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Modification of substrate specificity in single point mutants of *Agrobacterium tumefaciens* type II NADH dehydrogenase

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Abstract **Type II NADH dehydrogenases (NDH-2) are monomeric flavoenzymes catalyzing electron transfer from NADH to quinones. While most NDH-2 preferentially oxidize NADH, some of these enzymes have been reported to efficiently oxidize NADPH. With the aim to modify the NADPH vs NADH specificity of the relatively NADH specific *Agrobacterium tumefaciens* NDH-2, two conserved residues (E and A) of the substrate binding domain were, respectively, mutated to Q and S. We show that when E was replaced by Q at position 203 the enzyme was able to oxidize NADPH as efficiently as NADH. Growth on a minimal medium of an *Escherichia coli* double mutant lacking both NDH-1 and NDH-2 was restored more efficiently when mutated proteins able to oxidize NADPH were expressed. The biotechnological interest of expressing such modified enzymes in photosynthetic organisms is discussed.**

Keywords: NAD(P)H:quinone oxidoreductase; NDH-2; Hydrogen photoproduction; NADH/NADPH; Cofactor binding domain; Site directed mutagenesis

1. Introduction

Type II NAD(P)H:quinone oxidoreductases (NDH-2) are monomeric enzymes catalyzing a two-step electron transfer from NAD(P)H to quinones, without translocation of protons [1]. NDH-2 are found in respiratory chains of bacteria and archaea [2,3], as well as in eukaryotic organisms including fungi [4,5] and plants [6]. NDH-2 family is characterized by a wide range of physiological roles [1]. In *Saccharomyces cerevisiae*, as well as in other organisms lacking complex I (NDH-1), NDH-2 are the only components of the respiratory chain responsible for the oxidation of NAD(P)H [4]. But in most cases, NDH-2 are found together with NDH-1 and probably play an adaptative role as their synthesis might offer a fine-tuneable and rapid response to fluctuating energetic demand due to environmental modifications [1].

Most of the NDH-2 contain two typical nucleotide binding domains of 35–40 amino acids embedded in a β sheet–α helix–β sheet structure [7] both located in the N-terminus region of the protein [1] and both harbouring a conserved GXGXXG motif. From sequence homologies with lipoamide dehydrogenase [2] and structural modelling based on homologies with other FAD/NAD-linked reductases [8], it was proposed that the first GXGXXG motif of NDH-2 would bind FAD and the second NAD(P)H. Most of the NDH-2 characterized so far use NADH as a preferential substrate, but are also able to use NADPH, although with lower efficiency [1,9]. Two NDH-2 specifically oxidizing NADPH have been characterized on the outer surface of the inner membrane of mitochondria. The first one (NDE1) is the external calcium-dependent NADPH dehydrogenase from *Neurospora crassa* [10] and the other St-NDB1 from *Solanum tuberosum* [11]. Based on the alignment of NDH-2 sequences, Michalecka et al. [11] proposed that two residues located at the end of the second beta-sheet of the second nucleotide binding domain would determine the substrate specificity of the enzyme. In NADH-dependent NDH-2 these two positions are occupied by an acidic residue (Glu or Asp) followed by Ala or a Val. In contrast, in the two NADPH-dependent NDH-2 these residues are replaced by a neutral residue followed by a Ser.

Recently, a NDH-2 isolated from *Agrobacterium tumefaciens* has been purified and characterized in our laboratory [9]. This enzyme, which shows a higher affinity for NADH than for NADPH, contains GluAla at the end of the second nucleotide binding domain. In an attempt to investigate the role of these two residues in the substrate specificity of the enzyme, different AtuNDH-2 mutants have been generated. Whereas substitution of Ala by Ser had a limited effect on the enzyme specificity, replacement of Glu by Gln is reported to significantly enhance both affinity and catalytic efficiency for NADPH. The biotechnological interest of expressing such modified enzymes towards increasing biological hydrogen production in photosynthetic microorganisms such as unicellular algae is discussed.

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2. Material and methods

2.1. Strains and media

The *E. coli* strain DH10β was grown on a LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7) in presence of 100 μg μl^{-1} of ampicillin and 0.1% arabinose when appropriate. The *E. coli* mutant strain ANN0222 lacking NDH activities, generously provided by Prof. Thorsten Friedrich, was grown on the same LB medium. Complementation assays were performed in M9 medium (2 mM $MgSO_4$, 10 mM $CaCl_2$, 47 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 18 mM NH_4Cl, pH 7.2) supplemented with 0.4% mannitol.

