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In *Chlamydomonas*, the loss of ND5 subunit prevents the assembly of whole mitochondrial complex I and leads to the formation of a low abundant 700 kDa subcomplex

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Abstract

In the green alga *Chlamydomonas reinhardtii,* a mutant deprived of complex I enzyme activity presents a 1T deletion in the mitochondrial *nd5* gene. The loss of the ND5 subunit prevents the assembly of the 950 kDa whole complex I. Instead, a low abundant 700 kDa subcomplex, loosely associated to the inner mitochondrial membrane, is assembled. The resolution of the subcomplex by SDS-PAGE gave rise to 19 individual spots, sixteen having been identified by mass spectrometry analysis. Eleven, mainly associated to the hydrophilic part of the complex, are homologs to subunits of the bovine enzyme whereas five (including gamma-type carbonic anhydrase subunits) are specific to green plants or to plants and fungi. None of the subunits typical of the β membrane domain of complex I enzyme has been identified in the mutant. This allows us to propose that the truncated enzyme misses the membrane distal domain of complex I but retains the proximal domain associated to the matrix arm of the enzyme. A complex I topology model is presented in the light of our results. Finally, a supercomplex most probably corresponding to complex I–complex III association, was identified in mutant mitochondria, indicating that the missing part of the enzyme is not required for the formation of the supercomplex.

Keywords: *Chlamydomonas* mitochondria; ND5 subunit; Complex I mutant; NADH:Ubiquinone oxidoreductase

1. Introduction

NADH:ubiquinone oxidoreductase (complex I) is the largest and the less understood complex of the mitochondrial respiratory chain. It is an amphipathic enzyme located in the inner mitochondrial membrane and is composed of more than 40 subunits. Eukaryotic complex I components have a dual genetic origin: five to nine subunits (the ND subunits) are usually encoded in the mitochondrial genome whereas the remaining subunits are nuclear gene products. Because of this complexity, information on its structure and mechanism of action is limited. A simpler enzyme is found in bacteria with only 13–15 different subunits [1]. Low-resolution structures based on electron microscopy studies have revealed that mitochondrial NADH:ubiquinone oxidoreductase shares a common L-shape with its bacterial counterpart, with one arm embedded in the mitochondrial inner membrane and another arm protruding into the matrix [2,3]. In bacteria, the membrane arm is constituted by the homologues of the hydrophobic ND1, 2, 3, 4, 4L, 5 and 6 subunits, usually encoded in the mitochondrial genome of eukaryotes [4]; it also contains the proton-translocation machinery. The bacterial hydrophilic arm components contribute to the NADH dehydrogenase segment of the electron pathway by binding a flavin mononucleotide (FMN) and nine iron–sulfur clusters (up to eight in eukaryotes); it also contains the ubiquinone-binding site sensitive to rotenone [5].

Most of our knowledge of the catalytic function and location of the ND subunits in eukaryotes comes from bacteria. In particular, NUOL, NUOM and NUON bacterial subunits (corresponding to

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eukaryotic ND5, ND4, and ND2, respectively) are highly hydrophobic proteins, that contains around 15 transmembrane stretches, and are antiporter-like subunits probably involved in proton pumping activity [6]. The fact that ND5/NUOL and ND4/NUOM subunits are found located at the distal end of the membrane domain while the quinone binding pocket would be rather located at the interface between the two arms suggests that the coupling mechanism of complex I is likely to involve long range conformational changes [7,8]. However, the function of ND4 and ND5 subunits within complex I is far from being elucidated. In the case of ND5/NUOL, several authors argued that it could be involved in the proton translocation machinery of the enzyme in *Neurospora crassa* [9] or in the transport of Na^+ in bacteria [10]. Theoretical predictions of quinone binding sites [11] or photoaffinity labelling experiments [12] also suggested that the ND5 subunit is involved in the constitution of an inhibitor- and quinone-binding site(s).

A number of mutations in the human mitochondrial *nd5* gene have been identified. They are frequently associated with MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episode), LHON (Leber's hereditary optic neuropathy), idiopathic Parkinson disease, or Leigh's syndrome [13–22]. However the impact of the vast majority of these mutations on complex I activity and assembly is not well-characterized due to the variable degrees of heteroplasmy (simultaneous presence of wild-type and mutant mitochondrial DNA copies) in patient's tissues or to difficulties to collect samples for biochemistry analyses. In that respect, *Chlamydomonas reinhardtii* is a powerful experimental model since homoplasmic mitochondrial mutants can be isolated in this unicellular green alga [23]. In *Chlamydomonas*, only five *nd* genes (*nd1, 2, 4, 5* and *6*) are present on its small linear mitochondrial genome and several homoplasmic viable *nd* mutants have been characterized [24–27]. In particular, we demonstrated that the loss of *nd4* or the deletion of *nd4* and 3′ end of *nd5,* leads to the formation of a subcomplex of 700 kDa, missing 250 kDa compared to the size of the wild-type enzyme. In contrast, no complex or 700 kDa subcomplex is found when ND1, ND3, ND4L or ND6 subunits are lost [25,26,28].

