Biochimica et Biophysica Acta 1777 (2008) 453–461

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Purification of two putative type II NADH dehydrogenases with different substrate specificities from alkaliphilic *Bacillus pseudofirmus* OF4 ☆

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ARTICLE INFO

Article history:
Received 8 January 2008
Received in revised form 26 February 2008
Accepted 27 February 2008
Available online 5 March 2008

Keywords:
Type II NADH dehydrogenase
Flavoprotein
Deamino-NADH
NAD(P)H: menadione oxidoreductase
Alkaliphile
Na^+/H^+ antiporter

ABSTRACT

A putative Type II NADH dehydrogenase from *Halobacillus dabanensis* was recently reported to have Na^+/H^+ antiport activity (and called Nap), raising the possibility of direct coupling of respiration to antiport-dependent pH homeostasis. This study characterized a homologous type II NADH dehydrogenase of genetically tractable alkaliphilic *Bacillus pseudofirmus* OF4, in which evidence supports antiport-based pH homeostasis that is mediated entirely by secondary antiport. Two candidate type II NADH dehydrogenase genes with canonical GXGXXG motifs were identified in a draft genome sequence of *B. pseudofirmus* OF4. The gene product designated NDH-2A exhibited homology to enzymes from *Bacillus subtilis* and *Escherichia coli* whereas NDH-2B exhibited homology to the *H. dabanensis* Nap protein and its alkaliphilic *Bacillus halodurans* C-125 homologue. The *ndh-2A*, but not the *ndh-2B*, gene complemented the growth defect of an NADH dehydrogenase-deficient *E. coli* mutant. Neither gene conferred Na^+-resistance on an antiporter-deficient *E. coli* strain, nor did they confer Na^+/H^+ antiport activity in vesicle assays. The purified hexa-histidine-tagged gene products were approximately 50 kDa, contained noncovalently bound FAD and oxidized NADH. They were predominantly cytoplasmic in *E. coli*, consonant with the absence of antiport activity. The catalytic properties of NDH-2A were more consistent with a major respiratory role than those of NDH-2B.

1. Introduction

Recently a putative NADH dehydrogenase gene (designated *nap*) was reported from *Halobacillus dabanensis*, which conferred Na^+-resistance and Na^+/H^+ antiport activity when expressed in an antiporter-deficient mutant of *Escherichia coli* [1]. The possible coupling of redox and antiport capacities was intriguing since it could have important implications for the energetics of alkaliphilic bacteria. These organisms use robust Na^+/H^+ antiport activity to bring protons inside under very alkaline growth conditions to achieve a rather dramatic pH homeostasis, in which the interior of the cell is often maintained more than 2 pH units below the alkaline growth media [2]. Since typical secondary Na^+/H^+ antiport activity is energized by the transmembrane electrochemical proton gradient, protonmotive force, that is set up by respiration [3], there have been periodic suggestions of direct connections between respiration and antiport to carry out pH homeostasis when cells are thriving in extremely alkaline pH media, e.g., pH 10.5 to 11 at which the protonmotive force is low [2]. No compelling evidence of specific examples of such a connection has yet been presented. Rather, alkaliphile pH homeostasis thus far appears to depend upon secondary Na^+/H^+ antiporters that may be particularly adapted to achieve effective proton-gathering on their external surfaces [4,5]. Since alkaliphilic *Bacillus halodurans* C-125 has a homologue of the *H. dabanensis nap* gene [6], we anticipated that the genetically tractable alkaliphile *Bacillus pseudofirmus* OF4 was likely to have a homologue as well. If the enzyme proved to have both NADH dehydrogenase and antiport capacity, it would be possible to use targeted gene deletions and other genetic strategies to determine the physiological role of the enzyme/transporter. In this study we used the annotated alkaliphilic *B. halodurans* C-125 genome as a guide to finding two putative NADH dehydrogenase genes in an unpublished draft sequence of the *B. pseudofirmus* OF4 genome. One of these genes exhibited significant sequence similarity to the *ndh-2* gene of *B. subtilis*, and was designated *ndh-2A*, and the other exhibited significant sequence similarity to the *H. dabanensis nap* gene and was designated *ndh-2B*. The cloned genes were tested for the ability to complement *E. coli* mutants with deficiency in either NADH dehydrogenases or Na^+/H^+ antiporters and the enzymatic properties of the purified products of the genes were then determined. Both enzymes proved to be authentic NADH dehydrogenases that carried out NADH oxidation coupled to the reduction of several different substrates. Neither enzyme was able to complement an Na^+/H^+ antiporter mutant or confer membrane-associated antiport activity. Their catalytic properties suggested that NDH-2A is likely to be the physiologically important donor of electrons from NADH to the respiratory chain.

2. Materials and methods

2.1. Materials

Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. Nickel-nitrilotriacetic acid resin and plasmid kits were obtained from Qiagen.

☆ DNA sequences: new sequences for Bp *ndh-2A* and *ndh-2B* were deposited with GenBank accession no. EU030627 and EU030628.

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doi:10.1016/j.bbabio.2008.02.004

FAD, FMN, Riboflavin, NADH, deamino-NADH, NADPH, Q1 and menadione were purchased from Sigma Chemical Co. (U.S.A).

2.2. Bacterial strains and growth conditions

B. pseudofirmus OF4 strain 811M was grown at 30 °C in a semi-defined medium containing 50 mM sodium malate, 0.1% yeast extract with mineral salts and buffered with 0.1 M Na_2CO_3/$NaHCO_3$ at pH 10.5 [7]. *E. coli* KNabc (Δ*nhaA*Δ*nhaB*Δ*chaA*) lacking three major Na^+/H^+ antiporters was routinely grown in LBK medium consisting of 1% tryptone, 0.5% yeast extract, and 0.6% KCl, pH 7.5 with 50 μg/ml kanamycin (Km). *E. coli* ANN0222 (Δ*nuo*Δ*ndh*) without NADH dehydrogenase was grown in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.5) with 50 μg/ml kanamycin. *E. coli* strain Top 10 was grown in LB medium at pH 7.5. For complementation growth experiments, transformants of *E. coli* KNabc were grown in 2 ml of LBK with different concentrations of NaCl at 37 °C, with 250 rpm shaking, for 16 h. Arabinose (0.5%, w/v) was added in some experiments. Transformants of *E. coli* ANN0222 were grown on LB medium or M9 minimal medium (1× M9 salts, 2× 10^{-3} M $MgSO_4$, 10^{-4} M $CaCl_2$, 0.4% mannitol) with different concentrations of arabinose for induction [8]. The growth medium of plasmid transformants also contained 100 μg/ml ampicillin (Ap).

