Chloroplast NADH Dehydrogenase from *Pisum sativum*: Characterization of its Activity and Cloning of *ndhK* Gene

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The pea chloroplast *ndhK* gene coding for a component of a NADH-plastoquinone oxidoeductase has been cloned and sequenced. This gene codes for a polypeptide of 227 amino acids and a predicted molecular mass of 25,495 Da which belongs to the family of the 20 kDa PSST subunit of the bovine mitochondrial complex I. A fragment of this gene has been overexpressed in *Escherichia coli*, and antibodies against the expressed polypeptide recognize a protein of the predicted molecular mass from pea thylakoid membranes. This polypeptide is a component of a protein complex with NADH dehydrogenase activity and is not associated with ferredoxin-NADP\(^+\) reductase.

Key words: Chlororespiration — Complex I — Cyclic electron transport — NAD(P)H dehydrogenase (EC 1.6.99.3) — *ndh* gene — *Pisum sativum*.

Chloroplast genomes contain 11 reading frames (*ndhA-ndhK*) coding for homologues of components of mitochondrial NADH-ubiquinone oxidoeductase (complex I) (Sugiura 1992). Complex I catalyzes the electron transfer from NADH to ubiquinone linked to proton translocation across the membrane (Walker 1992). The mitochondrial complex I consists of at least 42 subunits (Walker et al. 1995), but eubacteria contain a much simpler version of the protein. For instance, complex I from *Escherichia coli* is composed of 14 subunits organized in three subcomplexes: a membrane fragment, containing seven subunits homologous to chloroplast *ndhA-ndhG* gene products, where the quinone is bound; a connecting fragment with four subunits homologous to chloroplast *ndhH-ndhK*; and a three subunit NADH-oxidizing fragment with no homologues encoded in chloroplast genomes (Leif et al. 1995, Friedrich and Weiss 1997).

The absence of genes encoding the NADH-oxidizing subcomplex in chloroplast genomes has led to different hypotheses. The polypeptides of the NADH-oxidizing subcomplex could be encoded by nuclear genes and imported to the chloroplast, constituting a NADH-quinone oxidoeductase. However, all efforts to demonstrate the existence of these three genes have failed so far, and the possibility of a chloroplast complex equipped with a different electron input device has been proposed (Friedrich et al. 1995). According to this proposal ferredoxin or NADPH could be the electron donor of the complex, in the latter case possibly mediated by association of the Ndh complex with FNR (Guedeney et al. 1996). Recently, the existence of a protein complex equipped with both FNR and the NADH-binding polypeptide has been proposed. In this way, the complex could use either NADH or NADPH as electron donor (Quiles and Cuello 1998).

Recently, a NADH-plastoquinone oxidoreductase has been purified from pea thylakoids (Sazanov et al. 1998a). This complex, which does not copurify with FNR, has a molecular mass of 530 kDa and is composed of at least 16 different polypeptides, including several *ndh* gene products. Although the presence in this complex of the three subunits of the NADH-oxidizing subcomplex has not been demonstrated yet, this complex contains three unidentified polypeptides with molecular masses very similar to the subunits of the NADH-oxidizing subcomplex of bovine complex I (Sazanov et al. 1998a). The complex seems to participate in cyclic electron transport around photosystem I in the light and in chlororespiration in the dark (Burrows et al. 1998, Kofer et al. 1998), and seems to play an important role in the response to heat stress in the dark (Sazanov et al. 1998b).

In this work we report on the complete sequence of *ndhK* from *Pisum sativum*, as well as the expression in *E. coli* of a fragment of its gene and the use of the expressed polypeptide to elicit antibodies that recognize a protein of the predicted molecular mass from pea thylakoid membranes. Moreover, we show that NdHk is a component of a protein complex with NADH dehydrogenase activity, and that it is not associated with FNR.

Materials and Methods

DNA cloning of pea chloroplast *ndhK*—DNA fragments of...
the pea ndhK gene were amplified from total chloroplast genomic DNA by PCR using, in the first instance, mixtures of synthetic oligonucleotides based on previously reported ndhK sequences from maize (Steinmetz et al. 1986), tobacco (Shinozaki et al. 1986), rice (Hiratsuka et al. 1989), and wheat (Nixon et al. 1989) as primers and hybridization probes. Further details are given in the legend to Fig. 1. The sequence was determined in both senses of DNA by the dideoxy chain termination method (Sanger et al. 1977). Recombinant DNA techniques were performed by standard methods (Sambrook et al. 1989).