In this paper, we report the genetic, biochemical and molecular characterization of a *Chlamydomonas* complex I mutant which presents a 1T deletion in the *nd5* gene. We show that the loss of ND5 subunit prevents the assembly of the whole complex I and leads to the formation of a 700-kDa membrane-associated subcomplex. The analysis of the subunit composition of this subcomplex brings new data on the possible location in the membrane domain of several subunits that are not present in mammal complex I. Our data finally suggest that the subcomplex is still able to associate with complex III into a supercomplex, indicating that the distal part of complex I membrane arm is not required for this interaction.

2. Material and methods

2.1. Strains and growth conditions

Strains used in this work are derived from the wild-type137c strain of *Chlamydomonas reinhardtii*. The following mitochondrial mutants have been used: *dum11, partial deletion of cob gene* [29]*; dum15*, double base-pair substitution in the *cob* gene, lacking complex III activity [30]; *dum18*, one T deletion in the *cox1* gene, lacking complex IV activity [30]; *dum20*, one T deletion in the *nd1* gene, lacking complex I activity [24]; *dum17*, one T deletion in the *nd6* gene, lacking complex I activity [26]. Some of the mitochondrial mutants were also auxotrophic for arginine, carrying the *arg7*, *arg7-2* and *arg7-8* nuclear mutations at the *ARG7* nuclear locus. Some of these mutations complement each other [31], allowing diploids to be selected on minimal medium.

Cells were routinely grown under white light (50 μE m^{-2} s^{-1}) on TAP (Tris–acetate–phosphate) or TMP (Tris–minimal-medium) agar medium supplemented with arginine 100 mg/l when required [32].

2.2. Mutagenesis and genetical analysis

Mutagenesis with acriflavine was performed on the wild-type mt^- strain as described in [27]. The transmission pattern of the mutations in crosses was determined by random analysis of the meiotic products. Recombinational analyses were performed as described before [26,33].

2.3. Whole-cell respiration

Measurements of whole-cell respiration were made using a Clark Electrode (Hansatech Instruments, King's Lynn, England) as described in Duby and Matagne (1999) [27]. Rotenone dissolved in dimethyl sulfoxide (DMSO, 15 mM) was used at a final concentration of 100 μM.

2.4. Enzyme activity analyses

Enzyme activity analyses were performed on total membrane fractions prepared as described by Remacle et al. [24]. NADH:ferricyanide oxidoreductase, complex I (rotenone-sensitive NADH:duroquinone oxidoreductase), complex II (succinate:2,6-dichlorophenol-indophenol (DCIP) oxidoreductase), complex II + III (succinate:cytochrome *c* oxidoreductase), complex III (decylbenzoquinone: cytochrome *c* oxidoreductase) and complex IV (cytochrome *c* oxidase) activities were measured following published procedures [24,26,34].

2.5. Isolation and subfractionation of mitochondria

Mitochondria were isolated from cell-wall less strains according to established procedure [26]. Mitochondria were disrupted by sonication (2×15 s, Vibra cell Sonicator, Danbury, CT) in 20 mM Tris–HCl buffer at pH 8.0 in presence of 0.5 mM phenylmethylsulphonyl fluoride. After removal of unbroken mitochondria (9500 ×*g* for 10 min), soluble (supernatant) and membrane (pellet) fractions were separated by centrifugation (20,000 ×*g*) for 20 min [35].

2.6. Protein electrophoresis

Complex I was analysed by Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) [26]. In-gel detection of NADH dehydrogenase activities was performed using a 10 mM MOPS-KOH pH 8.0 buffer containing 100 μM NADH and 0.1 % nitro blue tetrazolium (NBT). To resolve complex I into its constitutive subunits, the gel slice containing the complex was cut out from the BN-gel, treated 15 min at 60 °C in standard BN-buffer containing 1% SDS and 1% β-mercaptoethanol, washed in BN-buffer, sealed vertically on 8% (w/v) acrylamide SDS-PAGE stacking gel, and laid on separating SDS-gels. We used either 8–20% (w/v) acrylamide gradient Tris–Glycine SDS-gel or 15% (w/v) acrylamide Tris–Tricine SDS gel in presence or in absence of 6M urea. The molecular size of the proteins was calculated by comparison with known markers (Precision Plus Protein™ Dual Color Standards, BioRad). BN gels were electroblotted according to Duby et al. (2001) [36]. A polyclonal antiserum raised in rabbits against whole purified complex I from *N. crassa* was used (provided by Dr. H. Weiss and U. Schulte). Detection was performed using the ECL kit (Roche Molecular Biology) with anti-rabbit POD-conjugated antibodies.

2.7. Mass spectrometry analysis

Individual bands of the SDS-gels were excised and collected in individual Eppendorf tubes. The gel slices were then digested with trypsin and the resultant

peptides were analyzed on a 4700 Proteomics Analyzer, a MALDI tandem time-of-flight mass spectrometer (Applied Biosystems, Framingham, CA, USA) in both the MS and MS/MS modes. If MALDI-based protein identification failed, a nano LC-QTRAP MS/MS analysis was performed following published procedures [37,38]. Protein identification from mass spectral data was performed using an in-house Mascot server using the following settings: maximum number of missed cleavage: 1; peptide tolerance: 100 ppm; variable modifications: oxidation of methionines.

