Rotenone-insensitive NAD(P)H dehydrogenases in plants: Immunodetection and distribution of native proteins in mitochondria.

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Rotenone-insensitive NAD(P)H dehydrogenases in plants: Immunodetection and distribution of native proteins in mitochondria

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Abstract – Antisera produced against peptides deduced from potato *nda*1 and *ndb*1, homologues of yeast genes for mitochondrial rotenone-insensitive NADH dehydrogenases, recognise respective proteins upon expression in *Escherichia coli*. In western blots of potato (*Solanum tuberosum* L.) mitochondrial proteins, the NDB and NDA antibodies specifically detect polypeptides of 61 and 48 kDa, respectively. The proteins are found in mitochondria of flowers, leaves and tubers. Different signal intensities are seen relative to other respiratory chain components when organs are compared, indicating variations in relative abundance of dehydrogenases within the plant. The antibodies detect single polypeptides, of similar size as in potato, in mitochondria from several plant species. No specific cross-reaction was found in chloroplasts, but a weak NDA signal of 50 kDa was found in microsomes, possibly associated with peroxisomes. Two-dimensional native/SDS-PAGE analyses indicate that both NDA and NDB proteins reside as higher molecular mass forms, possibly oligomeric. The NDB immunoreactive protein is released by sonication of mitochondria, but resistant to extraction by digitonin and partially to Triton X-100. In comparison, the NDA protein remains bound to the inner membrane at sonication or digitonin treatment, but can be solubilised with Triton. Investigation of a beetroot (*Beta vulgaris* L.) induction system for external NADH dehydrogenase indicates that the NDB antibody does not recognise the induced external NADH dehydrogenase in this species, but possibly an external NADPH dehydrogenase.

KEY WORDS

Membrane Association / NAD(P)H dehydrogenase / plant mitochondria / potato / red beetroot / respiratory chain / rotenone

ABBREVIATIONS

PAGE, polyacrylamide gel electrophoresis / SDS, sodium dodecyl sulphate / SMP, sub-mitochondrial particle

1. INTRODUCTION

The mitochondrial respiratory chain of plants contains multiple NAD(P)H dehydrogenases [26, 31]. Distinct external, rotenone-insensitive enzymes oxidise cytoplasmic NADH and NADPH in a calcium-dependent manner [38]. Matrix NADH can be oxidised via two alternative enzymes. NADH oxidation through the rotenone-sensitive complex I (EC 1.6.5.3) mediates ATP production via proton pumping, whereas the activity of the rotenone-insensitive NADH dehydrogenase is not restricted by coupling to energy conservation [34]. Additionally, a calcium-dependent rotenone-insensitive NADPH dehydrogenase is present at the matrix surface of the inner membrane [24, 35]. Rotenone-insensitive NADH dehydrogenases, as detected by their enzymatic activities in isolated mitochondria, are present in all analysed plant species [26], whereas the occurrence of NADPH dehydrogenases have been investigated to a lesser extent.

Complex I in eukaryotic mitochondria is a very large transmembrane enzyme consisting of 30-42 subunits and several internal electron transfer groups [31]. By comparison, rotenone-insensitive NADH dehydrogenases, best studied in yeast and *Escherichia coli*, are encoded by single genes producing polypeptides of approximately 50 kDa [6, 20, 41]. None of these enzymes accept NADPH as a substrate. In *Saccharomyces cerevisiae*, the internal NDI1 catalyses the oxidation of matrix NADH whereas the external NDE1 and NDE2 oxidise NADH from the cytoplasm, as demonstrated by mutation analyses. [6, 20]. The proteins are firmly bound to the membrane by an unknown mechanism, as there are no long hydrophobic stretches in the sequences [6]. The rotenone-insensitive NADH dehydrogenases appear to have very high turnover numbers. The specific activity of the purified internal NADH dehydrogenase of yeast mitochondria is above 1 $mmol \cdot min^{-1} \cdot mg^{-1}$ [5]. As this activity is about 10000 times higher than the activities of rotenone-insensitive NADH dehydrogenases in potato mitochondria [35], one should expect very low protein amounts of these enzymes, so highly specific recognition is needed for their detection.

Recently, we described the presence of the *nda*1 and *ndb*1 genes in potato, homologues of internal and external rotenone-insensitive NADH dehydrogenases in yeast [36]. The protein products were competent for in vitro import into potato mitochondria where the NDA1 and NDB1 became associated with the internal and external surface of the inner mitochondrial membrane, respectively. On expression in *E. coli* the NDA1 hybrid protein was firmly bound to the cell membrane whereas the NDB1 hybrid could be released by sonication.

In order to enable specific detection and characterise the distribution of NDA and NDB type proteins in mitochondria from different sources, as well as by native size and membrane association after mitochondrial disruption, we have produced antibodies against the NDA1 and NDB1 gene products.