2.2. Cloning, expression and purification of (His)$_6$ native and mutant AtuNDH2 proteins

The three AtuNDH2 mutants were generated by using the PCR fusion technique as follows. The pSDN2Ag6H plasmid was used as a matrix to amplify the AtuNDH2 gene [9]. NcoI and XbaI restriction sites (indicated in bold) have been introduced, respectively, at the 5′ and 3′ end of the genes by using the pairs of primers N_2Ag-NcoI (F) (5′-GGC***CCATGG***AAGAACATCATGTTGTCGTC-3)/N_2Ag-XbaI (R) (5′-GGC***TCTAGA***TCAGGCCTCGTCCTTCAGCGT-3′) and N_2Ag-NcoI (F)/N_2Ag-PstI (R) (5′-AAAA***CTGCAG***TCAATGATGATGATGATGGGCCTCGTCCTTCAGCG-3′) as external primers. The primer N_2Ag-PstI was used to introduce the $(His)_6$-tag at the 3′ end of the genes. The forward sequences of the internal primers introducing the mutations (underlined codons) were, respectively: E203Q mutant: (5′-TTGCTTGTGCAGGCCGGCCCT-3′); A204S mutant: (5′-TTGCTTGTGGAGTCCGGCCCT-3′); E203Q/A204S mutant: (5′-TTGCTTGTGCAGTCCGGCCCT-3′).

The wild-type gene was cloned in parallel by using the N_2Ag-NcoI/N_2AgXbaI and N_2Ag-NcoI/N_2Ag-PstI pairs of primer. The various constructs were cloned in a pBAD24 vector, generously provided by Drs. P. Genevaux and C. Georgopoulos (University of Geneva, Switzerland). Protein expression was induced by addition of 0.1% arabinose when the culture reached 0.5 OD. Following 3 h of induction at 37 °C, t(His)$_6$-tagged proteins were purified as previously described [9].

2.3. Colony forming assays

The ANN0222 strain was chemically transformed with the different plasmids expressing both $(His)_6$-tagged and non-tagged version of the wild-type and mutants AtuNDH2 proteins. Transformants were selected and plated on LB or M9+mannitol medium as described previously [9].

2.4. Preparation of bacterial membranes

Fifty milliliters cultures of the various transformed ANN0222 strains were harvested by centrifugation (15 min, 3200 × *g*) at the end of exponential growth phase (OD = 1). Cells were disrupted by passing twice through a chilled French pressure cell maintained at 16000 p.s.i. [9]. Membrane fractions were collected by centrifugation (30 min, 4 °C, 48500 × *g*) and resuspended in 250 μl of analysis buffer B (50 mM phosphate buffer, pH 7.5 and 150 mM NaCl).

2.5. Immunological analysis

A specific antibody raised against the wild-type recombinant protein [9] was used to identify the wild-type and mutant AtuNDH2 proteins. The detection was performed by using an antibody Alexa 680 goat anti-rabbit (Invitrogen, Molecular Probes) and an Odyssey system (LI-COR Biosciences).

2.6. Spectrophotometric measurement of enzymatic activity

Assays of NAD(P)H oxidation were performed by monitoring the rate of absorbance decrease at 340 nm using Qo as an electron acceptor. Activities values were deduced from the measurements of the NAD(P)H concentration variations by applying the extinction coefficient of NAD(P)H at this wavelength (6.22 mM^{-1} cm^{-1}). NAD(P)H, acceptors, bovine erythrocytes superoxide dismutase (SOD, 500 U ml^{-1}) and bovine liver catalase (1000 U ml^{-1}) and protein extracts were added to the assay medium. SOD and catalase were purchased from Sigma. All activities were determined at room temperature using a Cary 50 bio UV-spectrophotometer.

2.7. O_2 uptake by purified enzymes and bacterial membranes

O_2 uptake rates were measured using a Clark electrode (DW2/2, Hansatech, King's Lynn, England). Membranes extracts from the various strains were introduced into the electrode chamber and suspended in buffer B equilibrated with air. O_2 consumption was measured at 25 °C in the presence of NADH or NADPH.