2.8. DNA analyses

C. reinhardtii total DNA was prepared according to the procedure of Newman et al. [39]. Segments of the *C. reinhardtii* mitochondrial genome (Genbank U03843: 1629–3301, 3236–5233, 6636–8593, and 10282–11283) containing *nd4*, *nd5*, *nd2–nd6*, and *nd1*, respectively, were amplified by PCR according to standard protocols. Amplified products were sent to Genome Express (Meylan, France) for automated sequencing on both strands.

2.9. In silico analyses

Protein sequences were obtained from the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/). Calculation of the hydropathy profile of amino acid sequences was done with the Plotscale tool using a window of 7 residues [40] from the ExPaSy molecular biology server (http://expasy.org/). Multiple sequence alignments were performed with the Clustal W tool (v1.8) [41] from the European bioinformatic Institute website (EMBL-EBI, http://www.ebi.ac.uk/), adjusted manually, and shaded with the BoxShade 3.21 tool (http://www.ch.embnet.org/).

3. Results

3.1. The mutation affects the mitochondrial nd5 gene

A new mutant (strain 673 hereafter called *dum23*) was isolated after acriflavine treatment of *Chlamydomonas reinhardtii* wild-type mating-type minus (mt^-) cells (strain 2). Like previously characterized complex I mutants [24,26], it grew very slowly in heterotrophic conditions (acetate-supplemented medium (TAP) in darkness) (dark+/− phenotype) whereas in mixotrophic conditions (TAP medium in the light), it produced colonies smaller than wild-type cells. The growth of the mutant cells was insensitive to rotenone, a classical complex I inhibitor, but highly sensitive to antimycin A and myxothiazol (inhibitors of complex III) (data not shown). To confirm a putative defect in complex I activity, dark respiratory rate of the mutant cells was measured. Cells from wild type and *dum17* complex I mutant [26] were used as controls (Table 1). The total respiratory rate of the 673 mutant strain represented about half the rate of wild-type and was very close to that of *dum17*. Addition of rotenone induced a reduction of 55% of the respiration of wild-type cells whereas the respiratory rates of 673 and *dum17* mutant cells were poorly sensitive to the inhibitor. This indicates that the new mutant strain behaves as a mutant deprived of complex I activity, with a respiration nearly insensitive to rotenone [24,26].

In *Chlamydomonas*, mitochondrial and nuclear mutations can be easily discriminated by analyzing the segregation of phenotypes after crosses. While a nuclear mutation is transmitted following a mendelian mode, a mitochondrial mutation is almost exclusively inherited from the mt^- parent [42]. In reciprocal croses between mutant and wild-type cells (mutant mt^- x wild-type mt^+, mutant mt^+ x wild-type mt^-), 90–95% of the meiotic progeny inherited the phenotype of the minus-type parent (data not shown). We thus concluded that the mutation responsible for the mutant phenotype affected the mitochondrial genome, and most probably one of the five mitochondrial *nd* genes (*nd1, 2, 4, 5, 6*) coding for subunits of complex I in *Chlamydomonas*. This new mutant was called *dum23* (**d**ark **u**niparental transmission by the **m**inus parent).

To determine which of the five *nd* genes was altered in the *dum23* mutant, a recombinational analysis was performed aiming to localize the mutation on the genetic map [24,33]. A strain auxotrophic for arginine and carrying the *dum23* mutation was crossed with arginine-dependent reference strains mutated in *cob* (*dum15*), *cox1* (*dum18*), and *nd1* (*dum20*) (see Materials and methods). By analyzing the phenotypes of the mitotic progeny issued from vegetative diploid zygotes, we determined the frequencies of wild-type recombinant cells as previously described [33]. As the frequency of recombination per physical distance unit is about 3% per kb [33], it can be deduced that the *dum23* mutation most probably affects the *nd5* gene sequence (Fig. 1A).

In *C. reinhardtii*, the mitochondria-encoded ND5 subunit is a 546 amino-acid protein (Genbank accession number P08739). The *nd5* gene sequence of the *dum23* mutant (strain 673) was amplified by PCR and fully sequenced. A deletion of one T was identified in a stretch of four T bases at codons 145–146 (Fig. 1B). This frameshift mutation generates a termination codon at position 151. The physical position of the *dum23* mutation was thus in accordance with the data obtained from the recombinational analysis. No sequence difference was found in the four other *nd* genes.

3.2. Impaired complex I activity and assembly are found in the dum23 mutant

The impact of the *dum23* mutation on the specific activities of several respiratory enzymes was investigated comparatively to

Table 1
Dark respiratory rates and mitochondrial specific respiratory enzyme activities of wild-type and mutant strains

	Wt	*dum23* (strain 673)	*dum17*
Whole cell respiratory rate			
Total	9.1±0.3	4.1±0.9	4.7±0.5
+ rotenone 100 μM	4.1±0.5	3.7±0.6	4.4±0.6
Specific activities on membrane fractions			
NADH:DQ oxidoreductase[a]	108±10	47±13	54±12
+ rotenone 100 μM[a]	66±15	46±11	53±10
NADH:Fe(CN)$_6^{3-}$ oxidoreductase[b]	2054±121	694±156	550±86
Complex II [c]	11±2	10±1	12±2
Complex III[d]	281±76	282±47	271±60
Complex II+III[e]	32±6	51±13	79±21
Complex IV[f]	313±86	354±44	265±65