2. Results

2.1. Antibodies against NDA and NDB proteins recognise single bands in mitochondria

Antisera raised against NDA1 and NDB1 proteins of potato was produced by immunisation of rabbits with conjugated peptides. The sequence of the NDA1 peptide was derived from a relatively unconserved segment immediately upstream of the second nucleotide binding motif whereas the NDB1 peptide corresponds to the more conserved extreme C-terminus of the NDB1 protein [36]. To investigate whether the antisera could detect the respective proteins, *nda*1 and *ndb*1 cDNA were expressed in *E. coli* as hybrid protein with N-terminal S-tags [36]. NDA1 hybrid protein is visualised by S-protein detection with a major band at 65 kDa, a larger form at approximately 170 kDa as well as degradation products below 65 kDa (*figure 1*). After stripping the membrane, consecutive detection with NDA antiserum reveals an almost identical pattern, except for small degradation products which by calculation from sequence should not contain the region against which the NDA antiserum was made. The NDB1 hybrid protein is detected by S-protein as a main 75 kDa band which is also recognised by NDB antiserum (*figure 1*). Additionally, a band at approximately 55 kDa is detected. This may partly be an N-terminally truncated degradation product of the NDB hybrid protein, lacking the S-tag domain. However, a band of similar size is detected also in *nda*1-expressing cells indicating that an endogenous *E. coli* protein is visualised. The antiserum against NDA does not recognise the NDB expression product and vice versa (*figure 1*).

The antisera were used for detecting NDA and NDB proteins in comparison with other respiratory chain components in potato. Mitochondria were isolated from unopened flowers, mature leaves and tubers of Desirée potato plants and protein analysed by SDS-PAGE and western blotting (*figure 2*). The NDA antibodies consistently detect a single polypeptide with an apparent molecular mass of approximately 48 kDa in leaves and flowers and slightly larger in tubers. The NDB antiserum recognises a 61 kDa band in mitochondria from all three organs. Whereas the NDB signal is more dense in tuber mitochondria than in flower and leaf, the NDA signal level is considerably elevated in leaves.

Antisera against wheat NAD9 and *Neurospora* 78 kDa complex I subunits specifically detect the potato homologues, the NAD9 and 76 kDa subunits, respectively as previously shown [10, 12, 30]. The complex I subunits show a relatively even distribution between different organs with highest signal intensities in tubers and lowest in leaves. This pattern is similar to that for the major 55 kDa band detected by spinach F_1-ATP synthase antiserum and cytochrome c oxidase activity (*figure 2*). The results suggest that NDA protein distributes differently as compared to the main respiratory chain components, whereas the NDB protein shows a profile more similar to the main chain enzymes.

2.2. Distribution of NDA and NDB homologues in other plants

The NDB antiserum detects a single 61 kDa polypeptide in mitochondria from several different plants sources (*figure 3*). Mitochondria from *Helianthus tuberosus* tubers, *Ipomea batatas* roots and *Zea mays* coleoptiles give a similar signal as in potato. *Arum* spadix mitochondria display a somewhat stronger band whereas a less intense band is seen in beetroot mitochondria. For NDA, *Arum* mitochondria show a very strong signal at 48 kDa. *Helianthus* mitochondria produce a 46 kDa band of similar density as in potato, whereas the 49 kDa band seen in beetroot mitochondria is less dense. Interaction is not detected in *Ipomea* and *Zea* mitochondria, indicating that the NDA1 protein segment used for producing antisera is not conserved in these species. No polypeptides could be detected by the antisera in rat liver mitochondria (results not shown).

2.3. Intracellular distribution in potato

In order to investigate the organellar specificity of the immunosignals, different cellular protein fractions, peroxisomes, chloroplasts, microsomal fraction and total leaf protein were analysed by western blotting (*figure 4*). The NDB antiserum detects a 61 kDa band only in mitochondria. A diffuse shadow of around 50 kDa is additionally present in chloroplasts, correlating to the position of the highly abundant large rubisco subunit, as seen by protein staining. The NDA antiserum recognises a 48 kDa polypeptide in mitochondria, but a broader, more diffuse band of similar molecular mass is seen in peroxisomes. The peroxisomal signal correlates strongly with the position of the catalase protein band, highly dominating this fraction [39], which may disturb the migration of an interacting protein, or be the target of a weak interaction.

The NAD9 antiserum detects a signal only in mitochondria, showing that mitochondria were absent, or present in minute amounts, in the other fractions. At much longer film exposure, a single, sharp 48 kDa band recognised by the NDA antiserum appears in the microsomal fraction, though without a concomitant signal detected by the NAD9 antibody, indicating that the NDA signal is not derived from mitochondrial membranes (results not shown).

The results above suggest that NDA and NDB antisera specifically detect respective target polypeptides in mitochondrial protein fractions of plants, and can be used for analysis of NDA- and NDB-type proteins in mitochondria.

2.4. The NDA and NDB proteins reside as higher molecular mass forms in the native state

In two-dimensional Blue native/SDS-PAGE gels of mitochondrial membrane proteins from Desirée tubers (*figure 5*), the 48 kDa protein recognised by the NDA antiserum migrates as an unsharp band in the first, native dimension. The location corresponds to a native molecular mass of 150-200 kDa, indicating a tri- or tetra-meric structure of the protein. No signal was detected migrating as a

monomer in the native dimension, even at higher exposures. The 48 kDa protein was absent from the mitochondrial soluble protein fraction, where only a faint interaction with a 94 kDa polypeptide was seen at similar exposure (*figure 5*).

To a larger extent than the NDA signal, the 61 kDa polypeptide recognised by the NDB antibody has a broad distribution in the native dimension. Four spots are detected, corresponding to native molecular masses of approximately 700, 600, 500 and 180 kDa (*figure 5*). All four NDB immunosignals could be detected in both membrane and soluble protein fractions with some variation in release between preparations, as also confirmed by one-dimensional analysis (results not shown).