2.3. Cloning of the putative NADH dehydrogenase genes

Genomic DNA of *Bacillus* OF4 was extracted with Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories). Primers were designed on the unpublished *ndh-2A* and *ndh-2B* nucleotide sequences (GenBank accession no. EU030627 and EU030628, respectively). The primers used for cloning the *ndh-2A* with an added C-terminal His_6-tag were 5′-ACCATGGAAGTGATTAGTTTGAAAAAG-3′ (NDH-2AF) and 5′-CAATGGGTTTTTCCCT-TTTTTC-3′ (NDH-2AH6R); the primers for the untagged version of *ndh-2A* were NDH-2AF and 5′-TTACAATGGGTTTTTCCCTTTTT-3′ (NDH-2AR); the primers used for cloning the *ndh-2B* with the additional C-terminal His_6-tag were 5′-GAGGAATAATAAATGAC-GATGAAATATGTAATTATC-3′ (NDH-2BF) and 5′-CCCTCTGCGCCTTGATGCC-3′ (NDH-2BH6R); the primers for the untagged form of *ndh-2B* were NDH-2BF and 5′-TTACC-CTCTGCGCCTTGATG-3′ (NDH-2BR). PCR reactions were carried out in a MJ mini thermal cycler (Bio-Rad) with a Taq DNA polymerase (TaKaRa, ExTaq hot start version). The resulting four PCR products were ligated with pBAD-TOPO vector (Invitrogen) and then transformed into *E. coli* Top10 on selective LB-ampicillin plates (pH 7.5) at 37 °C. The correct insert direction was identified by restriction analysis of the recombinant plasmids. These recombinant plasmids were designated as pBAD-NDH-2A-HM, pBAD-NDH-2A-NM, pBAD-NDH-2B and pBAD-NDH-2B-N respectively. The pBAD-NDH-2A-HM and pBAD-NDH-2A-NM were digested by NcoI and the resulting bands of about 5.3 kb were self-ligated respectively. These new constructs were designated as pBAD-NDH-2A and pBAD-NDH-2A-N. The pBAD cloning for the His-tagged constructs results in an additional 28 amino acids, including the six histidine residues, added to the C-terminus of the two proteins. The non-His-tagged NDH-2A has 405 amino acids and an Mr of 44,600. The native NDH-2B has 440 amino acids and an Mr of 48,600. With the His-tags, the sizes of NDH-2A and NDH-2B are 47,700 and 51,700, respectively. Sequence analyses were conducted in the Mount Sinai School of Medicine DNA Core Facility.

2.4. Complementation assays in E. coli mutant strains

pBAD-LacZ (pBAD-TOPO/*lac*Z/V5-His vector, Invitrogen), pBAD-NDH-2A, pBAD-NDH-2B and pBAD-OF4-Mrp were transformed into *E. coli* KNabc and *E. coli* ANN0222. The pBAD-OF4-Mrp from *B. pseudofirmus* OF4, provided by Dr. Masahiro Ito (Toyo University), served as a positive control for *E. coli* KNabc complementation and was tested for the ability to complement *E. coli* ANN0222. The plasmid pBAD-lacZ was used as a negative control for both strains. For complementation of *E. coli* KNabc transformants, 10 μl of overnight pre-culture grown in LBK was inoculated into 2 ml of LBK medium with different concentrations of NaCl, and after 16 h, the A_{600} was recorded. In some experiments, 0.5% of arabinose (w/v) was added. For complementation of *E. coli* ANN0222, transformants were grown on M9 minimal medium, with different concentrations of arabinose to vary the extent of induction and after 48 h, the A_{600} was recorded. As a control, the *E. coli* ANN0222 transformants were grown on LB medium with different concentrations of arabinose to induce and after 16 h, the A_{600} was recorded. All complementation assays were performed in duplicate in at least two separate experiments.

2.5. Isolation of everted membrane vesicles and assay of Na^+/H^+ antiporter activity

Liquid cultures were inoculated with 1% (v/v) of overnight pre-cultures of *E. coli* KNabc transformants. The pre-culture and final cultures were grown in LBK at 37 °C. When the cultures reached an A_{600} of 0.5, arabinose was added to the final concentration of 0.5% (w/v) except for the *E. coli* KNabc/pBAD-OF4-Mrp, in which 0.005% arabinose was used. Cells were harvested at an A_{600} of 2.5 to 3.0 after induction. Everted membrane vesicles were prepared from *E. coli* KNabc transformants as described [9]. The buffers used in the preparation were 10 mM Tris–HCl, pH 7.5, containing 140 mM choline chloride, 0.5 mM dithiothreitol, 10% glycerol, a protease inhibitor tablet (Roche), 1 mM phenylmethylsulfonylfluoride (PMSF) and a trace amount of DNase I (Roche). Protein content was measured by the Lowry method using bovine serum albumin as the standard [10]. Assays of monovalent cation/H^+ antiport were conducted using acridine orange (AO) as a fluorescent probe of the transmembrane pH gradient (ΔpH, acid in) as described [11]. The assay mixtures were made up to a total volume of 2 ml containing: 10 mM Tris–HCl (pH from 6.5 to 9), 140 mM choline chloride, 5 mM $MgCl_2$, 1 μM AO and 75 μg of vesicle protein. Respiration was initiated by the addition of Tris–succinate to a final concentration of 2.5 mM. Fluorescence was monitored with a Shimadzu RF-5301PC fluorescence spectrofluorophotometer at excitation and emission wavelengths of 420 nm and 500 nm, respectively. All assays were conducted in duplicate or triplicate in 2–3 independent experiments on different preparations.

2.6. Expression and purification of His-tagged NDH-2A and NDH-2B

An overnight culture of *E. coli* Top10/ pBAD-NDH-2A was inoculated (1%) into LB medium, with 225 rpm shaking at 30 °C. At an A_{600} of 0.8, 0.002% arabinose was added. The cells were harvested after 3 h induction, washed twice with a solution containing 50 mM Tris–HCl, pH 8, and stored at −80 °C. For the expression of NDH-2B, the *E. coli* Top10/ pBAD-NDH-2B was cultured at 37 °C with 225 rpm shaking. At an A_{600} of 0.5, 0.002% arabinose was added. The cells were harvested after 5 h induction, washed twice with a solution containing 50 mM Tris–HCl, pH 8, and stored at −80 °C.

After thawing, the cells were suspended in lysis buffer containing 50 mM Tris–HCl, pH 8, 300 mM NaCl, 10 mM imidazole, a protease inhibitor tablet (Roche), 1 mM PMSF and a trace amount of DNase I (Roche). The cells were broken in a French Press Cell under 10,000 p.s.i. pressure. The broken cell suspensions were centrifuged at 14,000 g for 15 min to precipitate unbroken cells and debris. The membrane vesicles were pelleted by ultracentrifugation at 250,000 g (Beckman Ti60 rotor) for 1 h at 4 °C and the resulting supernatant was subsequently used for purification. All subsequent steps of purification were performed at 4 °C. His-tagged protein was purified by chromatography using Ni-NTA resin (Qiagen). The resin was pre-equilibrated with lysis buffer containing 50 mM Tris–HCl, pH 8, 300 mM NaCl and 10 mM imidazole. Two milliliter of 50% Ni-NTA slurry was added to the lysate and then gently mixed for 1 h at 4 °C. The lysate–Ni-NTA mixture was transferred to a small column and washed twice with 6 ml wash buffer containing 50 mM Tris–HCl, pH 8, 300 mM NaCl, 20 mM imidazole. The bound enzyme was eluted with elution buffer containing 50 mM Tris–HCl, pH 8, 300 mM NaCl, 250 mM imidazole and the active, yellow fractions were pooled. Glycerol was added to 30% (w/v), and the enzyme was quick-frozen in liquid nitrogen and stored at −80 ° C. In some cases, during the purification of His_6-tagged NDH-2A protein, 20 μm FAD was added to all the buffers as detailed in the Results section. The NDH-2B was concentrated using Amicon Ultra-15 (Millipore) with a pore size of 10 kDa before the storage. It was found that concentration of NDH-2A resulted in loss of flavin, so the Ni-NTA pooled peak fraction was not concentrated before freezing.