Whole-cell respiratory rates (nmol O_2 min^{-1} 10^{-7} cells±SD) were measured in the absence or in the presence of 100 μM rotenone (means of three experiments). Specific activities (±SD from three to six experiments) were measured in crude membrane fractions: [a]NADH:duroquinone oxidoreductase (nmol of NADH oxidized min^{-1} mg $protein^{-1}$), [b]NADH:ferricyanide oxidoreductase (nmol of $K_3Fe(CN)_6^{3-}$ reduced min^{-1} mg $protein^{-1}$), [c]succinate: DCIP oxidoreductase (nmol DCIP reduced min^{-1} mg $protein^{-1}$), [d]decyl-benzoquinone: cytochrome *c* oxidoreductase (nmol Cyt *c* reduced min^{-1} mg $protein^{-1}$), [e]succinate:cytochrome *c* oxidoreductase (nmol Cyt *c* reduced min^{-1} mg $protein^{-1}$), and [f]Cytochrome *c* oxidase (complex IV) (nmol of cytochrome *c* oxidized min^{-1} mg $protein^{-1}$).

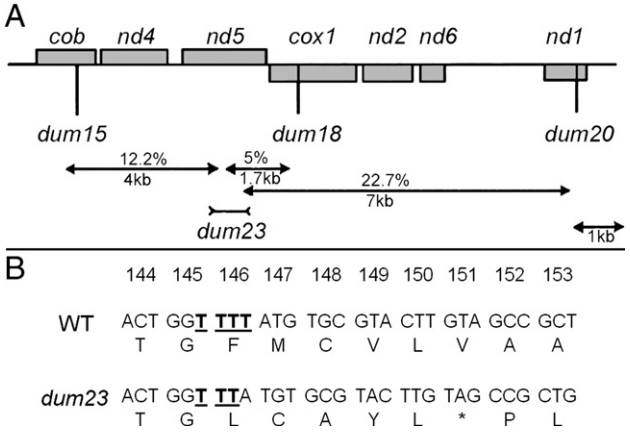

Fig. 1. Genetic and molecular analysis of the *dum23* mutation. A. Partial physical map of the 15.8 kb linear mitochondrial genome of *C. reinhardtii*. (adapted from [23].) The positions of genes encoding subunits of the respiratory chain complexes are indicated: *cob* (apocytochrome b, component of complex III); *cox1* (subunit 1 of cytochrome *c* oxidase or complex IV); *nd1, nd2, nd4, nd5, nd6* (subunits of the NADH:ubiquinone oxidoreductase or complex I). The position of *dum15*, *dum18*, and *dum20* point mutations, the frequencies of wild-type recombinant cells obtained and the deduced physical distances between *dum23* and the known mutations are indicated. (means of two experiments). B. Partial sequence of *nd5* gene (546 codons).

the wild-type and *dum17* complex I mutant strains (Table 1). As suspected from the data of whole-cell respiration (Table 1), complex I activity (rotenone-sensitive NADH:ubiquinone oxidoreductase) was null in *dum23* as in *dum17* while the activities of complex II, III, II+III and IV were not reduced. A diminished NADH:$K_3Fe(CN)_6$ oxidoreductase activity was also observed in mutants, which probably reflects a decrease of the NADH dehydrogenase activity associated to the peripheral arm of the enzyme [26].

In order to investigate the impact of the ND5-subunit loss on complex I assembly, mitochondria were purified from wild-type and *dum23* mutant cells and subjected to Blue Native polyacrylamide gel electrophoresis (BN-PAGE) analysis. Complex I was detected here by a NADH/NBT (nitro blue tetrazolium) staining reaction involving the NADH dehydrogenase activity of the peripheral arm of the enzyme (Fig. 2A, first lane). In wild-type mitochondria, three NADH dehydrogenase activities are detected at 200, 950, and 1500 kDa (Fig. 2A). The first two activities correspond to a soluble NADH dehydrogenase (200 kDa) related to a part of the complex I matricial arm and to the fully assembled complex I (950 kDa), respectively [26,37,43]. Also detected in previous works [37,43], the 1500 kDa activity was originally thought to be a dimeric form of complex I in *Chlamydomonas* [43]. However, while absent in *dum17* complex I mutant, the 1500 kDa activity is also absent in *dum11* complex III mutant which lacks the cytochrome *b* (Fig. 2B). This observation leads us to propose that the 1500 kDa activity most probably corresponds to a I+III_2 supercomplex resulting from the association of complex I (950 kDa) with complex III under dimeric form (500 kDa). Such a I+III_2 supercomplex has been previously identified in mammals [44,45] and land plants [46].

In *dum23* mitochondria, while the 200-kDa activity was detected, no signal corresponding to whole complex I and to I+III_2 supercomplex was ever found. Instead, a weak NADH dehydrogenase activity was visualized at 700 kDa (Fig. 2A, second lane). In immunoblot experiments, the 950 and 700-kDa bands also reacted with antibodies directed against complex I of *Neurospora crassa* (data not shown). With both methods, the intensity of the signal at 700 kDa was about 17±5 % relative to wild-type complex I. This estimation was confirmed by loading a lane with six times more proteins for the mutant (Fig. 2A, third lane). In these conditions, an additional faint activity was also visualized at 1200 kDa (Fig. 2A, third lane). Its apparent molecular mass is in good agreement with the theoretical mass of a subI+III_2 supercomplex (700 kDa+500 kDa) in the mutant.