3. Results

In order to test whether the nature of the two residues located at the end of the first nucleotide binding domain determines the substrate specificity of NDH-2 enzymes, as could be inferred from the comparison between sequences from enzymes showing NADH or NADPH substrate specificity, three different AtuNDH2 mutant proteins were constructed (E203Q; A204S and E203Q/A204S – see Fig. 1). To facilitate protein purification, an affinity tail consisting of 6 His residues was fused at the protein C-terminus. Recombinant proteins were purified by using a Ni^{2+}-affinity column and their purity was estimated higher than 90% (Fig. 2A). Immunodetection was performed (Fig. 2B) by using an antibody raised against AtuNDH2 [9]. We verified that the different mutations had no significant effect on the flavinic cofactor content and on its redox potential (Supplementary Fig. 1). CD and fluorescence emission spectra from wild-type and mutant proteins were similar (Supplementary Figs. 2 and 3). Slight differences were observed in absorption spectra (Supplementary Fig. 4), which might reflect the existence of interactions between the NADH binding domain and the FAD domain. Such interactions were proposed to favour electron transfer reactions [8].

In a first step, the ability of AtuNDH2 mutants to oxidize NADH and NADPH was studied using Qo as an acceptor. Fig. 3 shows that highest NADH oxidation activity of the wild-type enzyme was detected at pH ranging from 7 to 9 with an optimum at pH 7.5. This pattern is consistent with the pH dependency of NADH oxidation previously reported for other members of the NDH-2 family [10,12]. The NADH oxidation pattern of E203Q, A204S and E203Q/A204S mutants is very similar to that of the wild-type protein, but unexpectedly the three mutants exhibited a 2-fold activity increase compared to the wild-type protein. Note that such a phenomenon has already been described for similarly modified $NAD(P)^+$-dependent aldehyde dehydrogenases [13]. In the presence of NADPH, the A204S protein was slightly more efficient than AtuNDH2, maximal activity being reached at pH 7.0 (Fig. 3B). In contrast, E203Q and E203Q/A204S oxidized NADPH almost as efficiently as NADH, maximal NADPH oxidation activity being reached at neutral pH, as for NADH. Such a pH dependency of NADPH oxidation, which was observed both on wild-type and mutant AtuNDH2 proteins, strikingly differs from what was reported with other NDH-2, NADPH oxidation generally occurring at acidic pH [10] [12]. Catalytic properties of native and mutated proteins were determined at optimal pH by using NADH or NADPH as donors and Qo as acceptor (Table 1). Catalytic efficiencies were determined by the ratio k_{cat}/K_m (K_m referring to the Michaelis–Menten constant). As previously shown [9], the K_{mNADH} value of the wild-type protein is about 55-fold lower than the K_{mNADPH} reflecting its strong preference for NADH. Note that for unknown reasons, the V_m value measured for the wild-type protein was about half of that previously reported

Fig. 1. Sequence comparison of the NAD(P)H-binding domain of NADH and NADPH-dependent NDH-2. The nucleotide binding motif structure proposed by Wierenga et al. [7] is represented at the top (arrows: β sheet; cylinder: α helices). The three conserved glycine are shown in the "consensus" lane as well as the others conserved residues: B: basic or hydrophilic; H: hydrophobic; A: acidic. Two asterisks identify the two positions ending the nucleotide binding motif targeted by the mutagenesis. The name of each protein is indicated at the left of each line. Numbers in brackets refer to the position of aligned motifs in each protein sequence. The substrate preference of each protein is indicated at the right of each line. The three substitutions generated in AtuNDH2 are indicated below the sequence of the protein. Nc-NDI1: *Neurospora crassa* (EAA27430); Nc-NDE1: *N. crassa* (CAB41986); SC-NDE1: *Saccharomyces cerevisae* (NP-013865); SC-NDE2 : *S. cerevisae* (NP-010198); SC-NDI1: *S. cerevisae* (NP-013586); Ec-NDH: *Escherichia coli* (DEECR); St-NDB1: *Solamum tuberosum* (CAB52797); Y1-NDH2: *Yarrowia lipoytica* (CAA07265); AtuNDH2: *Agrobacterium tumefaciens* (AI2824).

