Disruption of iron-sulphur cluster N2 from NADH:ubiquinone oxidoreductase by site-directed mutagenesis

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We have cloned and inactivated, by repeat-induced point mutations, the nuclear gene encoding the 19.3 kDa subunit of complex I (EC 1.6.5.3) from Neurospora crassa, the homologue of the bovine PSST polypeptide. Mitochondria from mutant muo19.3 lack the peripheral arm of complex I while its membrane arm accumulates. Transformation with wild-type cDNA rescues this phenotype and assembly of complex I is restored. To interfere with assembly of a proposed bound iron-sulphur cluster, site-directed mutants were constructed by introducing cDNA with altered codons for two adjacent cysteines, Cys-101 and Cys-102.

The mutant complexes were purified and their enzymic activities and EPR and UV/visible spectra were analysed. Either of the mutations abolishes assembly of iron-sulphur cluster N2, showing that this redox group is bound to the 19.3 kDa protein. We also observed an interference with the reduction of redox group X, suggesting that cluster N2 is the electron donor to this high-potential redox group.

Key words: complex I, mitochondria, Neurospora crassa, respiratory chain.

INTRODUCTION

The proton-pumping NADH:ubiquinone oxidoreductase, also known as respiratory complex I (EC 1.6.5.3), consists of about 40 polypeptide subunits arranged in an L-shaped structure, with an intrinsic membrane arm extending into the lipid bilayer of the inner membrane and a peripheral arm protruding into the mitochondrial matrix [1,2]. It catalyses electron transfer from NADH to ubiquinone linked to the translocation of protons across the membrane. NADH oxidation by the peripheral arm of the complex employs one FMN and several iron-sulphur clusters for electron transport to the membrane arm, where ubiquinone reduction takes place. No prosthetic group has been identified in the membrane arm. The mechanism that couples proton translocation to electron transfer and the ubiquinone-binding site are unknown [3–5].

Six iron-sulphur clusters, designated N1a, N1b, N2, N3, N4 and N5, have so far been detected in complex I by means of EPR spectroscopy. N1a and N1b are binuclear iron-sulphur clusters, whereas the others are tetranuclear centres [6]. Two additional tetranuclear iron-sulphur clusters have been characterized by UV/visible spectroscopy, clusters N6a and N6b, with a midpoint potential of −250 mV [7]. Clusters N1a, N1b, N3, N4 and N5 as well as the FMN have midpoint potentials of between −400 and −300 mV, almost isopotential to the substrate NADH [6–8]. Cluster N2 has the highest redox potential, of −160 mV [8]. In contrast with most other clusters, its midpoint potential is pH-dependent [9]. It has been suggested therefore that cluster N2 is the electron donor to quinone and is directly involved in proton translocation [10,11]. Recently, the existence of an additional redox group in complex I with a midpoint potential above −100 mV was proposed [12,13]. The molecular nature of this redox group is unknown and has been designated redox group X for the time being.

The location of the iron-sulphur clusters on the subunits, a matter of controversy for some time, appears to be settled now. The isopotential iron-sulphur clusters N1a, N1b, N3, N4 and N5 are located on the 75, 51 and 24 kDa subunits, according to the bovine nomenclature [6]. These subunits form the NADH dehydrogenase fragment of Escherichia coli complex I [14]. Clusters N6a and N6b are bound by the ferredoxin-type TYKY subunit [7]. The location of cluster N2 on the PSST subunit, which has been debated for a long time [6,7,15–17], is addressed in this work.

In this study, we focus on the 19.3 kDa protein of Neurospora crassa, the homologue of the PSST subunit of bovine complex I. PSST is highly conserved among different species and belongs to the 14 subunits that constitute the minimal structural unit of complex I [4]. We constructed a mutant lacking this subunit which proved to be unable to assemble the peripheral arm of complex I. A more detailed insight into the function of the PSST subunit was obtained by site-directed mutagenesis of two conserved cysteines essential for binding of the iron-sulphur cluster. The altered protein complexes were purified and analysed by EPR and UV/visible spectroscopy. Our results show that both mutations abolish assembly of iron-sulphur cluster N2 and that the absence of this redox group interferes with the reduction of redox group X.

MATERIALS AND METHODS

N. crassa manipulations

Growth and handling of N. crassa were carried out according to standard procedures [18]. For expression of cDNAs under the control of the quinic acid promoter of pMYX2 [19], 10 mM quinic acid was added to the medium. Strains carrying pCSN44 or pMYX2 recombinant vectors were selected on plates con-

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taining 100 μg/ml hygromycin B or 0.5 μg/ml benomyl, respectively.