3.3. The 700 kDa subcomplex is poorly bound to the membrane owing to the lack of a membrane module

In terms of complex I assembly, the absence of ND5 thus leads to the formation of a 700-kDa subcomplex less abundant than the 950 kDa wild-type enzyme. To determine the location of the partially assembled complex I, mitochondria from wild-type and *dum23* cells were purified then disrupted into membrane and soluble fractions. Both the 950-kDa complex I and the 700 kDa subcomplex were clearly associated with mitochondrial membrane fractions (Fig. 3A). On the contrary, the 200-kDa NADH

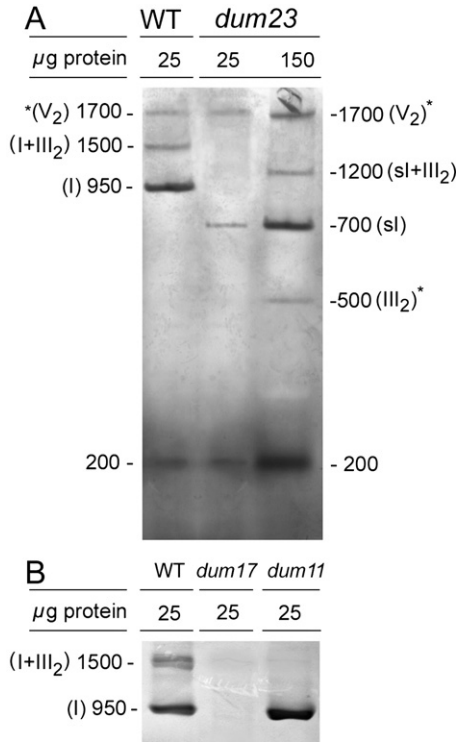

Fig. 2. Complex I assembly in *Chamydomonas reinhardtii* mutants. Blue-Native PAGE of proteins solubilized by addition of *n*-dodecyl maltoside 1.5% (w/v) to partially purified mitochondria from wild-type, *dum11* complex III mutant, *dum17* and *dum23* complex I mutant cells. Lanes were loaded with 25 or 150 µg proteins. The BN-gel was stained for NADH dehydrogenase activity using NBT as an electron acceptor (see materials and methods). Apparent molecular mass of protein bands are given in kDa. I, III_2, and V_2 correspond to mitochondrial respiratory-chain complexes I, III (dimeric state) and V (dimeric state), respectively. Asterisks indicate that the bands are not stained following the incubation with NADH/NBT reagent but are rather Coomassie-blue stained abundant complexes.

dehydrogenase activity belonged to the soluble fraction, as previously observed [26]. To determine whether there was a difference of anchorage to the inner membrane between the 950-kDa and the 700-kDa assemblies, mitochondrial membrane proteins were solubilized with different concentrations of *n*-dodecyl-β-D-maltoside (up to 2.5 % (w/v)) (Fig. 3B). While a 0.1% concentration (0.25 mg detergent per mg of proteins) was enough to release the 700-kDa subcomplex from *dum23* mitochondrial membranes, a much harsher treatment (0.5% or 1.25 mg detergent per mg of proteins) was required for the complete solubilization of wild-type complex I. In addition, the 1500-kDa supercomplex was stable at high concentrations of detergents. A similar observation has already been made for the *Arabidopsis* I+III_2 supercomplex [46]. Alternatively, aliquots of membrane fractions were subjected to salt extractions (up to 800 mM NaCl), washed and pelleted. The protein complexes that were still associated to membranes were then solubilized with 1.5 % detergent and loaded on a BN-gel. The Panel C in Fig. 3 shows that a 0.2 M NaCl concentration was sufficient to eliminate or dissociate the 700-kDa subcomplex from *dum23* mitochondrial membranes, whereas this treatment had no significant effect on the native complex I. Altogether, these results indicate that the 700-kDa complex I subcomplex is less bound to the mitochondrial membrane than the whole complex I. This experiment also indicates a strong sensitivity of the 1500 kDa supercomplex to the addition of sodium chloride at concentrations higher than 20–50 mM.

In order to determine the subunit composition of the 700-kDa enzyme, it was isolated in a first step by BN-PAGE from *dum23* mitochondrial extracts and then subjected to various SDS-PAGE analyses (see Materials and methods). Depending on the experiment, a 11 to 18 spot pattern was visualized for the mutant subcomplex after coloration of gels with Coomassie blue (data not shown). A subsequent mass spectrometry analysis allowed us to identify 16 different components for a total approximate molecular mass of 480 kDa (Table 2). Eleven of them (ND3, Nuo5, 6, 7, 8, 9, 10, 13, a9, s1, s4) are homologous to components of bovine complex I (ND3, 24, 51, 49, TYKY, 30, PSST, B17.2, 39, 75, AQDQ) [37]. Beside the typical mammal components, we found five proteins that have no counterparts in bovine complex I: Nuo21, homologous to a complex I subunit of fungi and land plants; Nuop3, Cag1, Cag2, and Cag3, complex I components typical of green photosynthetic organisms [37,47].