2.7. Flavin analysis: thin-layer chromatography, UV/visible spectra, fluorescence spectroscopy

For the spectral analysis and thin-layer chromatography, a larger scale preparation of NDH-2A was isolated without added FAD using a minimal volume of Ni-NTA resin so that it eluted in a concentrated form. It was then immediately used for the spectral analysis, due to the lability of its flavin moiety. As a consequence, the spectra of NDH-2A were recorded on samples in the Ni-NTA elution buffer, i.e., 50 mM Tris–HCl, pH 8, 300 mM NaCl, and 250 mM imidazole, at a concentration of 13.5 mg/ml. The spectra of NDH-2B, which was 2.65 mg/ml, were recorded from samples in 50 mM Tris–HCl pH 8.0 buffer that also contained 30% glycerol. UV-Visible spectra of the purified enzyme were recorded at room temperature in a final volume of 0.5 ml with a Shimadzu UV-2501PC UV-Vis recording spectrophotometer. Fluorescence spectra of the purified enzyme were also recorded at room temperature with a Shimadzu RF-5301PC fluorescence spectrofluorophotometer with a slit width of 5 nm for both excitation and emission wavelengths (2 ml volume). Fluorescence spectra of NDH-2A were obtained by excitation at 477 nm and monitoring of emission at 522 nm. Fluorescence spectra of NDH-2B were obtained by excitation at 475 nm and emission at 520 nm.

The type of flavin was determined by thin-layer chromatography (TLC). Two different methods were used to extract the flavin from the purified proteins. For one method, the protein (about 5 mg) was boiled for 5 min followed by centrifugation which resulted in a yellow supernatant and an uncolored pellet. For the second method, the protein was precipitated with 10% trichloroacetic acid (TCA) for 30 min on ice followed by centrifugation. The TCA was removed from the yellow supernatant by washing 3 times with 2 volumes of ether [12]. The supernatant samples were concentrated 15-fold under vacuum, and then were loaded onto silica gel plates (250 μm layer, 20×20 cm, catalogue no.4410222, Whatman, England). The plates were eluted in either solvent A (2% [w/v] Na_2PO_4 in water) or solvent B (*n*-butanol/glacial acetic acid/water, 2:1:1), and flavins were detected as fluorescent spots upon ultraviolet irradiation.

2.8. Enzyme assays

All enzyme assays were performed at room temperature in a Shimadzu UV-2501PC UV-Vis recording spectrophotometer. The assay volume was 1 ml and was buffered with either 50 mM BTP (bis-[tris(hydroxymethyl)methylamino]-propane) from pH 6-9.5 or citric acid-phosphate buffer from pH 3 to 7. The reductant and enzyme were added to different sides of the cuvette above the liquid and the reaction was initiated by rapid manual mixing. Between 1 and 10 μg of protein was used in the assays. In some assays, selected cations were added to determine if they affected activity. FAD (20 μM) was added in assays of NDH-2A. With the exception of ferricyanide oxidoreductase activity measurements, reactions were followed by the decrease in A_{340}, reflecting the

oxidation of the electron donor (NAD(P)H or deamino-NADH, which were used at 200 μM). With each set of assays, the background activity in the absence of protein was determined and the background was subtracted from the activity observed in the presence of protein with oxygen as the electron acceptor [13]. The corrected oxidase activity was then subtracted from the rates observed with enzyme and menadione or Q1 (50 μM) as the electron acceptor. For the electron acceptor ferricyanide, the