**Mutant isolation**

The *nuo19.3* gene, encoding the 19.3 kDa subunit of complex I, was isolated from a *N. crassa* genomic library in phage λ J1 by hybridization with the corresponding cDNA [20]. A 3 kb genomic DNA fragment with EcoRV ends was isolated and inserted into the *SmaI* site of pGEM4 [21]. The resulting plasmid was digested with *SacI* (located in the polylinker region of the vector and in the genomic DNA) and a 2 kb band containing the entire coding region of *nuo19.3* was cloned into pCSN44 [22]. This recombinant vector, pNuoU19.3, was used for transformation of *N. crassa* 74-OR23-1A spheroplasts as described in [23]. Genomic DNA from transformants was analysed by Southern blotting to identify strains with single-copy integration. One transformant carrying a duplication of the *nuo19.3* gene was crossed with strain 74-OR8-1a in order to inactivate this gene by repeat-induced point mutations [24]. Random progeny from the cross were germinated and their mitochondrial proteins were analysed by Western blot with an antiserum against the 19.3 kDa protein, leading to the identification of mutant *nuo19.3*.

**Site-directed mutagenesis**

The cDNA coding for the 19.3 kDa subunit cloned in pGEM4 [20] was cleaved with EcoRI, treated with Klenow to create blunt ends and then ligated into the *SmaI* site of the expression vector pMYX2, downstream of the qa-2 inducible promoter. Site-directed mutagenesis was achieved using the QuikChange™ Site-Directed Mutagenesis kit according to manufacturer’s procedures (Stratagene). Briefly, the pMYX2-recombinant vector and two pairs of synthetic complementary oligonucleotide primers containing the desired mutation were used in a PCR reaction to create a mutated plasmid. The mutagenic oligonucleotide primers used were 5′-GGCCTCGCCCTGC-3′ (C101S) and 5′-GGCCCTGCCTGCGCTGG-3′ (C102A) and their complementary strands. The underlined nucleotides represent substitutions that change codons within the cDNA, resulting in the replacement of Cys-101 by Ser or of Cys-102 by Ala, respectively. The mutagenesis was confirmed by complete sequencing of the cDNA constructs. The mutated plasmids were transformed into the *nuo19.3* mutant. Transformants expressing higher amounts of the mutated proteins were selected.

**Isolation and characterization of mutant complexes**

Mitochondria were isolated from hyphae grown in the presence of 10 mM quinic acid. Water-suspended organelles (27 ml; 1.5 g of protein) were solubilized with 16 ml of 20 mM Tris/HCl, pH 7.5/50 mM NaCl/0.1% Triton X-100. After centrifugation for 20 min at 20000 g, the supernatant was applied to sucrose gradients (6 × 30 ml of 10–30% sucrose in 50 mM Tris/HCl, pH 7.5/50 mM NaCl/0.1% Triton X-100). Mitochondrial proteins were separated at 130000 g for 21 h. The gradients were fractionated and analysed for NADH:ferricyanide reductase activity. Fractions corresponding to the lower quarter of the gradients showing high activity were pooled and applied to a source Q anion-exchange column (16 mm × 95 mm; Amersham Biosciences). Complex I was eluted by means of a 50 ml salt gradient (50–400 mM NaCl in 50 mM Tris/HCl, pH 7.5/0.05% Triton X-100). Fractions with high NADH:ferricyanide oxidoreductase activity were pooled, concentrated by ultrafiltration on a Diaflo XM 300 filter and applied to a gel-filtration column (Superdex 200; Amersham Biosciences) in 50 mM Tris/HCl/0.05%, Triton X-100. Peak fractions were pooled and concentrated again by ultrafiltration. The NADH:decylquinone reductase activity was determined after reconstitution of the enzyme in phospholipids [25]. To obtain EPR spectra, complex I at 5 mg/ml was reduced by a 1000-fold molar excess of NADH in the presence of rotenone mediators [8]. EPR measurements were conducted with a Bruker EMX 1/6 spectrometer operating at X band (9.2 GHz). The sample temperature was controlled with an Oxford Instrument ESR-9 helium-flow cryostat. The magnetic field was calibrated using a strong or a weak pitch standard. UV/visible spectra of complex I in the oxidized and NADH-reduced forms were taken with a Zeiss S100 spectrophotometer. To 0.6 ml of complex I at 1 mg/ml in a stirred cuvette, 1 μl of NADH solution of varying concentrations was added while spectra from 285 to 600 nm were recorded every 200 μs. Analysed spectra were baseline-corrected at 600 nm, and traces at fixed wavelength were corrected by subtracting the trace at 600 nm.