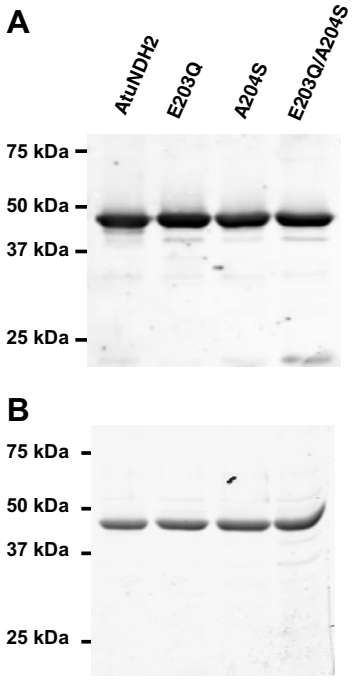

Fig. 2. Ni^{2+} affinity purification and immunological characterization of wild-type AtuNDH2 and E203Q, A204S and E203Q/A204S mutated enzymes. (A) Separation of purified proteins using a 10% SDS–PAGE. (B) Immunological characterization of purified proteins by using an antibody raised against AtuNDH2 [9].

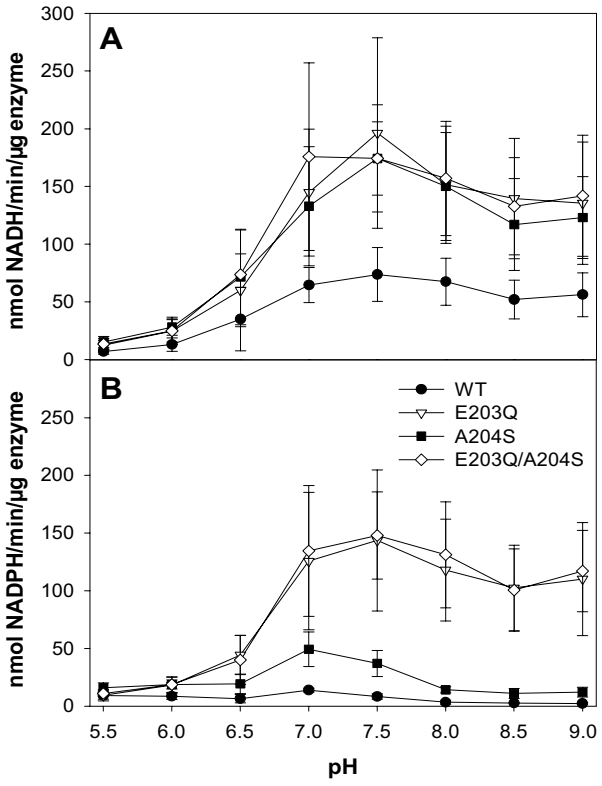

Fig. 3. pH dependence of NADH (A) and NADPH (B) oxidizing activities of wild-type (●), E203Q (△), A204S (■) and E203Q/A204S (◇) AtuNDH2 proteins. Initial concentrations of NADH and NADPH were 200 μM. Average values and error bars were calculated from triplicate measurements realized on three independent samples.

[9]. The K_{mNADH} value of the three mutated proteins was close to that of wild-type AtuNDH2. E203Q and E203/A204S respectively exhibited 2.5 and 1.8-fold increases of the catalytic efficiency when compared to WT, which was explained by the 3-fold increase of their k_{cat} value, their K_m value being almost unchanged. In the presence of NADPH, E203Q and E203Q/A204S mutants exhibited strong decreases of their $K_m$value together with an increase of their catalytic efficiencies (160 and

Table 1
Kinetic parameters of NADH and NADPH oxidation by wild-type and mutated AtuNDH2 proteins

	NADH				NADPH			
	K_m (μM)	V_m (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (μ M^{-1} s^{-1})	K_m (μM)	V_m (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
AtuNDH2	4.5 ± 1.1	74 ± 23	57 ± 18	12.7	244 ± 88	31 ± 4	24 ± 3	0.1
E203Q	4.9 ± 0.9	196 ± 83	150 ± 64	30.8	6.9 ± 4.3	144 ± 61	112 ± 47	16.4
A204S	7.2 ± 2.6	174 ± 46	133 ± 35	18.5	243 ± 86	108 ± 33	83 ± 25	0.34
E203Q/A204S	5.8 ± 1.9	174 ± 32	133 ± 25	23	4.5 ± 0.5	148 ± 38	114 ± 29	25.2

Kinetic parameters were measured at optimal pH (pH 7.0 for NADH and pH 7.5 for NADPH), using Q_0 (200 μM) as an acceptor, in the presence of SOD (500 U ml^{-1}) and catalase (1000 U ml^{-1}) in the reaction medium. Enzyme activity was measured by monitoring NADH consumption expressed as μmol NADH min^{-1} mg^{-1} protein. Average values and error bars were calculated from triplicate measurements realized on three independent samples.