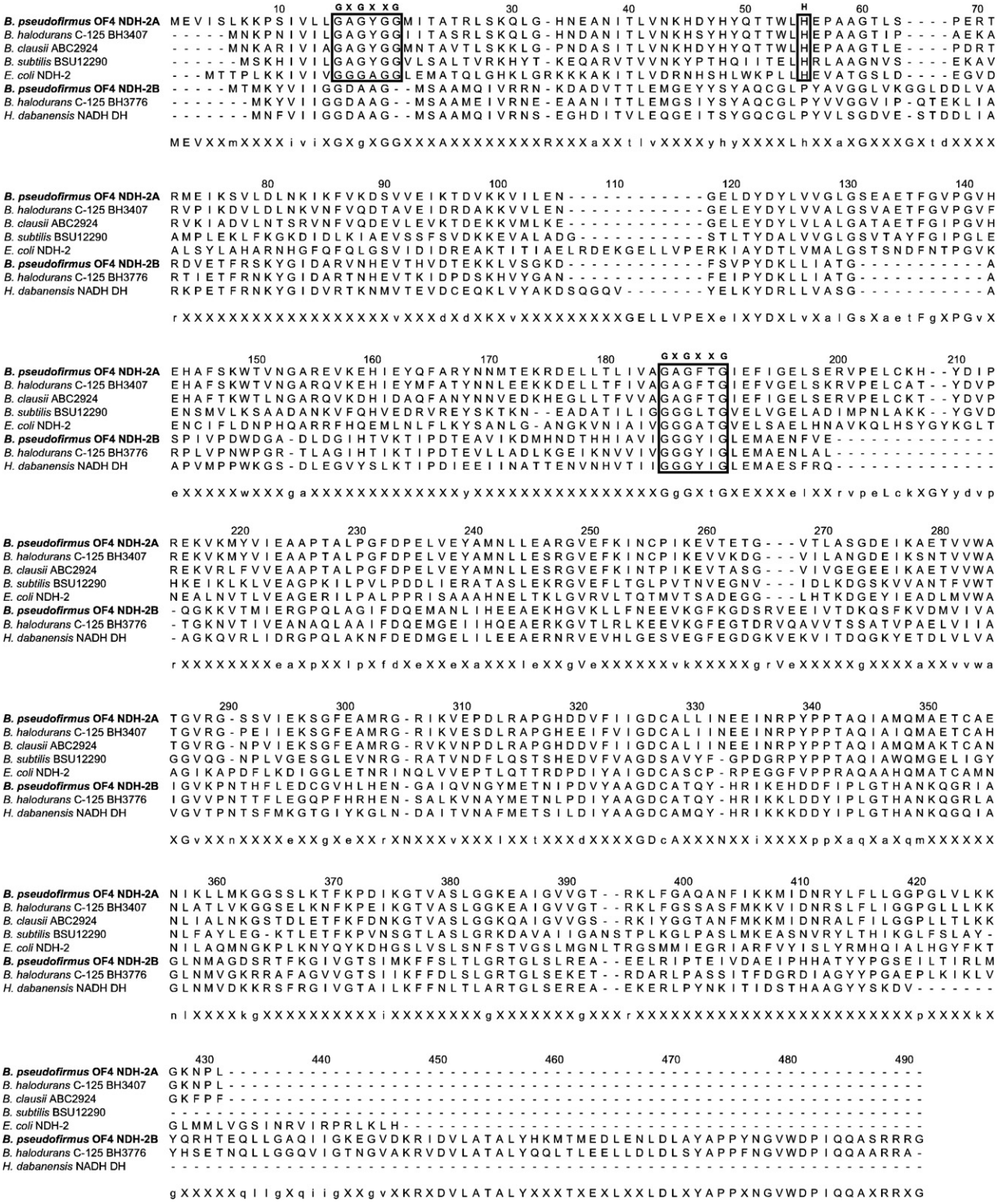

Fig. 1. Sequence alignment of NDH-2A and NDH-2B with selected bacterial NADH dehydrogenases. The alignment was created using ClustalW. The large boxed sequences show the GXGXXG motifs, which for NDH-2A are at residues 15–20 and 168–173. Note that NDH-2B lacks a complete N-terminal GXGXXG motif. Also boxed is a conserved histidine, equivalent to *E. coli* NDH-2 His52, which has been modeled as a potential quinone-binding residue [20]. Interestingly, that histidine (His54) is conserved in *B. pseudofirmus* OF4 NDH-2A, which has robust quinone oxidoreductase activity, but is not conserved in NDH-2B, which has extremely low quinone-dependent activity. The consensus sequence below the alignment shows the amino acid in lower case when more than half of the proteins have that residue in that position, while the amino acid is capitalized where the residue is 100% conserved. *B. pseudofirmus* OF4 NDH-2A, accession #EU030627, *B. pseudofirmus* OF4 NDH-2B, accession #EU030628, *B. halodurans* C-125 BH3407, accession #NP_244273, *B. clausii* ABC2924, accession #YP_176419, *B. subtilis* BSU12290, accession #NP_389111, *E. coli* NDH-2, accession #NP_415627, *B. halodurans* C-125 BH3776, accession #NP_244643, and *H. dabanensis* NADH DH, accession #ABF60143.

reactions were monitored at A_{420}, using 1 mM NAD(P)H or deamino-NADH and 1 mM $K_3Fe(CN)_6$. For this set of reactions, the background activity without protein was subtracted from the enzymatic rates to yield the true enzyme activities. The following extinction coefficients (mM^{-1}, cm^{-1}) were used in the calculations: NADH (O_2 or menadione as electron acceptor), 6.2 at 340 nm; NADH (Q1 as electron acceptor) [14], 6.81 at 340 nm; ferricyanide, 1.0 at 420 nm [15]. However, since we are defining 1 U of enzyme activity as 1 μmol of NADH oxidized per minute, we have divided the ferricyanide reductase activity by 2 to reflect the activity on the basis of 2 electrons, i.e., per molecule of NADH. All assays were conducted in duplicate or triplicate in 2–3 independent experiments on different preparations.

2.9. Other biochemical analyses

Protein content was determined by the method of Lowry et al. [10], using bovine serum albumin as a standard. Proteins were resolved on 12% SDS-PAGE gels [16]. The gels were stained with colloidal Coomassie Brilliant Blue G (National Diagnostics) or transferred to nitrocellulose membranes, and His-tagged proteins were detected by chemiluminescence (Pierce) with the INDIA-anti-His probe (Pierce).

2.10. Bioinformatics

Secondary structure predictions were done with the meta-search engine PredictProtein (http://cubic.bioc.columbia.edu/predictprotein/) and with AMPHIPASEEK (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_amphipaseek.html). Algorithms for topology predictions for the presence of transmembrane helices were done through the meta-search engine ExPasy-tools (http://www.expasy.org/tools/#ptm).

3. Results and discussion

3.1. Identification, sequence analyses of NDH-2A and NDH-2B and topological predictions on their products

Type II (NDH-2) NADH dehydrogenases have one or more GXGXXG motifs that are adenine dinucleotide-binding motifs used in the non-covalent binding of flavin and NAD(P)H [17]. As a guide for finding putative *ndh-2* genes in a draft genome sequence from *B. pseudofirmus* OF4, we first analyzed the completed genome of the alkaliphile *B. halodurans* C-125 [6] for genes whose predicted products contained this motif. Three such genes were identified, with locus tags of BH3407, BH3415, and BH3776. One of the three genes, BH3415, has a predicted product with three GXGXXG motifs. This protein does not fall directly into any of the three groups of NDH-2 enzymes as described by Melo et al. [17]. We found an incomplete open reading frame in the *B. pseudofirmus* OF4 genome whose predicted product similarly has three motifs that correspond to those in the *B. halodurans* C-125 gene BH3415 product. These unusual genes are intriguing but for the purposes of this project we decided not to pursue them at this time. Two complete putative NADH dehydrogenase-encoding genes in the *B. pseudofirmus* OF4 genome were homologues of BH3407 and BH3776, with predicted products containing 2 and 1 of the GXGXXG motifs respectively. The BH3407 homologue was designated *ndh-2A* and the BH3776 homologue was named *ndh-2B*. An alignment of the two alkaliphile proteins with some related NADH dehydrogenases is shown in Fig. 1. NDH-2A has strong sequence similarity to the predicted product of BH3407 of *B. halodurans* C-125 (81% identity; 325/400 aa) as well as an NADH dehydrogenase gene product from another alkaliphile, *Bacillus clausii* KSM-K16 (ABC2924) (75% identity; 301/400 aa). NDH-2A also shows significant sequence similarity with YjlD, the proposed NDH-2 of *Bacillus subtilis* [18] (locus tag BSU12290) (37% identity; 143/386 aa) and to the well-studied NDH-2 of *E. coli* (29% identity; 118/397 aa) [15]. NDH-2B shows a high sequence similarity to the predicted product of BH3776 from *B. halodurans* C-125 (62% identity; 272/436 aa) and to the putative NADH dehydrogenase (Nap) from *H. dabanensis* (56% identity; 207/367 aa) that prompted this study.

The function of type II NADH dehydrogenases requires that they interact with the membrane in order to transfer electrons from NADH to the quinone pool during respiration. The specific nature of the membrane-association for many of the NDH-2 proteins is not well-established. Many of the enzymes in this group lack a transmembrane helix (according to topology prediction programs) that would anchor them to the membrane. Although the NDH-2 proteins lacking a transmembrane helix may be peripheral membrane proteins, they generally have been purified as if they were integral membrane proteins in the sense that they have been extracted from everted vesicles with detergent and detergent was included in all purification steps; for examples see references [8,14,15,19]. It has been proposed that amphipathic α-helices may mediate membrane-association [19–21] for the category of NDH-2 proteins that do not appear to have a transmembrane helix, a group that also includes the *B. pseudofirmus* OF4 NDH-2A and NDH-2B proteins as predicted by a number of programs (PSORTb, HMMTOP, DAS, SOUSI). According to secondary structure predictions, the alkaliphile proteins contain a number of α-helices, some of which exhibit amphipathic character that may be involved in membrane-association, although these potential helices do not fall neatly into the classes of amphipathic helices reviewed in [22]. Consistent with the predicted absence of a transmembrane helix, the localization of both overexpressed NDH-2A and NDH-2B in an *E. coli* host is predominantly cytoplasmic, as described in Section 3.3.