Expression of ndhK and preparation of antisera—For the expression of a non-fusion form of NdhK, an internal ndhK fragment (coding for amino acids 40 to 169) was amplified by PCR and cloned into the expression vector pKN172 (Way et al. 1990). Competent E. coli BL21 (DE3) (Studier and Moffatt 1986) cells were transformed with the resulting recombinant plasmid, which contained no errors in the nucleotide sequence of the insert. The cells were grown in LB supplemented with 0.1 mg ml⁻¹ ampicillin to an absorbance of 0.3 at 600 nm. IPTG was then added to the culture to a final concentration of 0.6 mM, and incubation continued for another 3 h. Cells were collected by centrifugation, and the pellet, resuspended in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, was disrupted by a single passage through a French press (Aminco) operated at a pressure of 140 MPa. Insoluble material containing the expressed polypeptide was recovered by centrifugation at 20,000 × g for 30 min. The identity of the expressed NdhK protein was confirmed by N-terminal sequencing. The expressed protein was recognized by an antiserum raised against tobacco (Schmid and Radunz 1974) and radish (Morigasaki et al. 1993) leaf-FNR were kindly provided by Prof. G. H. Schmid (University of Bielefeld, Germany) and Dr. K. Wada (Kanazawa University, Japan), respectively. Antiserum against NdhK from Synechocystis (Berger et al. 1988) was kindly donated by Dr. K. Steinmüller (University of Düsseldorf, Germany).

Preparation of plant material—Chloroplasts were obtained from 15 day-old pea leaves (Pisum sativum var. Lincoln) using the method described by Lægsgaard and Malkin (1986). Chloroplasts were lysed by freezing and thawing and then washed 3 times with 20 mM Bistris (pH 6). The pellet (2 mg Chl ml⁻¹) was finally washed with 20 mM Bistris (pH 6), 0.3 M NaCl, and solubilized in 20 mM Bistris (pH 6), 1 mM EDTA, 8% glycerol, 1 mM PMSF, 50 mM sucrose containing either 0.7% β-dodecylmaltoside or 0.5% Triton X-100 for 30 min at 4°C.

Protein was determined by the biocinchonic acid method (Smith et al. 1985) and Chl according to Arnon (1949).

Gel electrophoresis—BN-PAGE was carried out as described by Schägger (1994). Coomassie blue was added to the solubilized proteins to produce a detergent/Coomassie ratio of 4:1 (w/w/w), and samples were loaded onto a 4–20% acrylamide gradient gel made in a buffer containing 50 mM Bistris (pH 7), 0.5 M 6-aminocaproic acid. Gels were run at 4°C using 50 mM Tri-

Results and Discussion

Cloning and sequence analysis of the pea chloroplast ndhK gene—Based on previously published ndhK sequences, the pea chloroplast ndhK gene has been cloned by a PCR strategy. The pea ndhK gene has a length of 684 bp, and codes for a polypeptide of 227 amino acids with a predicted molecular mass of 25,495 Da (Fig. 1).

Analysis of the 5’ end of the ndhK gene shows that, compared with the tobacco sequence, the pea ndhK gene has a 14 bp deletion between the two ATG coding for M1 and M23 in the tobacco sequence (Fig. 2). This deletion causes a frameshift and, as a result, the first ATG is not in frame. Moreover, the presence of stop codons in the reading frame of the first ATG also argues against the possibility of this ATG to be the initiation codon. Therefore, this sequence shows that pea NdhK starts with a methionine corresponding to M23 in tobacco, eliminating the partial overlap of ndhK and ndhC genes that exists in other chloroplast genomes. Recent N-terminal sequencing of NdhK from pea has confirmed the DNA sequence presented in this work (Sazanov et al. 1998a). A similar situation has been reported in the ndhK gene from lupin, where a deletion of 13 bp causes the same effects as in pea (Oczkowski et al. 1997). These findings suggest that for most of the chloroplast ndhK genes sequenced so far, the initiation codon may have been wrongly assigned about 22 codons upstream of the true one giving rise to the overlap of ndhC and ndhK genes, although this should be confirmed by N-terminal sequencing of their products. Nevertheless, the two cyanobacterial ndhK genes studied are 15–19 amino acids longer in its N-terminal end (Steinmüller et al. 1989, Howitt et al. 1996), causing a partial overlap of ndhC and ndhK in Anabaena (Howitt et al. 1996).

Comparison of deduced amino acid sequences encoded by the ndhK gene from pea, maize (Steinmetz et al. 1986), tobacco (Shinozaki et al. 1986), rice (Hiratsuka et al. 1989), wheat (Nixon et al. 1989), lupin (Oczkowski et al. 1997), soybean (Whelan et al. 1992), Marchantia poly-
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Fig. 1 Nucleotide sequence of the chloroplast ndhK gene from Pisum sativum and the deduced amino acid sequence. Overlapping DNA fragments were isolated from total chloroplast DNA by a strategy based on PCR. The boxed protein sequences IF, IRA and 1RB represent the conserved regions of the previously reported sequences that were used to design synthetic oligonucleotide mixtures which were employed as forward and nested reverse primers, respectively. A partial DNA fragment was detected in the reaction products by hybridization with a third mixture of oligonucleotides based on conserved amino acids corresponding to positions 82-87 in the pea ndhK sequence. This DNA fragment was found to encode amino acids 35-177 of the complete protein. The boxed nucleotide sequences served as unique primers in two further reactions in which the sequence was extended to the 3' and 5' extremities. Forward primer 2F was used with degenerate primer 2R which was based on a conserved region (DWHSIA) of the flanking ndhJ gene in the extension to the 3' end. A unique 3F primer designed from the sequence of a DNA fragment containing the 3' end of the flanking ndhC gene, which was amplified in similar PCR experiments, was used with reverse primer 3R in the extension to the 5' extremity. Therefore, the complete DNA sequence was compiled from three partial sequences.