2.5. Membrane association

Bintje tuber mitochondria were subfractionated by sonication, separating the membranous SMP from soluble proteins, and digitonin which primarily disrupts the outer membrane, separating mitoplasts from outer membranes and intermembrane space proteins. On analysis of the protein fractions by SDS-PAGE and western blotting (*figure 6*), the NDA signal is found only in intact mitochondria, mitoplasts and SMP. The NDB signal is visible in mitochondria, and after sonication, only in the soluble protein fraction. However, after digitonin treatment the immuno-detected polypeptide remains in the mitoplast fraction with some staining visible also in the outer membrane fraction, and none in the soluble intermembrane space fraction. The control for inner membrane integral proteins, the 76 kDa subunit of complex I, is present in mitochondria, mitoplasts and SMP, similar to NDA. The matrix protein, NAD-malic enzyme (EC 1.1.1.39), is found in mitochondria, mitoplasts and in the soluble protein fraction, but also in the intermembrane space fraction, indicating that some inner membrane has been permeabilised by the digitonin treatment allowing a minor release of matrix content.

Triton X-100 was additionally tested for the ability to solubilise the immunodetected NDA and NDB proteins from Desirée tuber mitochondria (*figure 7*). After treatment with different concentrations of detergent, the solubilised proteins were separated by pelleting the membranes. The 61 kDa NDB signal is progressively solubilised over a wide range of detergent concentrations with a slight signal in the supernatant already at the lowest detergent concentration. Some protein remains associated with the membranes also at the highest concentration used. The NDA signal is detected in the supernatant only after treatment with 1.0 % Triton (*figure 7*). Similar results were seen with Bintje tuber mitochondria (results not shown).

2.6. The NDB signal is not increased upon beetroot induction

Red beetroots constitute the only known plant tissue where mitochondrial rotenone-insensitive NADH dehydrogenases can be induced [3, 26, 37]. On washing sliced beetroots, the external NADH dehydrogenase in isolated mitochondria increased 3.5-fold whereas the internal rotenone-insensitive NADH

dehydrogenase increased only 1.8-fold (*figure 8*). Also the external oxidation of NADPH was only slightly increased by the treatment. The immunodetected NDA and NDB both showed little increased staining in mitochondria from induced tissue as compared to control beetroots. The level of the NAD9 subunit of complex I was unchanged by the treatment.

3. DISCUSSION

NDA and NDB antisera recognise *E. coli*-expressed *nda*1 and *ndb*1, respectively, with no cross-reactivity (*figure 1*). The NDA antiserum, produced against a peptide sequence of potato NDA1, detects an approximately 48 kDa large polypeptide in potato mitochondria (*figure 2*). The molecular mass of the detected protein is similar to the apparent size of the imported NDA1 and somewhat smaller than the theoretical molecular mass, 55 kDa, of the NDA1 precursor protein as calculated from sequence. The latter is however processed upon mitochondrial import into a slightly shorter polypeptide [36]. The antiserum against the C-terminal peptide sequence of NDB1 recognises a polypeptide of 61 kDa in potato mitochondria, similar to the theoretical molecular mass of the NDB1 and the apparent mass of in vitro translation products from the *ndb*1 cDNA [36]. The results thus suggest that the NDA and NDB antibodies recognise the NDA1 and NDB1 proteins in mitochondria, respectively. However, isoenzymes with identical molecular masses to the two proteins may also be detected by the antisera if epitopes are conserved. In the newly sequenced *Arabidopsis thaliana*, 2 and 4 genes show strong similarity to potato *nda*1 and *ndb*1, respectively (Agius S.C., Rasmusson A.G., unpublished). Additional NDA and NDB isoenzymes are also likely to be found in potato.

The NDA and NDB immunosignals are seen in potato mitochondria from three major organs indicating that NDA1 and NDB1 proteins or close homologues are constitutively present in the plant. A comparison of signal intensities indicates a general pattern of protein abundance shared by the immunorecognised NDB protein and other main respiratory chain components (*figure 2*). The immunodetected NDA signal, however, varies considerably from this pattern by a stronger signal in leaves, indicating a metabolical function for the NDA1 gene product individually regulated as compared to the enzymes of the main respiratory chain. The presence of different NDA isoenzymes in the different organs is also indicated, i.e. the polypeptide recognised in tubers is consistently seen at a slightly higher apparent molecular mass when compared to the signal in leaves (*figure 2*). The observed variation in signal intensity may be a consequence of differences in the affinity of the antibodies for different isoenzymes that may be expressed in different tissues.

The presence of single bands cross-reacting with the antisera in mitochondria from several plant species indicates that NDA and NDB homologues are generally present in plants, and that the segments of NDA1 and NDB1 used for antibody production are differently conserved (*figure 3*). Interestingly, though the NDA antiserum cannot detect a homologue in the monocotyledonous maize, a very strong signal is seen in *Arum* spadix. The latter tissue display very high respiratory chain capacity and insensitivity to rotenone as compared to other sources [17], indicating that high protein amounts of internal rotenone-insensitive NADH dehydrogenase is present.