3.2. Complementation studies with the cloned NADH dehydrogenase genes and antiport assays

To test whether the *ndh-2A* and *ndh-2B* of *B. pseudofirmus* OF4 encode NADH dehydrogenases that are functional *in vivo*, a strain of *E. coli* lacking both NDH-1 and NDH-2 (*E. coli* ANN0222) was used for complementation assays. This strain is unable to grow on a minimal medium (M9) containing mannitol as the sole carbon source [23]. Four transformants were examined for complementation of this phenotype. The *ndh-2A* and *ndh-2B* genes were cloned in the pBAD vector and studied in comparison with two control plasmids, pBAD containing *lacZ* as an insert and pBAD into which the full *mrp* operon of *B. pseudofirmus* OF4 was cloned. Mrp, the major Na^+/H^+ antiporter of alkaliphilic *Bacillus* species, has subunits with sequence similarity to membrane subunits of proton-pumping NADH dehydrogenases (type I) but does not have NADH oxidation capacity [5,24,25]. Since Mrp has

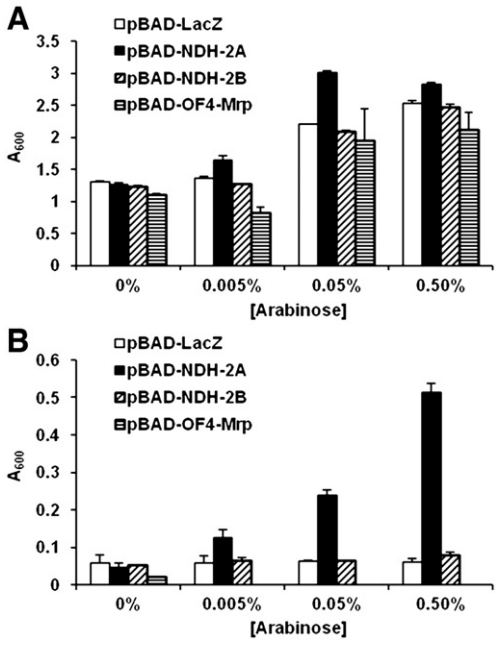

Fig. 2. Growth of transformants of *E. coli* mutant ANN0222 (Δ*ndh*Δ*nuo*) with pBAD-lacZ, pBAD-OF4-Mrp, pBAD-NDH-2A and pBAD-NDH-2B on (A) LB medium or (B) M9 minimal medium containing mannitol as the major carbon source with various concentrations of arabinose (i.e., different levels of induction). The A_{600} were recorded after 16 h at LB medium or 48 h at M9 medium. Values are the average of duplicate determinations from at least 2 independent growth experiments and the error bars show the standard deviation.

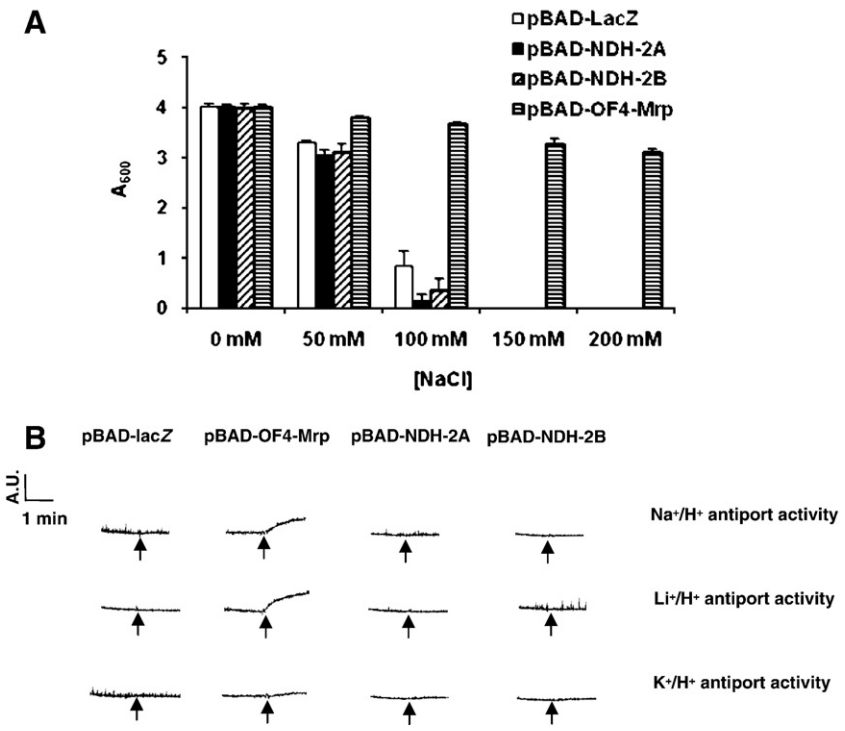

Fig. 3. A. Complementation assays of *E. coli* KNabc transformants with pBAD-lacZ, pBAD-OF4-Mrp, pBAD-NDH-2A and pBAD-NDH-2B. *E. coli* KNabc transformants were grown on LBK medium, pH 7.5 in the presence of various concentrations of NaCl. The A_{600} values were recorded after 16 h. Values are the average of duplicate determinations from at least 2 independent growth experiments and the error bars show the standard deviation. B. Assay of Na^+/H^+, Li^+/H^+, and K^+/H^+ antiport activity of *E. coli* KNabc transformants with pBAD-lacZ, pBAD-OF4-Mrp, pBAD-NDH-2A and pBAD-NDH-2B. Assays were conducted at 10 mM Tris–HCl, pH 8. The arrows indicate the addition of 5 mM NaCl, LiCl or KCl after the acridine orange quenching had leveled off. A.U. represents the arbitrary units of fluorescence, where 1 A.U. as shown represents 20% dequenching.

no NADH oxidative activity, it was anticipated that it would not complement *E. coli* ANN0222 and thus would yield the same results as the negative control, pBAD with *lacZ*. As shown in Fig. 2A, the four types of transformants exhibited comparable growth in LB medium with different amounts of the inducer molecule, arabinose. By contrast, only the *ndh-2A* transformant showed significant growth on M9-mannitol minimal medium and, unlike the other transformants, its growth increased as the arabinose concentration was increased (Fig. 2B).

To assess whether NDH-2A or NDH-2B has Na^+/H^+ antiport activity, complementation studies of the sodium-sensitive triple antiporter mutant *E. coli* KNabc (Δ*nhaA*Δ*nhaB*Δ*chaA*) were carried out. Since the Mrp antiporter confers Na^+-resistance and Na^+/H^+ antiport activity on this strain [26], the pBAD-*mrp* plasmid was a positive control in these experiments. The sensitivity of *E. coli* KNabc to concentrations of sodium chloride at 100 mM or higher was complemented by *mrp* expression but *ndh-2A* and *ndh-2B* expression resulted in no increase in sodium-resistance (Fig. 3A). The results shown in Fig. 3A were obtained with no arabinose induction, because arabinose severely inhibited the growth of the *mrp* transformant in LBK with no added sodium. In experiments not shown, neither *ndh-2A* nor *ndh-2B* conferred any sodium-resistance when 0.5% arabinose was added to the medium even though they both grew well in LBK under these conditions.