4. Discussion

In 1995, the isolation of a mammal cell line carrying a nearly-homoplasmic frameshift ND5 mutation (C9T) revealed that the capacity to assemble the mtDNA-encoded subunits of the enzyme seemed to be preserved in the absence of ND5, although with a decreased efficiency or a reduced stability [48]. The authors concluded in the ability of cells to build a whole complex I without ND5. This assumption was later supported by the isolation of

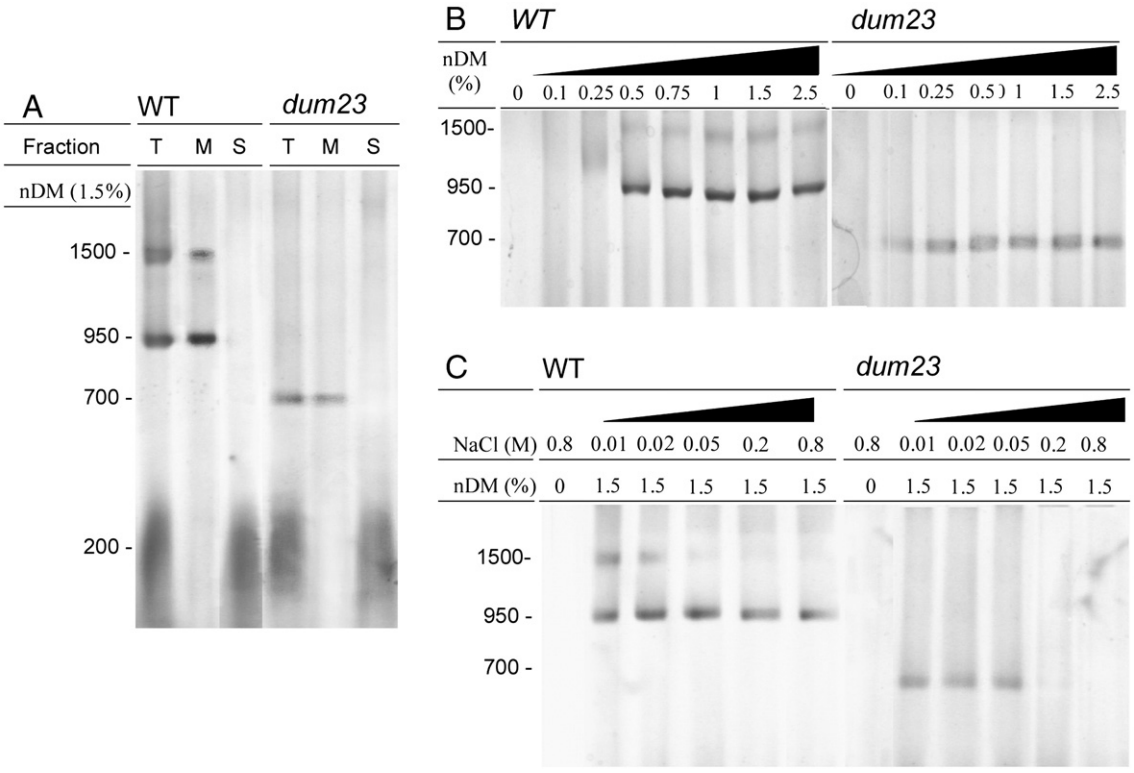

Fig. 3. Topology and membrane anchorage of 950 and 700 kDa complexes I in wild-type and *dum23* complex I mutant mitochondria. BN gels of membrane solubilized proteins stained for NADH dehydrogenase activity using NBT as an electron acceptor (see materials and methods). Proteins were solubilized by treatment with *n*-dodecyl-maltoside (nDM; concentrations are given in % (w/v)) of (A) total (T), membrane (M) and soluble (S) fractions obtained from 25 μg mitochondrial proteins; (B) membrane fractions obtained from 25 μg mitochondrial proteins; (C) membrane fractions obtained from 25 μg mitochondrial proteins that were treated with NaCl, washed and pelleted prior solubilization with nDM.

Table 2
Partial subunit composition of the 700-kDa subcomplex I

Molecular mass (kDa)[a]	*C. reinhardtii* protein name	Generic name[b]	Genbank accession number	Number of matching peptides, coverage (%) and start-end of peptides along the proteins
76	Nuos1	75 kDa	AAQ73136	4 (11%) 399–424, 535–549, 594–606, 693–727
52	Nuo6	51 kDa	AAQ63696	6 (11%) 51–61, 62–69, 279–288, 365–380, 381–390, 408–419
44	Nuo7	49 kDa	AAQ63700	5 (13%) 209–217, 233–246, 259–270, 409–417, 430–444
39	Nuoa9	39 kDa	AAQ55458	4 (14%) 166–179, 264–276, 338–355, 356–366
34	Cag3	CAG3	AAS48197	4 (24%) 38–59, 60–73, 124–137, 286–310
30	Cag2	CAG2	AAS48196	5 (21%) 42–52, 122–136, 137–146, 186–198, 229–238
28	Cag1	CAG1	AAS48195	2 (7%) 32–41, 61–70
28	Nuo5	24 kDa	AAQ63695	2 (10%) 53–71, 221–230
26	Nuo9	30 kDa	AAQ55457	2 (9%) 239–249, 257–269
26	Nuo8	TYKY	AAQ63697	2 (7%) 101–109, 100–117
23	*n.i.*	*n.i.*		
19	Nuos4	AQDQ	AAQ64640	5 (36%) 33–48, 49–53, 82–93, 158–170, 171–183
18	Nuop3	NUOP3	AAS58502	1 (8%) 115–130
16	Nuo10	PSST	AAQ63698	1 (9%) 67–80
16	Nuo13	B17.2	AAQ64638	1 (5%) 50–60
14	ND3	ND3	AAQ55461	1 (5%) 116–129
13	Nuo21	20.9 kDa	AAQ64641	2 (25%) 2–14, 68–84
10	*n.i.*	*n.i.*		
9	*n.i.*	*n.i.*		

MS/MS spectra derived from trypsinated peptides of proteins were matched at local Mascot against a custom protein database and against the Chlamydomonas V3.0 predicted proteins database (available at http://genome.jgi-psf.org/Chlre3/Chlre3.download.ftp.html).