Analysis of different cellular membranous fractions for antibody interaction yielded a pattern of high specificity of NDB protein for mitochondria (*figure 4*). Also the NDA antiserum shows a major interaction with mitochondria. A signal

of the same molecular mass is, however, also detected in peroxisomes. Although this fraction is highly dominated by catalase, producing a very broad dense band at the same molecular mass in Coomassie stained gels [39], an NDA-type protein may be present in this organelle. At much higher exposure, a 50 kDa polypeptide was specifically detected in the microsomal fraction, a signal that may be derived from peroxisomes. A β-specific NADH: ferricyanide oxidoreductase activity correlating with a 53 kDa polypeptide has previously been reported present in vesicles of the peroxisomal membrane [39]. Apart from the potential peroxisomal signal, the results suggest that immunodetected NDA and NDB proteins are specifically localised in mitochondria, though we cannot exclude that homologues without conserved epitopes are targeted to other organelles.

The singularity of NDA and NDB immunorecognised signals in mitochondria from several sources, and complete lack of interaction with any protein in others, e.g. rat liver mitochondria, indicates that the antibody recognition is highly specific, and that the sera can be used with confidence for detection of the proteins in isolated mitochondria.

Resolution of Desirée potato mitochondria by Blue native-PAGE on gradient gels separates the protein complexes especially of the respiratory chain in a highly intact form and according to their native sizes. This can be seen by Coomassie staining after a second dimension of SDS-PAGE (*figure 5*) as has been reported previously [13]. Under these conditions, the immunorecognised NDA protein of tuber mitochondria migrates in the native dimension mainly as a 150-200 kDa species, indicating a possible tri- or tetra-meric structure of the enzyme. This is similar to the condensed high molecular mass form produced on expression in *E. coli*, though the latter could not be resolved into its monomer by SDS-PAGE (*figure 1*). The 61 kDa polypeptide detected by the NDB antiserum has a broader distribution in the native dimension. This may suggest the presence of several native mass forms of the NDB1 enzyme composed of different numbers of polypeptide units. Alternatively, the enzyme may be partially split up during the gel run from a large oligomeric species. Irrespectively, the results clearly indicate that the NDA and NDB are present in mitochondria as larger molecular mass forms, probably oligomers. The native molecular masses of the homologues of *E. coli* and fungi have to our knowledge not been investigated.

In comparison to the partial release in Desirée, sonication released the NDB completely from Bintje tuber mitochondria, where the immunorecognised protein was found only in the soluble protein fraction (*figure 6*). However, upon treatment with digitonin the protein was retained by the mitoplasts, as well as by outer membranes. It should however be noted, that at this protein loading of the two fractions (*figure 6*), the outer membrane is highly overrepresented as compared to the ratio in intact mitochondria, where typically only 5 % of the total protein is present in the outer membrane [7]. It is highly unlikely that the signal in the outer membrane fraction should derive from the outer membrane NADH:cytochrome c reductase, as this protein has very different enzymatic and structural properties as compared to the NDB homologues in yeast and *E. coli* [5, 20, 26, 41]. The immunodetected NDB remains completely bound to the membranes, though some matrix proteins are clearly released by a limited

permeation of the inner membrane, as seen from the presence of NAD-malic enzyme in the inter-membrane space fraction, (*figure 6*). Also Triton X-100 is less efficient than sonication in solubilising the immunorecognised NDB protein. The protein is partially released over a wide Triton concentration range from Desirée mitochondria (*figure 7*). A similar result was seen also with Bintje mitochondria (results not shown). The apparent stabilisation of membrane interactions by low concentrations of detergents may be a property also of the external NADPH dehydrogenase, p64, of *Neurospora crassa*. This enzyme is, similar to the immunodetected NDB protein, retained by membranes at digitonin treatment [22, 23]. However, whether the p64 protein is released by sonication has not been investigated. In comparison to NDB, the immunorecognised NDA protein behaves as a firmly bound membrane protein, similar to the yeast internal NADH dehydrogenase [5]. NDA is retained by the membrane at sonication or digitonin treatment, and become solubilised at higher Triton concentrations (*figures 6, 7*).

The results suggest that previous analyses in more artificial systems of the NDA1 and NDB1 proteins [36] are valid also for the proteins as present in isolated mitochondria. In the previous investigation, in vitro synthesised NDB1 was shown after mitochondrial import to remain bound to membranes at digitonin treatment. On expression as hybrid proteins in *E. coli*, the NDB1 protein was, unlike NDA1, released by sonication of cells [36]. An important difference to the bacterial system, however, resides in that the native immunorecognised NDB protein in tuber mitochondria is released by sonication also in the presence of divalent salts (*figure 6*). Release of external NAD(P)H dehydrogenases from plant mitochondria by osmotic swelling or sonication has previously been reported [8, 19, 25], a characteristic that may be general for the external enzymes. The presence of immunorecognised NDB protein also in the outer membrane fraction indicates that the protein has access to the outer membrane before the digitonin treatment, consistent with the assignment of the NDB1 as an enzyme facing the inter-membrane space [36], if the enzyme is loosely bound to the membrane, as discussed above. Additionally, it indicates that the protein in vivo may be able to move between the outer surface of the inner membrane and the inner surface of the outer membrane. Whether this may be a point of regulation, however, remains to be investigated.

