase. The K_m of the *A. vinelandii* complex I for NADH (24.2 μM) is higher than that determined for NDH-2 (13 μM), in contrast to what is observed in the respiratory chains of most studied organisms. This peculiarity emphasizes the idea that the oxidation of NADH by the respiratory chain of *A. vinelandii* may occur mainly via the alternative NADH dehydrogenase (6). It is suggested that the NDH-2A from *Synechocystis* spp. are redox sensors that play a regulatory role responding to the redox state of the plastoquinone pool and the cytosolic levels of NADH (28). The *N. crassa* NDH is the sole NDH-2 reported to be responsible for matrix NADH oxidation; it is functionally complementary to complex I and may play an important role during spore germination (17). Recent studies of potato leaves revealed that the expression of *nda1* is sensitive to cold and is light dependent; the later effect suggests that it could be related to photosynthesis (81).

From the examples considered in the present study, the presence of non-proton-pumping NAD(P)H dehydrogenases varies among different organisms, where examples of enzymes from groups A, B, and C can be observed. Their specific role is still unclear in many cases, but they might be involved in situations of NAD(P)H stress. In addition to metabolic functions, a regulatory role in response to the quinone pool redox state (28) or to the oxygen concentration (6) has been proposed. In *N. crassa*, these enzymes might play an important role in metabolic adaptation of the organism to different carbon sources when sucrose, the most effective substrate, is not available (17). In *Y. lipolytica*, the external NDH-2 was redirected to the matrix, where it was able to rescue complex I mutants which were lethal in its absence (34). Different organisms have different roles for NDH-2, suggesting that these enzymes give them the plasticity to better adapt to their environmental conditions.

A Therapeutic Application of NDH-2

In humans, complex I is solely responsible for the oxidation of matrix NADH, and shuttle mechanisms exist for the oxidation of cytosolic NADH. Mutations in mtDNA genes, for instance complex I genes, can be responsible for drastic changes is phenotypes (88); the same happens with nuclear gene mutations (84). Complex I defects associated with human pathologies failed to be healed by chemotherapy (12). The capacity of type II NADH:quinone oxidoreductases to perform the turnover of NADH may be more important to health than their inability to pump protons. As previously suggested, it is also possible that the oxidation of NADH via NDH-2 can contribute to the formation of an electrochemical potential difference of sufficient magnitude to fulfill the cellular requirements if the enzyme turnover number is large, thus increasing the rates of NADH oxidation and, concomitantly, the flux over the respiratory chain.

An approach to overcoming complex I defects is to introduce in patient cells a type II NADH:quinone oxidoreductase to restore the function of oxidizing NADH in their mitochondria (36). The internal NADH dehydrogenase from *S. cerevisiae* (NDI1) was expressed in *E. coli* (36) and in complex I-deficient Chinese hamster cells (70), where they functioned as a member of the respiratory chain in the host cells. NDI1 was able to restore the NADH oxidase activity in the latter case. Human kidney cells were also transfected by the gene

encoding NDI1. The enzyme was successfully transcribed and translated to produce a functional enzyme linked to the electron transport chain of the host cell mitochondria (71). More recent experiments revealed that the *S. cerevisiae* NDH was expressed successfully in human embryonic kidney 293 cells and that it could complement complex I activity in cells with respiratory deficiencies caused by NDH-1 defects. Moreover, the enzyme was functionally active in nonproliferating human cells, which were able to grow in the presence of the complex I inhibitor rotenone (72). A very encouraging observation was the fact that the expression of NDI1 in human cells lacking the mitochondrially encoded ND4 subunit of complex I, which is essential for enzyme activity, completely restored NADH dehydrogenase activity. In addition, when the gene encoding NDI1 was introduced into the nuclear genome of a human cell line, the protein was located in mitochondria and had its binding site facing the matrix (1). These achievements confirm the potential of the NDH-2 enzymes to eventually cure human complex I-mediated diseases.

FINAL REMARKS

Type II NAD(P)H:quinone oxidoreductases are an example of apparently redundant proteins, which end up playing crucial roles with such plasticity that the roles can change according to which organisms they belong and to which conditions they are submitted, thus contributing to their robustness. Knowledge about these enzymes has extended the possibilities available to to overcome complex I failures in human patients, thus encouraging fundamental research in this field.

Finally, the study of rotenone-insensitive NADH dehydrogenases, started in the laboratory bench, has moved to computers in the genomic era. The large amount of precious information available in the genomes must be converted into knowledge in a post-genomic age; this can be done only by going back to the laboratory and testing the genomic suggestions.

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