[a]The observed molecular mass are somewhat slightly different from those previously published [37,43], especially concerning the small molecular mass proteins. However, the use of different molecular mass markers may explain the differences in protein band mass estimations. [b]The complex I subunits are named according to the bovine nomenclature, when available. *n.i.*: not identified.

bovine and bacteria complexes missing the ND5/NUOL subunit but containing both the peripheral arm and the membrane arm of the enzyme [8,49]. However, the ability to assemble complex I in the C9T mutant was recently reassessed by analysing native protein complexes by electrophoresis followed by staining or immunological detection studies. No whole complex I could be identified but ND2 and several complex I nuclear-encoded subunits were found to be attached to the mitochondrial inner membrane [50]. The analysis of the homoplasmic *dum23* mitochondrial mutant of *Chlamydomonas* isolated in this work unambiguously demonstrates that the lack of ND5 prevents the assembly of the whole complex I, but allows the assembly in low amount of a subcomplex of 700 kDa with NADH dehydrogenase activity. This subcomplex is loosely bound to the mitochondrial membrane which could determine its instability. The reduced amount of the subcomplex could also result from a down-regulation of other component synthesis, or to a low assembly rate of remaining complex I subunits.

Mitochondrial complex I is currently seen as the assembly of several domains [51–53]. Subcomplex Iλ, which is predominantly hydrophilic and contains all of the bound redox cofactors of the complex, constitutes the globular matricial arm. Subcomplex Iα includes subcomplex Iλ and several subunits mainly hydrophobic (e.g. 39 kDa, ND6). These latter components constitute along with the small hydrophobic domain Iγ (including ND1, ND2, ND3, and ND4L subunits) the proximal part of the membrane arm whereas Iβ constitutes the distal part of the membrane arm. Previous structural studies associated both NUOM (ND4) and NUOL (ND5) subunits at the distal end of the membrane domain in *E. coli* [7,8]. Similarly, in the bovine complex, ND4 and ND5 belong to the larger subcomplex Iβ which contains 12 additional subunits [51,52]. The 700-kDa subcomplex detected here was already observed in other *Chlamydomonas* mutants deprived of ND4 or both ND4 and ND5 subunits [26,36], but it had not been investigated further because mitochondrial preparations of the deletion mutants were always highly contaminated by chloroplastic membranes, resulting in green protein complexes in the range of 500–800 kDa. Similarly, loss of ND4 in *Zea mays* also induces a reduced complex I activity and the formation of an incomplete complex I [35]. Together, these data point out the crucial role of ND5 and ND4 in the assembly of complex I, and most probably of its Iβ component. They also suggest that the presence of Iβ is critical for anchorage of the whole complex within the mitochondrial inner membrane.

Chlamydomonas reinhardtii complex I was found to possess counterparts to seven nuclear-encoded subunits belonging to the bovine Iβ subcomplex (B12, B15, B18, B22, ESSS, SDAP, and PDSW) [37,54]. The 250-kDa domain (putatively Iβ) lost in the ND5 and ND4 *Chlamydomonas* mutants could thus comprises ND4 (49 kDa), ND5 (59 kDa) and the seven homologous polypeptides (Nuob12, 7 kDa; Nuop2, 16 kDa; Nuob18, 10 kDa; Nuob22, 14 kDa; Nuo17, 20 kDa; Acp1, 14 kDa; Nuob10, 18 kDa) representing a theoretical total molecular mass of circa 210 kDa. As a matter of fact, none of these seven subunits have been identified as components of the 700-kDa subcomplex by mass spectrometry, while 16 other complex I subunits were found, for a total estimated molecular mass of 480 kDa. Besides three spots unidentified spots by mass spectrometry, very small or highly hydrophobic components probably escaped our analysis owing to technical limitations [55]. Conversely, among identified subunits, eleven have counterparts in bovine complex I: nine (24 kDa, 30 kDa, 49 kDa, 51 kDa, 75 kDa, TYKY, PSST, AQDQ, and B17.2) are associated to the matricial subcomplex Iλ and two (ND3 and 39 kDa) belongs to the membrane domain that makes the junction between Iλ and Iβ [51,56].