In order to analyse the presence of immunorecognised proteins in correlation to NAD(P)H dehydrogenase activities we investigated red beetroot induction (*figure 8*). Beetroots are known to induce external NADH oxidation, to a smaller extent the rotenone-insensitive internal NADH oxidation, but not external NADPH oxidation, during washing of tissue slices [1, 3, 37]. A similar induction, specific for external NADH oxidation and not involving the external NADPH dehydrogenase is seen upon long-term storage of intact beets at 10 °C [9]. In this investigation, beetroot polypeptides recognised by both NDA and NDB antisera are increased relatively little upon washing beetroot slices (*figure 8*). Correlation to activities clearly indicate that the induced external NADH dehydrogenase in beetroots is not recognised by the NDB antibodies. The serum may instead recognise an external NADPH dehydrogenase. Unfortunately there is no plant

system available where external NADPH oxidation is known to be induced, so the reverse experiment can presently not be made. Recently, the p64 homologue in *N. crassa* was shown by directed mutation to be an externally facing NADPH dehydrogenase [22], whereas all previously investigated homologues of fungi [5, 15, 20] have been shown only to interact with NADH. Though the phylogenetic relationship between the NDB1 of potato and the p64 of *N. crassa* is presently unclear, the sequences show similarities as compared to all other investigated homologues, especially by carrying inserts with potential calcium-binding motifs at similar locations [14, 23, 36], a property that may be typical for the external enzymes of multicellular organisms. As the protein superfamily now contains both NADH and NADPH dehydrogenase members, it will be necessary to investigate the substrate specificity of each homologue in plants separately.

4. METHODS

4.1. Material and extractions

Expression of *nda*1 and *ndb*1 hybrid constructs were induced for 2.5 h at 37 °C in *Escherischia coli*, total protein extracted into SDS-PAGE loading buffer and S-tagged proteins detected as previously described [36]. Potato plants (*Solanum tuberosum* L. cv. Desirée) were grown in greenhouse with supplemental light. Potato tubers cv. Bintje were from the local market. Leaf and flower mitochondria were prepared principally according to Boutry et al. [2]. The method was scaled up for 5-10 g of tissue and disruption was made in a household blender fitted with razor-blades. Tuber mitochondria and peroxisomes were prepared according to Struglics et al. [39]. Jerusalem artichoke (*Helianthus tuberosus* L.) mitochondria were isolated according to Lidén and Møller [18]. Mitochondria were purified from *Arum italicum* Miller spadices, collected in the Royal Botanic Gardens, Adelaide, Australia in the late ε stage, according to Day et al. [4]. Seven-day old etiolated maize (*Zea mays* L.) coleoptiles were used for mitochondrial extraction according to Moore and Proudlove [27]. Crude mitochondria [40] from sweet potato (*Ipomoea batatas* L.) roots were purified as described in Struglics et al. [39]. Red beetroots (*Beta vulgaris* L.) were induced according to Rayner and Wiskich [37], and mitochondria purified according to Rasmusson et al. [32]. Mitochondria were subfractionated by sonication, and sub-mitochondrial particles (SMP) taken as the 105,000 x *g* minus 12,000 x *g* pellet, according to Rasmusson and Møller [34]. Digitonin fractionation was according to Ragan et al. [29]. Soluble protein fractions were concentrated in 10 kDa cut-off micro concentrators (Pall-Filtron) before gel analysis. Total leaf protein was extracted according to Martinez-Garcia et al. [21]. Potato chloroplasts were prepared according to Guedeney et al. [11]. Microsomal membranes were prepared by centrifuging the supernatant, after pelleting of chloroplasts, for 10 min at 10,000 x *g*, discarding the pellet, and then pelleting microsomes at 46,000 x *g* for 20 min. Solubilisation of mitochondria was done for 10 min on ice at a protein concentration of 10 $mg \cdot mL^{-1}$. The incubation medium contained 0.3 M mannitol, 10 mM 3-[N-Morpholino]propanesulfonic acid buffer (pH 7.2), 1 mM ethylenediaminetetraacetic acid and Triton X-100 as denoted. The suspension was centrifuged at 100,000 x *g* for 30 min to separate solubilised proteins released into the supernatant, from unsolubilised membranes remaining in the pellet.

4.2. Gel electrophoresis and blotting

One-dimensional SDS-PAGE of mitochondrial proteins were made according to Laemmli [16] using a polyacrylamide concentration of 10 % in separation gels. For two-dimensional analysis, Desirée mitochondria were separated into membrane and soluble protein fraction and resolved by Blue Native/Tricine Tris SDS-PAGE according to Jänsch et al. [13]. Western blotting was carried out as in Moos et al. [28], and detection of antibody labelling, and membrane stripping between consecutive detections, was performed according to the ECL system

(Amersham). Unless otherwise denoted, in each lane equal protein amounts were loaded. For production of antisera, peptides were synthesised according to potato NDA1 and NDB1 deduced sequences [36, EMBL accession numbers AJ245861 and AJ245862). The NDA1 and NDB1 peptides, D_{215}VPGVSEEEKRRLLHC$_{230}$ and (C)D_{563}WVRRYIFGRDSSRI$_{577}$, respectively, were coupled via the cysteines to keyhole limpet haemocyanine and the conjugates used for immunisation of rabbits. The antisera were used for western blotting at a titre of 1:1000. For the antiserum against NDA1, an occasional additional interaction with a 52 kDa protein could be seen in potato tuber mitochondria, especially from Bintje. Blue native/SDS-PAGE analysis showed this to be due to an interaction with the abundant core subunits of complex III, and the signal could be avoided by incubating the antibody solution with a blotting membrane carrying purified complex III [13].