250-fold, respectively) when compared to wild-type protein. A204S showed a 3.4-fold increase in NADPH turnover compared to wild-type protein but the K_m value for NADPH was unaffected.

3.1. Interaction of AtuNDH2 with the E. coli respiratory chain

In a second step, the ability of the different AtuNDH2 mutants to interact with the respiratory chain of *E. coli* was compared. Wild-type and mutated AtuNDH2 proteins were expressed in the *E. coli* mutant strain ANN0222 lacking NDH-1 and NDH-2. Membrane and soluble fractions from the various strains were analyzed by immunodetection (Fig. 4A). As previously reported for AtuNDH2 [9], the three modified enzymes were predominantly present in the membrane fraction. Two lower mass polypeptides, likely corresponding to degradation products, were observed in soluble fractions. Although A204S was expressed at a similar level as the wild-type protein, E203Q and particularly E203Q/A204S mutants were much less abundant, indicating that these mutations may affect protein stability.

Respiratory O_2 uptake rates were measured in the presence of 200 μM NADH (saturating concentration) or 200 μM NADPH (non-saturating concentration for the wild-type enzyme) in membrane fractions prepared from strains expressing the different AtuNDH2 recombinant proteins. Fig. 4B shows the ratio between the rate of O_2 consumption in the presence of NADH and NADPH for each membrane fraction. Membranes prepared from cells expressing E203Q and E203Q/A204S proteins could oxidize NADPH as well as NADH while the wild-type and A204S proteins exhibited a strong preference for NADH. Therefore, modifications in substrate specificity previously observed in vitro with purified His-tagged proteins can be reproduced ex vivo with the non-tagged proteins interacting with the respiratory chain of *E. coli*.

3.2. Growth complementation of the E. coli ANN0222 mutant

In a third step, the ability of the three mutated proteins to complement growth of an a *E. coli* strain deficient in both NDH-1 and NDH-2 was compared. The ANN022 *E. coli* strain is unable of growth on a minimal medium (M9) supplemented with mannitol as the sole source of carbon [14]. AtuNDH2 has previously been reported to restore the growth of the ANN0222 strain under such conditions [9]. No growth differences among the five strains were detected when plated on LB medium either in absence or in the presence of inductor (arabinose). Partial growth restoration was observed on a minimal medium in the absence of induction for wild-type and