It was possible that the products of the cloned genes catalyzed Na^+/H^+ antiport activity without conferring observable sodium-resistance or used a different monovalent cation such as potassium in the antiport reaction. To assess these possibilities, everted vesicles from the *E. coli* KNabc transformants were prepared and assayed for Na^+/H^+, Li^+/H^+ and K^+/H^+ antiport activity as detailed in the Materials and methods. A range of pH values from 6.5 to 9 was used for these assays and 5 or 50 mM monovalent cation was tested for dequenching activity (a measure of antiport activity). Only the *mrp* transformant showed $Na^+(Li^+)/ H^+$ antiport activity, and neither the *ndh-2A* nor the *ndh-2B* transformant exhibited $Na^+(Li^+, K^+)/H^+$ antiporter activity under any of the tested conditions. This is illustrated in Fig. 3B, at pH 8.0 and 5 mM monovalent cation.

3.3. Expression, purification of NDH-2A and NDH-2B

Before undertaking purification efforts on His-tagged NDH-2A and NDH-2B, preliminary experiments were carried out on the *E. coli* ANN0222 transformants expressing these proteins to assess whether localization of the gene products was primarily cytoplasmic, as suggested by the topology predictions (Section 3.1.). As detailed in Materials and methods, quantitative Western blots of both proteins showed that they were predominantly in the cytoplasmic fraction. Only 18±7% (n=4 independent preparations) of the total NDH-2A and 5±2% (n=3 independent preparations) of the total NDH-2B was found in the membrane fraction even without salt washes of the membrane

Table 1
Effects of FAD or salts on the activity of NADH: menadione oxidoreductase activity of purified NDH-2A and NDH-2B

	NDH-2A		NDH-2B	
20 μM FAD added	Specific activity	% of no addition	Specific activity	% of no addition
None	9.48±1.68	100	0.69±0.08	100
Yes	32.85±2.43	347	0.77±0.09	112
Salt added [a]				
None	32.85±2.43	100	0.69±0.08	100
NaCl, 100 mM	46.38±0.41	141	0.76±0.08	110
KCl, 100 mM	48.20±2.00	147	0.71±0.06	103
LiCl, 100 mM	45.41±1.21	138	0.81±0.06	117
$CaCl_2$, 5 mM	51.67±0.90	157	0.70±0.08	101
$MgCl_2$, 5 mM	43.10±2.69	131	0.74±0.02	107

[a] All NDH-2A assays contained 20 μM FAD in addition to the indicated salt. No FAD was added to the NDH-2B assays. Specific activity is expressed as U/min mg.

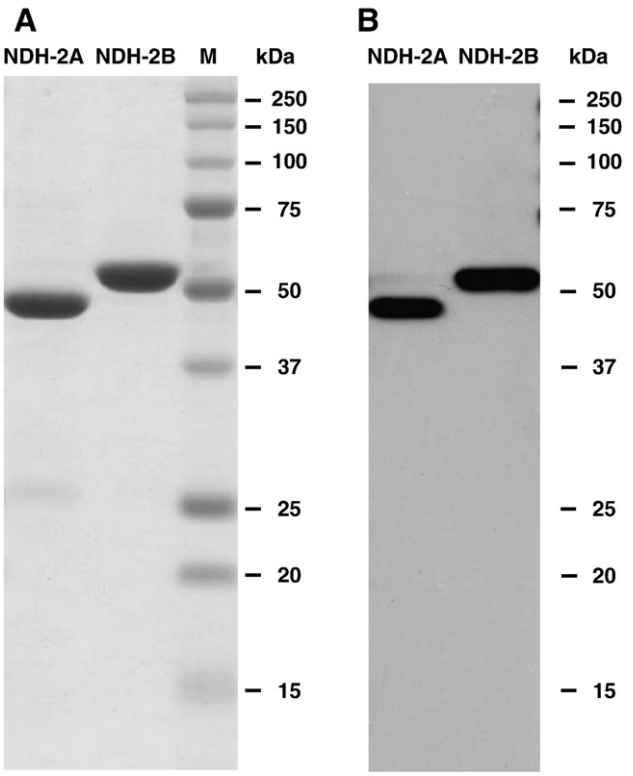

Fig. 4. A.SDS-PAGE of the purified NDH-2A and NDH-2B. Three micrograms of either NDH-2A or NDH-2B were loaded on an SDS 12% acrylamide gel, and the gel was stained with Coomassie blue. B. Western blotting analysis of the purified NDH-2A and NDH-2B, in which 0.2 μg of purified protein was loaded for each lane.

fraction. The low membrane-association of NDH-2B was consistent with the absence of antiport activity conferred by expression of that protein since all known antiporter proteins are polytopic membrane proteins that would associate strongly with the membrane fraction. To account for the complementation of the NADH dehydrogenase-deficient *E. coli* ANN0222 mutant by NDH-2A, we hypothesize that it transiently binds to the membrane during electron transfer in order to carry out the reduction of the membrane quinone pool. The membrane-binding ability of NDH-2A may be different, and possibly stronger, in *B. pseudofirmus* OF4 than in *E. coli* since the alkaliphile has membrane phospholipids that are particularly anionic, with strikingly high concentrations of phosphatidyl glycerol and cardiolipin [27].

Using the optimal growth conditions for the expression of the His-tagged NDH-2A and NDH-2B as detailed in Materials and methods, the enzymes were purified from the cytoplasmic fraction by metal-chelating chromatography. The yield of NDH-2A was 3–4 mg/l of culture; NDH-2B yields were 13–17 mg/l. Purified NDH-2A and NDH-2B were both intensely yellow. Assays of NADH:menadione oxidoreductase activity showed that activity of NDH-2A was consistently much higher than that of NDH-2B but the activity of NDH-2A, only, was labile and the enzyme also became noticeably less yellow when it was concentrated by ultrafiltration. Addition of FAD to the reaction mixture stimulated activity of NDH-2A more than 3-fold whereas it had only a modest effect on NDH-2B (Table 1, top). Therefore, FAD (20 μM) was included throughout the purification of NDH-2A, except for the preparations used for flavin and spectral analysis (see below) where no FAD was used in the purification. FAD was not added during purification of NDH-2B. Since this enzyme did not appear to appreciably lose its flavin, it was routinely desalted and concentrated by ultrafiltration but this was not done for NDH-2A. The purification of NDH-2A and NDH-2B were assessed by SDS-PAGE gel electrophoresis. Both proteins were specifically recognized by the anti-His probe (Fig. 4B). His-tagged NDH-2A migrated as a band of 49.0 kDa, a little larger than its predicted size of 47.7 kDa. The preparation had a small amount of a 25 kDa contaminant (Fig. 4A). NDH-2B migrated as a single band of about 53.5 kDa, approximately 2 kDa larger than the predicted size of the His-tagged protein (51.7 kDa) (Fig. 4A).