4.3. Assays

Cytochrome c oxidase (EC 1.9.3.1) activity was determined according to 33]. External NAD(P)H oxidation was determined in an oxygen electrode (Rank Brothers), in assay medium, 0.3 M sucrose, 20 mM 3-[N-Morpholino]propanesulfonic acid/KOH (pH 7.2), 5 mM K-PO_4, 2.5 mM $MgCl_2$, 0.5 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid, supplemented with 1 mM $CaCl_2$, 0.4 µM carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone, and 1 mM NADH or NADPH. Malate oxidation was measured in assay medium with addition of 10 mM glutamate, 0.5 mM coenzyme A, 0.25 mM ATP, 0.5 mM NAD^+. The reaction was started with 25 mM malate and 0.375 mM ADP and 20 µM rotenone was added in order to measure the rotenone-insensitive state 3 activity. Protein was determined by the bicinchoninic acid reagent according to the manufacturers instructions (Sigma).

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FIGURE LEGENDS

Figure 1. Detection of NDA1 and NDB1 hybrid proteins upon expression in *E. coli*. Total protein was extracted from cells expressing S-tagged hybrid proteins of NDA1 (**a**) and NDB1 (**b**). The proteins were resolved by SDS-PAGE, electroblotted, and the expressed proteins detected consecutively by S-protein binding (S-tag), NDA antiserum (NDA) and NDB antiserum (NDB). The sizes of molecular mass markers are shown on the left.

Figure 2. Detection of NDA and NDB proteins in mitochondria from potato organs. Mitochondria of flower (F), leaves (L) and tuber (T) from Desirée were resolved by SDS-PAGE and western blotted. Membranes were treated with different antisera. **A**, the F_1 domain of spinach ATP synthase (ATP-F_1) of which the major signal at 55 kDa is shown, the 78 kDa subunit of *Neurospora crassa* complex I detecting the 76 kDa potato homologue (76 kDa), the NAD9 subunit of wheat complex I detecting the potato polypeptide at 27 kDa. The specific cytochrome c oxidase activity of the isolated mitochondria (CCO) is denoted above the blots in $\mu mol \cdot min^{-1} \cdot mg^{-1}$. **B**, antiserum against NDA1. **C**, antiserum against NDB1. Molecular mass markers are denoted at the left of the gels in **B** and **C**.

Figure 3. Western blotting of mitochondria from different species. Mitochondria isolated from red beetroot (*Beta vulgaris; B.v.*(r)), Sugarbeet (*B.v.*(s)), *Helianthus tuberosus* tubers (*H.t.*), *Ipomea batatas* roots (*I.b.*), *Zea mays* coleoptiles (*Z.m.*), *Arum maculatum* spadix (*A.m.*) and *A. italicum* spadix (*A.i.*) was compared to Bintje potato tuber mitochondria (*S.t.*). Hundred µg of protein was loaded per lane of an SDS-PAGE gel. The NDB antiserum recognises single 60-62 kDa polypeptides in all the mitochondrial samples. A less intense signal is detected for beetroot. The NDA antibodies recognise single polypeptides of 47-50 kDa in several species. Highest signal intensity is found in *Arum* sp., followed by Jerusalem artichoke, potato and beetroot. No signal is detected for sweet potato nor for maize.

Figure 4. Antibody signals in different cellular compartments. Cell fractions were resolved by SDS-PAGE, western blotted and analysed with NDA, NDB and NAD9 antisera. Potato leaf mitochondria (Mi), microsomal fraction (MF), tuber peroxisomes (PX) and total leaf protein extract (T) were loaded at 30 µg protein per lane. For potato chloroplasts (CP), organelles containing 4 µg chlorophyll were analysed. Molecular mass markers are denoted at the left.

Figure 5. Two-dimensional resolution of tuber mitochondria. Desirée mitochondria were sonicated and separated into membranes and soluble proteins. The protein fractions, 200 µg of each, were analysed according to native size by blue native-PAGE (horizontal). Proteins in gel strips were denatured and resolved by SDS-PAGE according to polypeptide molecular mass (vertical). Gels were stained with Coomassie R 250 or analysed by western blotting. The NDA

antibodies detect a 48 kDa polypeptide only in membranes, with migration in the native dimension as a 150-200 kDa protein. The NDB antibodies recognise a 61 kDa polypeptide distributed in several spots between 150 and 700 kDa in the native dimension of both membranes and soluble mitochondrial proteins. Native and denatured molecular mass standards are denoted on top and at the sides, respectively.

Figure 6. Subfractionation of mitochondria. Tuber mitochondria (Mit) from Bintje were separated into SMP and soluble protein (Sol) by sonication, and into mitoplasts (Mpl), intermembrane space fraction (IMS) and outer membrane (OM) by digitonin treatment. Fractions were analysed by SDS-PAGE and western blotting. Of each fraction, 20 µg protein was loaded, except for soluble protein (37 µg) and intermembrane space (13 µg). Antibodies detecting the 76 kDa subunit of complex I was used as control for inner membrane, and antibodies against potato NAD-malic enzyme (NAD-ME) for matrix.