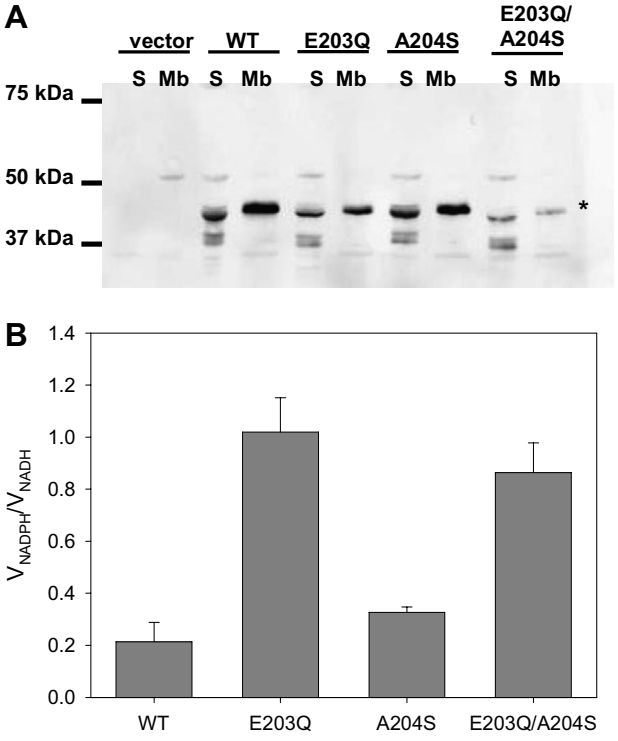

Fig. 4. Expression and activity of wild-type and mutated AtuNDH2 enzymes expressed in an *Escherichia coli* (ANN022) mutant lacking both NDH-1 and NDH-2. (A) Immunodetection of soluble (S) and membrane (Mb) fraction extracted from ANN022 strain expressing wild-type AtuNDH2 or mutated proteins E203Q, A204S and E203Q/A204S by using an antibody raised against AtuNDH2. Protein synthesis was induced by addition of 0.1% arabinose. Gel loading was approximately 2.5 and 30 μg proteins in Mb and S lanes, respectively. The ANN022 strain carrying the empty vector is shown as a control. (B) Ratios between O_2 uptake rates measured in the presence of 200 μM NADPH and 200 μM NADH in membrane fractions of the ANN022 strain expressing wild-type AtuNDH2, and E203Q, A204S, E203Q/A204S mutant proteins. In order to perform experiments with similar protein levels, membrane amounts were adjusted according to AtuNDH2 content as deduced from immunodetection using the AtuNDH2 antibody. Average values and error bars were calculated from measurements performed on four independent membrane preparations.

A204S, but not for E203Q mutant proteins. Such an observation has previously been reported for wild-type AtuNDH2 and was attributed to a promoter leakage [9]. Western blotting analysis revealed that it was also the case in these experiments (data not shown). When induced by arabinose, expression of

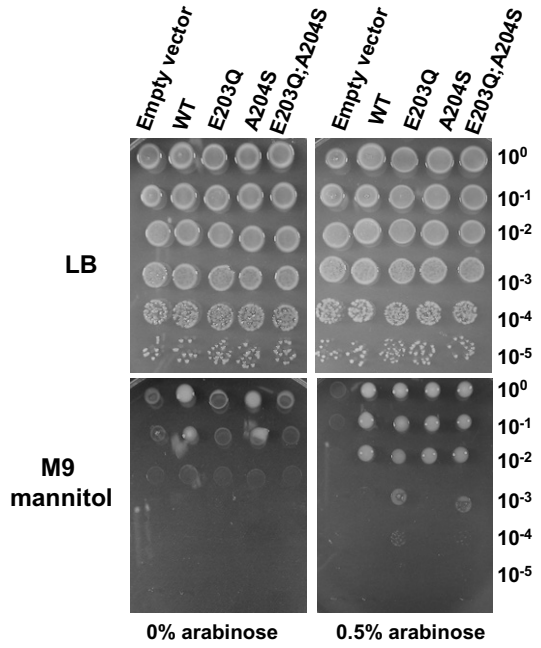

Fig. 5. Growth complementation of the *Escherichia coli* strain ANN0222 deficient in NDH-1 and NDH-2 by wild-type AtuNDH2, and mutated proteins E203Q, A204S and E203Q/A204S. Colony forming assays were performed on a rich medium (LB) or minimal medium (M9) supplemented with 0.4% mannitol as the sole source of carbon. Numbers on the right refer to 10-fold serial dilutions of bacterial cultures spotted on plates and grown at 37 °C.

the wild-type and mutated proteins significantly improved growth of the ANN0222 strain. Interestingly, although amounts of expressed proteins were significantly lower in strains expressing E203Q and E203Q/A204S, growth was significantly higher (see Fig. 5). Similar growth differences between strains were also observed in liquid cultures (data not shown). This suggests that the ability of E203Q and E203Q/A204S enzymes to oxidise both NADH and NADPH may turn out to be a selective advantage under particular growth conditions.

4. Discussion

We show here that substituting a Glu to a Gln residue at the end of the second nucleotide binding domain (position 203) of the *A. tumefaciens* type II NADH dehydrogenase significantly alters the substrate specificity of the enzyme. While native AtuNDH2 preferentially uses NADH, the modified enzyme with a Gln replacing Glu is able to oxidize both NADPH and NADH with similar efficiencies. This effect is shown to result from a strong decrease of the K_{mNADPH} value combined with a moderate increase of the NADPH catalytic efficiency. This results confirms the prediction of Michalecka et al. [11] who concluded from sequence comparisons that the negative charge provided by the Glu203 residue would impair the accessibility of NADPH to the catalytic site in AtuNDH2. It is also in agreement with the structural modelling proposed by Schmid et al. [8] for the *E. coli* NDH-2 enzyme. In this study, the authors suggested that the *E. coli* E214 residue, corresponding to E203 in AtuNDH2, would be in direct contact and possibly forming H-bond with the ribose 2′-OH of NADH adenosine moiety. Nevertheless, the substitution of E203 does not diminish the affinity of AtuNDH2 for NADH, suggesting that other residues or structural features could be responsible for NADPH preference in enzymes such as NDE1 from *N. crassa* [8] and St-NDB1 from *S. tuberosum* [11]. Interestingly, similar conclusions have been raised in the case of other FAD/NAD^+ enzymes, such as the L-lactate dehydrogenase from *Bacillus stearothermophillus,* where inversion of the specificity of the enzyme was shown to require multiple amino acid substitutions [15].