3.4. Identification of the prosthetic group

Absorption and fluorescence spectra of the two enzymes were consistent with the presence of flavin. NDH-2A exhibited absorption peaks at 371 nm and 446 nm (Fig. 5A), while NDH-2B gave peaks at 377 nm and 455 nm (Fig. 5C). The emission maxima of NDH-2A and

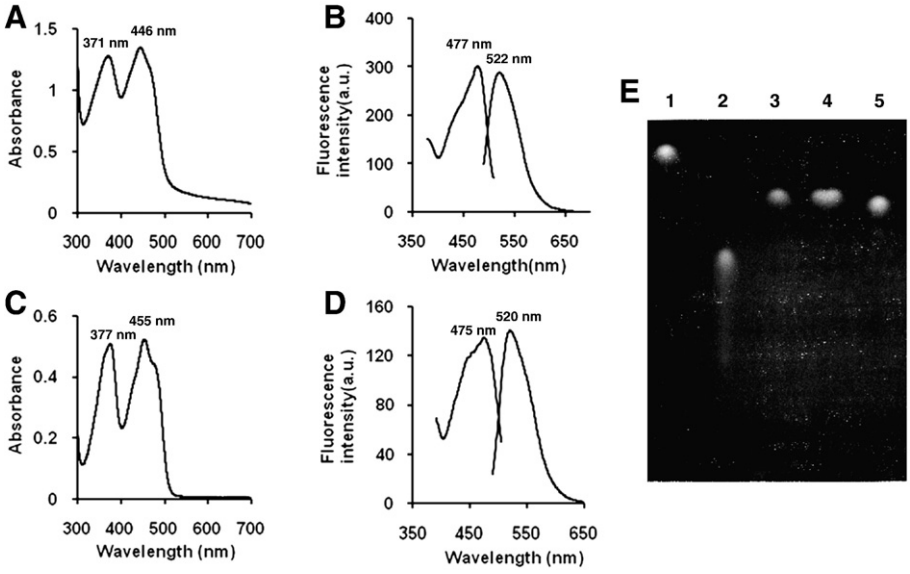

Fig. 5. Flavin analysis of purified proteins. A and C are absorption spectra of the purified NDH-2A and NDH-2B, respectively. B and D are fluorescence spectra of the purified NDH-2A and NDH-2B, respectively. Details can be found in Materials and methods. E. Thin-layer chromatogram of NDH-2A and NDH-2B chromophores. Thin-layer chromatography was performed as described in Materials and methods. Lane 1, Riboflavin; lane 2, FMN; lane 3, FAD, lane 4, extracted NDH-2A chromophore; lane 5, extracted NDH-2B chromophore.

NDH-2B in the fluorescence spectra were 522 nm (Fig. 5B) and 520 nm (Fig. 5D), when excited at 477 nm and 474 nm, respectively. The excitation peaks for NDH-2A and NDH-2B were 477 nm (Fig. 5B) and 475 nm (Fig. 5D), when the emission wavelengths were set at 522 nm and 520 nm, respectively.

The identity of the prosthetic group of NDH-2A and NDH-2B was determined by thin-layer chromatography. For both enzymes, the yellow color could be readily separated from the protein either by boiling the enzyme or by denaturation with trichloroacetic acid, indicating that the flavin is noncovalently bound to the enzyme. In solvent system A (2% [w/v] Na_2PO_4 in water), the R_f values of riboflavin, FMN, FAD, NDH-2A ligand and NDH-2B ligand were 0.79, 0.55, 0.69, 0.69 and 0.67, respectively (Fig. 5E). In a different solvent system, solvent B (*n*-butanol/glacial acetic acid/water, 2:1:1), in which the relative migrations of FMN and FAD are reversed, the R_f values of riboflavin, FMN, FAD, NDH-2A ligand and NDH-2B ligand were 0.84, 0.49, 0.36, 0.35, and 0.35, respectively (data not shown). The elution behavior of the chromophores from NDH-2A and NDH-2B were identical or very similar to FAD, indicating that both NDH-2A and NDH-2B contain FAD as the prosthetic group. The results shown in Fig. 5E were obtained from the supernatant after boiling the purified proteins; the results were the same when the flavin was released from purified proteins by TCA treatment (data not shown). Thus like most reported type II NADH dehydrogenases [8,14,17,28,29], both NDH-2A and NDH-2B contain noncovalently bound FAD as a prosthetic group.

3.5. Enzymatic properties of purified NDH-2A and NDH-2B

Although neither enzyme had exhibited Na^+/H^+ antiport activity, the reported association of the *H. dabanensis* Nap homologue of NDH-2B with Na^+-resistance [1] made it of interest to test the effect of different salts on the NADH:menadione oxidoreductase activity of both NDH-2A and NDH-2B. The activities of sodium ion-linked enzymes are often affected by Na^+, as illustrated by the NADH dehydrogenase activity of Nqr, a sodium ion-translocating enzyme from *Vibrio cholerae*, which is stimulated 5-fold by sodium chloride [30].

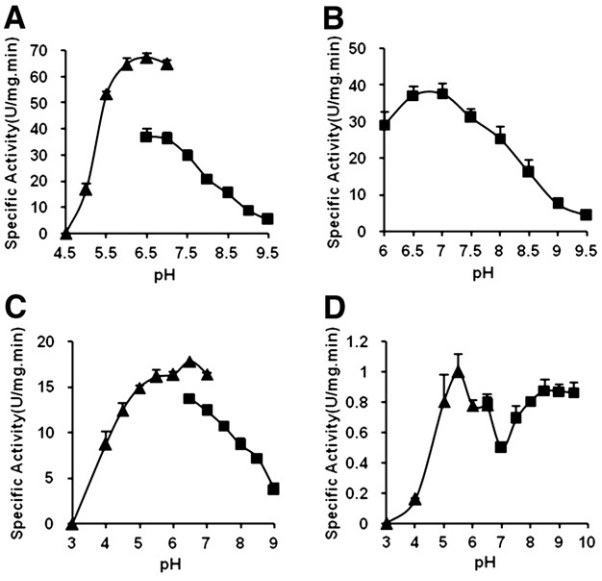

Fig. 6. Effect of pH on NADH:ferricyanide oxidoreductase activity of purified NDH-2A (A) or NDH-2B (C), and NADH:menadione activity of NDH-2A (B) or NDH-2B (D). Enzymatic activity was measured at different pH values as described in Materials and methods. The closed triangle sign indicates assays performed with citric acid-phosphate buffer and the closed square sign indicates assays performed with 50 mM BTP buffer. Values are the average of duplicate determinations from at least 2 independent protein preparations and the error bars show the standard deviation.