Fig. 2 Analysis of the adjacent regions of ndhC and ndhK genes from pea, lupin (Oczkowski et al. 1997), tobacco (Shinozaki et al. 1986), maize (Steinmetz et al. 1986), rice (Hiratsuka et al. 1989), and wheat (Nixon et al. 1989). The end of the ndhC gene is indicated by the arrow. Gaps introduced for alignment are indicated by asterisks. The two potential initiation codons of the ndhK gene are boxed. In pea and lupin these two codons are not in frame due to deletions of 14 bp and 13 bp, respectively. Stops codons that are in frame with the first potential initiation codon in pea and lupin are written in lower case.
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It is interesting to note the conservation of the four cysteine residues present in the sequence throughout the range of species that have been investigated. Even a homologous subunit of a formate hydrogen lyase from \textit{E. coli} presents three of the four conserved cysteine residues (Böhm et al. 1990). These residues are part of a sequence that follows very closely the pattern CxxCxCxxGxCxxxGx\textsubscript{m}-GCPP present in the small subunit of nickel hydrogenases (Albracht 1993). As far as the role of these conserved cysteine residues is concerned, one possibility is that they are involved in liganding a Fe-S cluster. Respiratory complex I and the small subunit of nickel hydrogenases have several Fe-S clusters. Although their presence in the chloroplast homologue has not been demonstrated yet, it is likely that the chloroplast protein has Fe-S clusters.

\textit{Isolation of a thylakoid NADH dehydrogenase containing NdhK}—The antiserum obtained against the expressed product of the pea \textit{ndhK} gene fragment was able to recognize a 27 kDa polypeptide in Western blot analysis of pea thylakoid membranes solubilized by SDS. This molecular mass agreed with that deduced from the sequence of the \textit{ndhK} gene. This polypeptide was not recognized by the antibodies when the soluble fraction of chloroplasts or mitochondrial membranes were tested. This band was not recognized either by the preimmune serum (data not shown).

Pea thylakoid membranes were selectively solubilized by 0.7% β-dodecylmaltoside. The solubilized proteins were separated according to their molecular masses by BN-PAGE in a 4-20% gradient gel. The presence of Coomassie blue in the samples and in the cathode buffer as well as the Chl bound to some proteins allowed the visualization of
several bands without the need of any staining method (Fig. 3A).

NAD(P)H dehydrogenase activities were assayed in the gel using NBT as the electron acceptor. Six activity bands were detected, the four bands corresponding to the larger proteins being stained only with NADH as the electron donor (Fig. 3).

The presence of NdhK and FNR in the activity bands was checked by a second-dimension SDS-PAGE of the excised bands, followed by a Western blot analysis using anti-NdhK and anti-FNR antibodies. NdhK was only present in band 1 and 2, which showed NADH dehydrogenase activity. Moreover, FNR was absent from these two bands, and its presence was increased in the smaller complexes (Fig. 4). Similar results were obtained when 0.5% Triton X-100 was used for solubilization of membranes (data not shown). However, band 1 was only detected with 0.7% β-dodecylmaltoside, and its presence varied from one preparation to another.

These results do not agree with those obtained by Guedeney et al. (1996) who reported an association of ndh gene products with FNR to form a protein complex with NADPH dehydrogenase activity in potato thylakoids solubilized by 4% Triton X-100. In their work, two bands with NADPH (rather than NADH) dehydrogenase activity were separated by native electrophoresis in a homogenous polyacrylamide gel. Several ndh gene products were present in the slow-migrating band while FNR appeared in both of them. Therefore, an association of the ndh gene products with FNR was proposed to form a protein complex with NADPH dehydrogenase activity. Our results are also in contrast to those recently obtained by Quiles and Cuello (1998), who reported on the existence of a NAD(P)H dehydrogenase containing both FNR and the NADH-binding subunit in barley thylakoids solubilized by deoxycholate. Differences between the plant species studied, the sticky nature of FNR, the use of different solubilizing conditions and the different non-denaturing electrophoresis systems used might be the reasons for discrepancy among the results obtained in these studies.

Nevertheless, our results obtained in pea thylakoids support the hypothesis of Nixon and coworkers that NADH is the electron donor of the thylakoid protein complex formed by ndh gene products and that FNR is not one of its components (Sazanov et al. 1996, 1998a, Burrows et al. 1998).

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