In contrast to Glu203, Ala204 does not appear to be involved in NADH vs NADPH specificity, as substitution to a Ser residue did not affect the substrate specificity. Surprisingly, as the Gln substitution at position 203, Ser substitution at position 204 increased the catalytic efficiency for NADH. A substantial stimulation of catalytic efficiency has also been reported in aldehyde dehydrogenase in response to a single point mutation immediately downstream of the β_B strand in the Rossmann fold [12]. This phenomenon might be related to weak structural perturbations induced by these substitutions.

In addition to its effect on the affinity of the enzyme for NADPH, the E203Q mutation resulted in a lower expression of the protein, particularly in the membrane fraction, this effect being enhanced in the E203Q/A204S double mutant. Although thermal stability of purified proteins does not seem to be decreased by mutations (data not shown), this lower amount in vivo might be linked to an increased instability of these proteins in the cellular context which may result from structural changes affecting binding of the enzyme to the bacterial membrane. Sequence analysis revealed the presence in AtuNDH2 of putative amphipatic helices located in the C-terminal region of the protein [9]. Such 2D structures are conserved among NDH-2 of the prokaryotic group B and have been proposed to be involved in the membrane docking [9,16,17]. AtuNDH2 also contains a domain enriched in aromatic residues, located between the two Rossman folds, which was proposed to bind the quinones [18,16]. Interestingly, the structural modelling proposed by Schmid et al. [8] suggests that the second nucleotide binding motif would be physically close of these two amphipathic domains. In this context, it is reasonable to think that structural perturbations close to the catalytic site of the enzyme might affect membrane binding. No NDH-2 X-ray structure being available and E203Q, A204S and E203Q/A204 constituting the first modified NDH-2, this hypothesis remains difficult to validate at the moment. The recent report by Brito et al. [19] on the crystallization of a NDH-2 from *Acidianus ambivalens* suggests that structure of the first NDH-2 might be obtained soon and supply a structural basis to better understand the mechanisms underlying NDH-2 activity.

It was previously proposed that over-expressing a NDH-2 in *Chlamydomonas reinhardtii* plastids would be an interesting strategy to optimize the capacity of this alga to photo-produce molecular hydrogen [9]. Recently, a NDH-2 type enzyme has been proposed to be responsible for the non-photochemical reduction of the pool of PQ, which is an essential, and most likely limiting step for the hydrogen production in the absence of PSII [20]. AtuNDH2 has been reported as able to interact with the algal photosynthetic chain and to stimulate the non-photochemical reduction of the pool of PQ [9]. We have shown

here that expression of a mutated NDH-2 able to oxidize NADPH in *E. coli* resulted in significant physiological effects since growth of a double mutant lacking both NDH-1 and NDH-2 was restored more efficiently. The capacity to oxidize NADPH may turn out to be also an interesting feature in photosynthetic organisms, particularly in the context of hydrogen production, NADPH being generally recognized as the main pool of reducing equivalents in chloroplasts [21].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.07.035.