Figure 7. Solubilisation of mitochondria by Triton X-100. Detergent extracts of potato tuber mitochondria were resolved by SDS-PAGE, western blotted and immunodecorated with NDA and NDB antisera. For supernatant (S) and pellet (P) from each detergent incubation, the sum of loaded protein corresponded to 40 µg of mitochondria.

Figure 8. Induction of NAD(P)H dehydrogenases in red beetroot. Mitochondria were isolated from control and washed beetroot slices and immunodetected signals for NDA, NDB and NAD9 were compared to rotenone-insensitive NAD(P)H dehydrogenase activities. Specific activities in $nmol \cdot min^{-1} \cdot mg^{-1}$ are denoted on top and molecular masses to the left. NADHex; external NADH oxidation, NADPHex; external NADPH oxidation, Malate+Rot.; rotenone-insensitive malate oxidation.

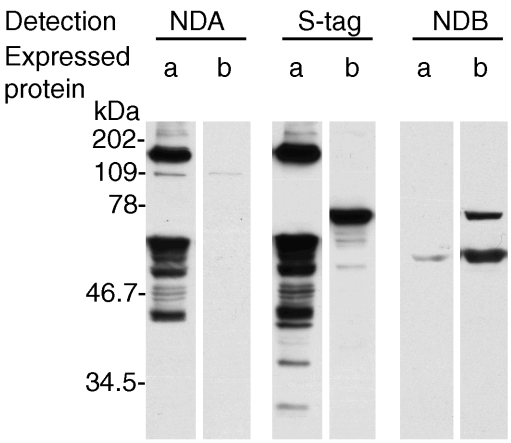


Figure 1

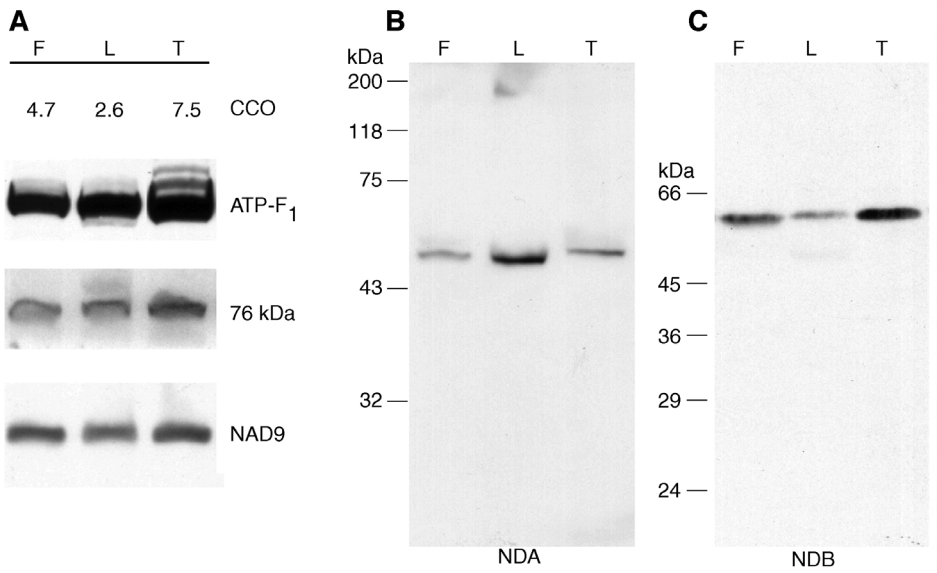


Figure 2

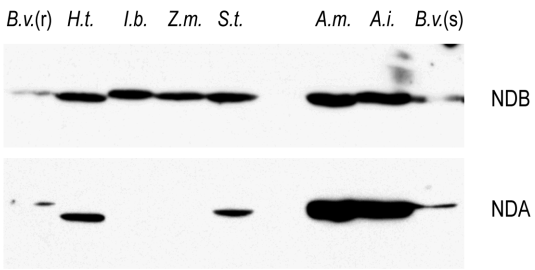


Figure 3

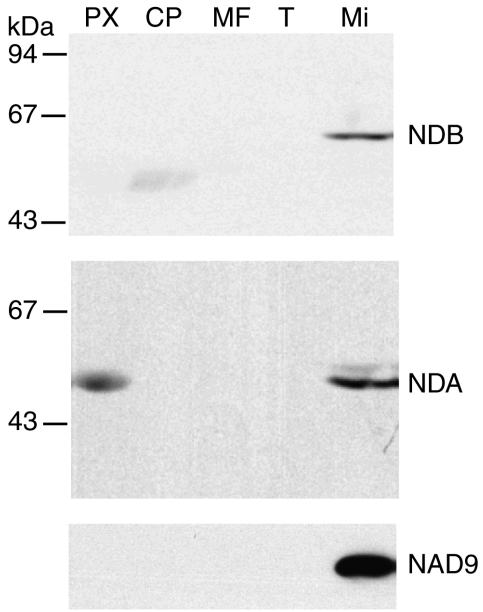
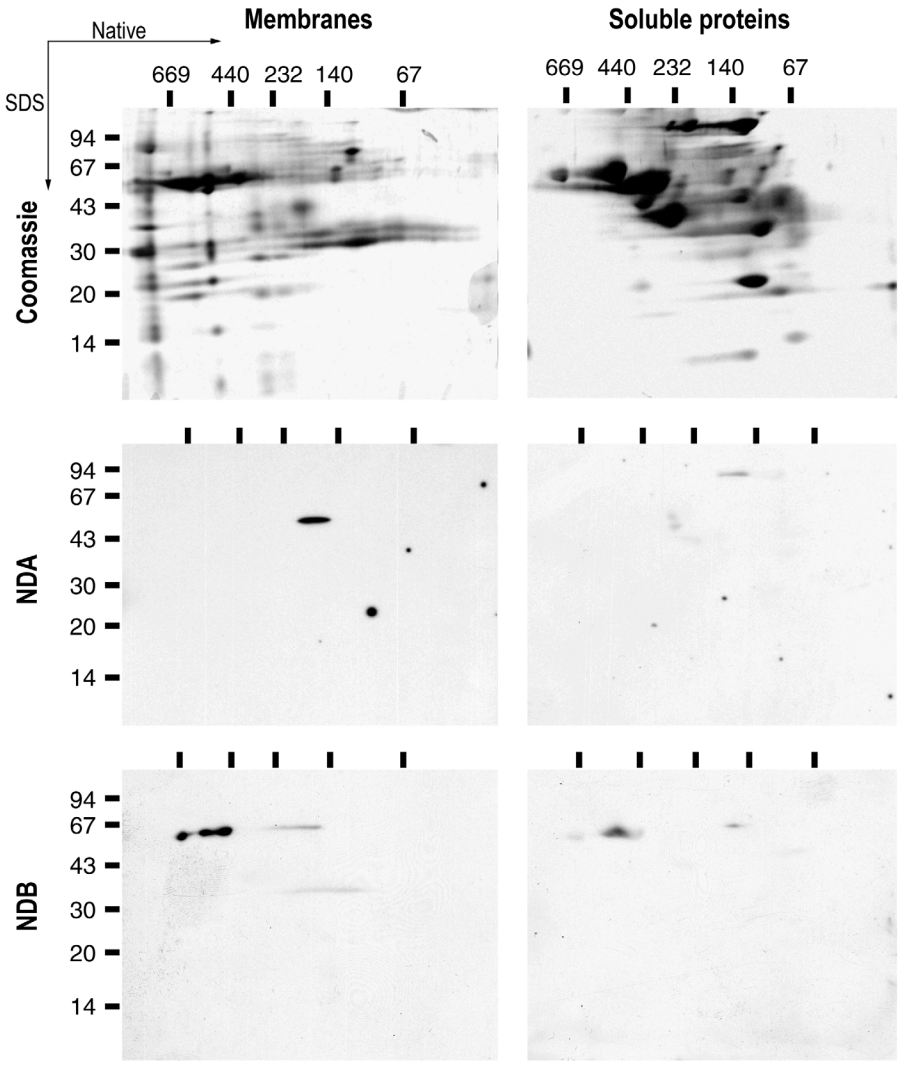


Figure 4


Membranes
Soluble proteins
Native
SDS
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669 440 232 140 67
Coomassie
NDA
NDB
94
67
43
30
20
14

Figure 5

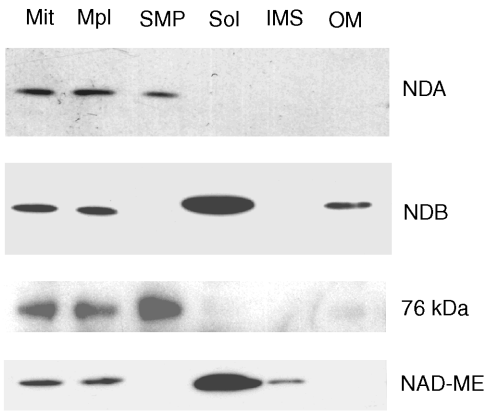

Figure 6

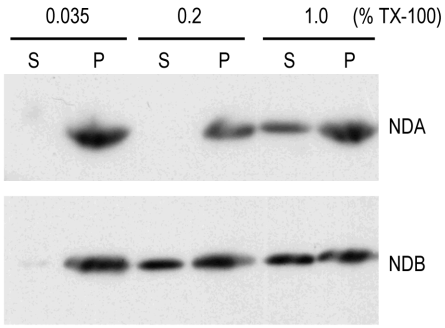

Figure 7

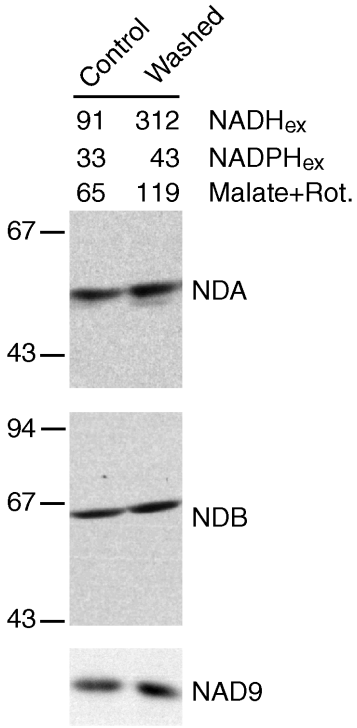
Control
Washed
91 312 NADH$_{ex}$
33 43 NADPH$_{ex}$
65 119 Malate+Rot.
67
43
NDA
94
67
43
NDB
NAD9

Figure 8