Table 2
Enzyme activities of purified NDH-2A and NDH-2B with different electron donors and acceptors

Electron donor	Electron acceptor	NDH-2A		NDH-2B	
		pH 7.5	pH 8.2	pH 7.5	pH 8.2
NADH	O_2	3.37±0.24 [a]	1.56±0.09	0.39±0.06	0.35±0.06
	Menadione	32.85±2.43	18.26±1.48	0.69±0.08	0.89±0.06
	Ferricyanide	29.93±1.88	17.79±0.76	10.74±0.05	7.73±0.05
	Q_1	47.07±4.78	21.37±3.26	0.46±0.05	0.70±0.01
d-NADH	O_2	0.35±0.02	0.15±0.07	0.95±0.05	0.70±0.03
	Menadione	0.32±0.12	0.02±0.03	0.16±0.03	0.09±0.01
	Ferricyanide	0.54±0.05	0.35±0.10	7.10±0.99	5.08±0.04
	Q_1	0.39±0.10	0.04±0.02	0.08±0.04	0.02±0.02
NADPH	O_2	0.07±0.01	0.06±0.03	0.93±0.03	0.30±0.03
	Menadione	0	0	0.08±0.02	0.02±0.03
	Ferricyanide	0	0	8.11±0.61	2.31±0.20
	Q_1	0	0	0.01±0.01	0

[a] μmol NADH oxidized/min mg protein.

The NADH:menadione oxidoreductase activity of NDH-2A was slightly increased, in a non-specific manner, by different salts (NaCl, KCl, LiCl, $CaCl_2$, $MgCl_2$), whereas the same activity of NDH-2B was almost not affected by these salts (Table 1, bottom).

The activities of the two enzymes were pH-dependent and had optima that were somewhat below neutral pH. NDH-2A exhibited a pH optimum for NADH:ferricyanide oxidoreductase activity at pH values between 6 and 7 (Fig. 6A), whereas NDH-2B showed an optimum at pH 5.5–7 (Fig. 6C). NDH-2A catalyzed NADH:menadione oxidoreduction with a pH optimum of 6.5–7 (Fig. 6B), whereas NDH-2B showed two pH optima, at 5.5 and 8.5–9.5 (Fig. 6D).

In considering the appropriate pH value(s) to use for assays of substrate specificity, we decided to focus on the cytoplasmic pH that has been observed under the two growth conditions used for most of the physiological studies of *B. pseudofirmus* OF4. The two values of growth pH that are most commonly used for studies of this alkaliphile are pH 7.5 and pH 10.5, at which the internal pH is 7.5 or 8.2, respectively [31]. Therefore the activity of NDH-2A and NDH-2B with NADH, deamino-NADH, or NADPH as reductants was assayed at pH 7.5 and 8.2 with the electron acceptors menadione, ferricyanide, Q1, or oxygen. Many type II NADH dehydrogenases are able to reduce oxygen, and the alkaliphile enzymes are no exception (Table 2). The activities with the added substrates listed in Table 2 were calculated after

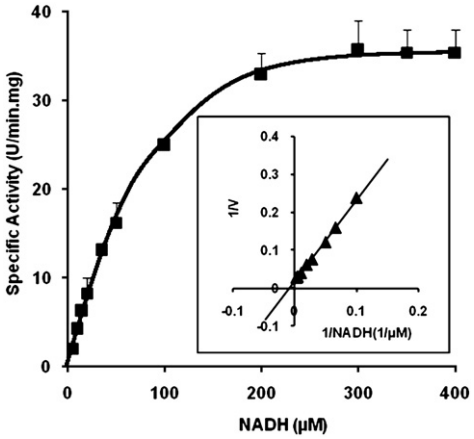

Fig. 7. Kinetic parameters for the NADH:menadione oxidoreductase activity of NDH-2A at pH 7.5. A velocity vs. substrate concentration plot is shown and, in the inset, the double reciprocal plot is shown. The assay protocol is described in Materials and methods. Values are the average of duplicate determinations from at least 2 independent protein preparations and the error bars show the standard deviation.

subtraction of the oxygen-dependent activity. For NDH-2A, the activity with oxygen is about 8–10% of the activity with menaquinone. NDH-2A is very active either with the quinone analogs or ferricyanide as the acceptor, but is quite narrow in its ability to use reductants, with NADH being the only robust reductant. No activity was observed with NADPH and d-NADH exhibited marginal activity (less than 2% of the activity observed with NADH). Kinetic parameters for NDH-2A at pH 7.5 were determined for the NADH:menadione oxidoreductase reaction (Fig. 7). The enzyme had a K_m of 114 μM and a V_{max} of 53.2 μmol NADH oxidized/min mg protein under these reaction conditions. Values from the literature vary widely depending on substrate, pH, temperature, and source of the enzyme but the measured K_m values generally range from 5 to ≥100 μM for NADH [12,14,15,32,33].

The NDH-2A of *B. pseudofirmus* OF4 is likely to play a physiological role in providing the entry point of electrons from NADH to the respiratory chain. This likelihood is based on: (i) the ability of *ndh-2A* to complement the NADH dehydrogenase-deficient *E. coli* strain and (ii) the ability of NDH-2A to use NADH to reduce quinone analogs at relevant pH values.

NDH-2B presented a very different spectrum of catalytic activities (Table 2). The oxygen-dependent activity of NDH-2B is about 4–5% of the activity with ferricyanide as acceptor. However, note that in relation to the very low activities seen with quinone analogs, the activity with oxygen of NDH-2B is quite high, anywhere between 35% and over 90% (in the case of quinone reduction with either d-NADH or NADPH). The only truly effective acceptor for this enzyme is ferricyanide but it is promiscuous with respect to electron donor (NADH>NADPH>d-NADH). The ability of type II NDH-2B to use d-NADH as reductant almost as well as NADH and NADPH is notable since use of d-NADH is often used to monitor type I, proton-pumping NADH dehydrogenases [17]. We cannot propose a physiological role for NDH-2B on the basis of the findings in this study. No antiport activity was detected and although both NADH and NADPH served as electron donors in reduction of ferricyanide by NDH-2B, the observation that this enzyme reduced quinone analogs poorly makes it unlikely that quinones are the physiological electron acceptors. With its much higher specific activities, NDH-2A would more readily transfer electrons to the quinones of the respiratory chain than NDH-2B. Perhaps the *in vivo* role is related to the capacity to transfer electrons from NADPH, as this was only found with NDH-2B. We note that thus far neither the membrane localization of *H. dabanensis* Nap nor its oxidoreductase activities have been characterized yet. If *H. dabanensis* Nap localization resembles that of the *B. pseudofirmus* OF4 enzymes studied here, it will raise the possibility that it is not a primary pump with antiport capacity. Instead, the effect of the Nap on Na^+-resistance and flux could be a secondary effect of a Nap-dependent oxidoreductase activity on one of the residual antiporters in the *E. coli* KNabc strain in which it was characterized, e.g. one of the CPA-1 type antiporters or the KefFC antiporter that was recently shown to have Na^+/H^+ as well as K^+/H^+ capacity [25,34].

Acknowledgements

This work was supported by research grant R01-GM28454 from the National Institute of General Medical Sciences. We thank Masahiro Ito for providing the recombinant pBAD vector expressing the *mrp* operon.

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Glossary

AO: acridine orange
BTP: bis-[tris(hydroxymethyl)methylamino]-propane
NDH-1: type I NADH dehydrogenase
NDH-2: type II NADH dehydrogenase
Q1: coenzyme Q1
PMSF: phenylmethylsulfonyl fluoride
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA: trichloroacetic acid