References

[1] Melo, A.M., Bandeiras, T.M. and Teixeira, M. (2004) New insights into type II NAD(P)H:quinone oxidoreductases. Microbiol. Mol. Biol. Rev. 68, 603–616.
[2] Bjorklof, K., Zickermann, V. and Finel, M. (2000) Purification of the 45 kDa, membrane bound NADH dehydrogenase of *Escherichia coli* (NDH-2) and analysis of its interaction with ubiquinone analogues. FEBS Lett. 467, 105–110.
[3] Gomes, C.M., Bandeiras, T.M. and Teixeira, M. (2001) A new type-II NADH dehydrogenase from the archaeon *Acidianus ambivalens*: characterization and in vitro reconstitution of the respiratory chain. J. Bioenerg. Biomembr. 33, 1–8.
[4] de Vries, D.J., Herald, C.L., Pettit, G.R. and Blumberg, P.M. (1988) Demonstration of sub-nanomolar affinity of bryostatin 1 for the phorbol ester receptor in rat brain. Biochem. Pharmacol. 37, 4069–4073.
[5] Weiss, H., von Jagow, G., Klingenberg, M. and Bucher, T. (1970) Characterization of *Neurospora crassa* mitochondria prepared with a grind-mill. Eur. J. Biochem. 14, 75–82.
[6] Moller, I.M., Kay, C.J. and Palmer, J.M. (1986) Chlortetracycline and the transmembrane potential of the inner membrane of plant mitochondria. Biochem. J. 237, 765–771.
[7] Wierenga, R.K., Terpstra, P. and Hol, W.G. (1986) Prediction of the occurrence of the ADP-binding beta alpha beta-fold in proteins, using an amino acid sequence fingerprint. J. Mol. Biol. 187, 101–107.
[8] Schmid, R. and Gerloff, D.L. (2004) Functional properties of the alternative NADH:ubiquinone oxidoreductase from *E. coli* through comparative 3-D modelling. FEBS Lett. 578, 163–168.
[9] Bernard, L., Desplats, C., Mus, F., Cuine, S., Cournac, L. and Peltier, G. (2006) *Agrobacterium tumefaciens* type II NADH dehydrogenase. Characterization and interactions with bacterial and thylakoid membranes. FEBS J. 273, 3625–3637.
[10] Melo, A.M., Duarte, M., Moller, I.M., Prokisch, H., Dolan, P.L., Pinto, L., Nelson, M.A. and Videira, A. (2001) The external calcium-dependent NADPH dehydrogenase from *Neurospora crassa* mitochondria. J. Biol. Chem. 276, 3947–3951.
[11] Michalecka, A.M., Agius, S.C., Moller, I.M. and Rasmusson, A.G. (2004) Identification of a mitochondrial external NADPH dehydrogenase by overexpression in transgenic *Nicotiana sylvestris*. Plant J. 37, 415–425.
[12] Edman, K., Ericson, I. and Moller, I.M. (1985) The regulation of exogenous NAD(P)H oxidation in spinach (*Spinacia oleracea*) leaf mitochondria by pH and cations. Biochem. J. 232, 471–477.
[13] Zhang, L., Ahvazi, B., Szittner, R., Vrielink, A. and Meighen, E. (1999) Change of nucleotide specificity and enhancement of catalytic efficiency in single point mutants of *Vibrio harveyi* aldehyde dehydrogenase. Biochemistry 38, 11440–11447.
[14] Howitt, C.A., Udall, P.K. and Vermaas, W.F. (1999) Type 2 NADH dehydrogenases in the cyanobacterium *Synechocystis* sp. strain PCC 6803 are involved in regulation rather than respiration. J. Bacteriol. 181, 3994–4003.
[15] Flores, H. and Ellington, A.D. (2005) A modified consensus approach to mutagenesis inverts the cofactor specificity of *Bacillus stearothermophilus* lactate dehydrogenase. Protein Eng. Des. Sel. 18, 369–377.
[16] Bandeiras, T.M., Salgueiro, C., Kletzin, A., Gomes, C.M. and Teixeira, M. (2002) *Acidianus ambivalens* type-II NADH dehydrogenase: genetic characterisation and identification of the flavin moiety as FMN. FEBS Lett. 531, 273–277.
[17] Rapisarda, V.A., Chehin, R.N., De Las Rivas, J., Rodriguez-Montelongo, L., Farias, R.N. and Massa, E.M. (2002) Evidence for Cu(I)-thiolate ligation and prediction of a putative copper-binding site in the *Escherichia coli* NADH dehydrogenase-2. Arch. Biochem. Biophys. 405, 87–94.
[18] Kerscher, S.J., Okun, J.G. and Brandt, U. (1999) A single external enzyme confers alternative NADH:ubiquinone oxidoreductase activity in *Yarrowia lipolytica*. J. Cell Sci. 112, 2347–2354.
[19] Brito, J.A., Bandeiras, T.M., Teixeira, M., Vonrhein, C. and Archer, M. (2006) Crystallisation and preliminary structure determination of a NADH:quinone oxidoreductase from the extremophile *Acidianus ambivalens*. Biochim. Biophys. Acta 1764, 842–845.
[20] Mus, F., Cournac, L., Cardettini, V., Caruana, A. and Peltier, G. (2005) Inhibitor studies on non-photochemical plastoquinone reduction and H_2 photoproduction in *Chlamydomonas reinhardtii*. Biochim. Biophys. Acta 1708, 322–332.
[21] Peltier, G. and Cournac, L. (2002) Chlororespiration. Annu. Rev. Plant Biol. 53, 523–550.