Besides these components, Nuo21, the homolog of a complex I subunit only found in fungi (*Neurospora crassa* 20.9 kDa, *Yarrowia lipolytica* NUXM) and higher plants (*Arabidopsis* At4g16450) [37] has been also identified in the 700-kDa subcomplex. Nuo21 homologs share a similar hydropathy profile

Fig. 4. Clustal W partial multiple aligments of Arabidopsis Nuop3 homolog (At_Nuop3, NP_566309) and ferredoxin-like proteins (FDX): *Arabidopsis thaliana* (At, AAM62925) *Drosophila melanogaster* (Dm, NP_523993), *Magnetospirillum magneticum* (Mm, YP_422386). Amino acids conserved in the four sequences are shown on a black background; similar residues are shown on a light-grey background. Position of Cys residues are indicated by asterisks.

with two conserved hydrophobic stretches (Supplemental Fig. 1) but their function within complex I remain largely unknown. Interestingly, disruption of the NUO20.9 gene in *N. crassa* impairs the assembly of the membrane arm of complex I [57] and recently, the *Arabidopsis* homologous protein has been identified in a 600-kDa subcomplex related to the membrane arm [58]. Altogether, these data indicate that Nuo21 probably belongs to the proximal membrane part of complex I.

Of particular interest is the identification of four complex I components shared only by green plants: Nuop3, Cag1, Cag2, and Cag3. Nuop3 homologs are found in complex I of *Arabidopsis thaliana* and *Oriza sativa* [37,47]. A search performed in databases moreover showed us that putative homologs are widely present among photosynthetic organisms, including mosses and diatoms (Supplemental Figure 2). Interestingly, the Nuop3 subunit of land plants complex I shares also some similarity with ferredoxin and ferredoxin-like proteins (e.g. from plants, arthropods and α-proteobacteria, see Fig. 4), with three conserved cysteine residues. On the other hand, Cag proteins are related to putative gamma-type carbonic anhydrases, a protein family whose five members have been assigned to the membrane arm of complex I in *Arabidopsis* [58,59]. One of them (*Arabidopsis*/At1g47260 gene product) has been shown to be important for complex I assembly and to be possibly involved in mitochondrial one-carbon metabolism [60]. Single particle electron microscopy analysis of complex I from the colorless alga *Polytomella sp*. and from *Arabidopsis thaliana* also showed that these gamma-type carbonic anhydrase subunits constitute a plant-specific globular matrix-exposed domain attached to the central part of the complex I membrane arm [58,60]. Our results thus suggest that this domain is attached to the proximal membrane domain (i.e. Iγ or the hydrophobic part of Iα) and does not require Iβ subunits for association to complex I. One may also hypothesize that recruitment of a ferredoxin-like protein within plant complex I (Nuop3, see above) could drive electrons to this domain. The schematic Fig. 5 summarizes our model of subunit localization within Complex I of *Chlamydomonas*.

Since the first description of the association of respiratory-chain complexes into supramolecular structures by Schägger and Pfeiffer in 2000 [45], the occurrence of so-called supercomplexes is now well-documented in various prokaryotes and eukaryotes. For instance, complex I and complex III are associated into supercomplex in mammals [44,45], and land plants [46]. In wild-type *Chlamydomonas*, the 1500 kDa band showing NADH/NBT enzyme activity (Fig. 2A) most probably corresponds to such a I+III_2 supercomplex. Deprived of the distal part of its membrane arm, complex I of *Chlamydomonas* seems still to be able to interact with dimeric complex III since a 1200 kDa band with NADH dehydrogenase activity was detected in the *dum23* mutant. Recently, a structural characterization by single-particle EM of Arabidopsis I+III_2 supercomplex showed that complex I and complex III interacts within the plane of the membrane [61]. In addition, the authors demonstrated that the tip of the complex I hydrophilic arm is essential for the formation of the I+III_2 supercomplex even if the interaction between matrix-exposed domains of both complexes is probably not strong [61]. In *dum23* mitochondrial membrane, the occurrence of a supercomplex, whose subcomplex I component is probably constituted of a whole hydrophilic arm and a truncated membrane arm, strongly suggests that the distal part of the membrane is not required in the supercomplex formation process. The junction between complexes I and III could thus occur through proximal membrane fraction subunits.

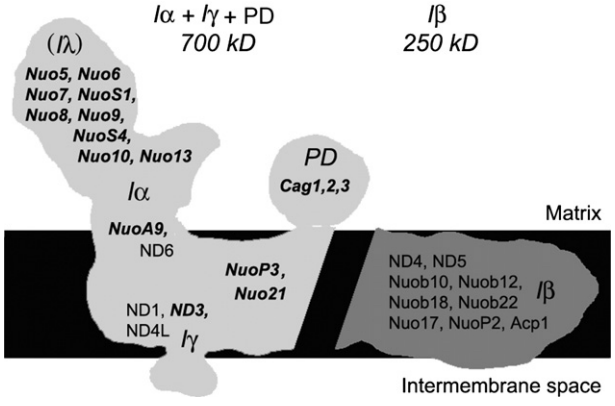

Fig. 5. Schematic model representation of *Chlamydomonas reinhardtii* complex I. Outline shape of complex I side-view is drawn from Arabidopsis projection map obtained by EM single-particle analysis [61]. The light-grey and dark-grey shaded portions correspond to the 700-kDa subcomplex I and to the distal part of membrane arm that is putatively lost in the ND5-null mutant, respectively. Location of subunits within subcomplexes is also indicated (see text for further details). Subunits identified by mass spectrometry in the 700 kDa subcomplex are marked in bold and italics (see Table 1). ND1, ND4L and ND6 subunits are also located in the proximal membrane module, accordingly to a previous model [28]. PD: Plant-specific domain.

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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.bbabio.2008.01.001.

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