**General protein analysis**

The techniques used for the preparation of *N. crassa* mitochondria [26,27], SDS/PAGE [28], production of rabbit antibodies [29,30], blotting and incubation of blots with antisera [31], detection of alkaline phosphatase/secondary antibody conjugates [32], sucrose-gradient centrifugation analysis of detergent-solubilized mitochondrial proteins [33] and NADH:ferricyanide reductase activity [34] have been described elsewhere.

**RESULTS**

**Site-directed mutagenesis of the 19.3 kDa subunit**

By the generation of repeat-induced point mutations in the *nuo19.3* gene, we constructed the *N. crassa* mutant *nuo19.3*. This strain lacks the 19.3 kDa subunit of complex I, the homologue of bovine PSST protein. To analyse the impact of the mutation on complex I assembly, mitochondrial proteins from wild-type and mutant *nuo19.3* were solubilized and fractionated by sucrose-gradient centrifugation. Wild-type complex I migrated about two-thirds of the way through the gradient, as indicated by the NADH:ferricyanide reductase activity and the immunological detection of complex I subunits (Figures 1A and 1B). In contrast, no NADH:ferricyanide reductase activity could be detected in fractions obtained from mutant mitochondria (Figure 1A). Analysis of the distribution of several complex I subunits in this gradient showed the accumulation of the peripheral subunits (30.4 kDa) in the top of the gradient. This and the lack of any NADH:ferricyanide reductase activity indicate that the peripheral arm is not assembled. The 12.3 and 20.8 kDa subunits of the membrane arm show a peak in fraction 9, most likely representing the assembled membrane arm (Figure 1C).

Analysis of the function of the 19.3 kDa subunit was advanced further by constructing site-directed mutants that affect the assembly of the iron-sulphur cluster. To change Cys-101 into a Ser and, in a different mutant, Cys-102 into an Ala, the cDNA encoding the 19.3 kDa polypeptide was cloned into the vector pMYX2 and the two missense mutations were introduced individually by site-directed mutagenesis. The pMYX2-recombinant plasmids carrying either wild-type cDNA or mutant cDNAs C101S or C102A were expressed in the *nuo19.3* mutant. Sucrose-gradient centrifugation analysis of mitochondrial proteins from any of these strains revealed that assembly of complex I was restored. NADH:ferricyanide reductase activity as well as...
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Figure 1  Complex I assembly in mutant *nuo19.3*

Mitochondria were isolated, solubilized with Triton X-100 and centrifuged in sucrose gradients. Fractions of the gradients (numbered 1–12 from top to bottom) were collected and analysed for NADH:ferricyanide reductase activity, shown in (A): ●, wild type; ○, mutant *nuo19.3*. The same fractions from the wild-type strain (B) and mutant *nuo19.3* (C) were analysed by Western blotting with a mixture of individual antisera against the subunits of complex I indicated on the left.

the distribution of complex I subunits resembled that of the wild type (Figure 2).

To characterize complex I formed in the mutants C101S and C102A, we isolated complex I by a three-step procedure involving sucrose-gradient centrifugation and anion-exchange and gel-filtration chromatography. Upon SDS/PAGE analysis, complex I from the strain rescued by wild-type cDNA showed the same polypeptide pattern as the wild-type complex I and thereafter was used as a control. The mutants C101S and C102A revealed a similar polypeptide pattern, confirming the presence of fully assembled and stable complex I in these strains. In addition, we isolated complex I from each mutant by immunoprecipitation with a specific antiserum [29]. Western-blot analysis of the immunoprecipitated material with antiserum against the 19.3 kDa protein confirmed its presence in complex I of the mutants (results not shown).

Enzymic analysis

The NADH dehydrogenase activity of complex I measured as NADH:ferricyanide reductase activity is catalysed solely by the peripheral arm. With respect to this activity, complex I from C101S as well as C102A does not differ from wild-type complex I. To determine the rotenone-sensitive NADH:decylquinone oxidoreductase activity, which resembles the native NADH:ubiquinone activity and requires interaction of the peripheral and the membrane arm, the isolated enzymes were reconstituted into soya-bean phospholipids. Whereas wild-type complex I

**Figure 2  Identification of complex I in cDNA-complemented mutants**

Mitochondrial proteins were analysed in sucrose gradients as described in Figure 1. NADH:ferricyanide reductase activity is shown in (A): ●, cDNA-rescued strain; ○, C101S. Western blots are from the cDNA-rescued (B) and C101S (C) strains.

**Figure 3  EPR spectra of complex I isolated from the strain rescued by wild-type cDNA (trace A) and mutants C101S (trace B) and C102A (trace C)**

Spectra were recorded at 13 K and at 5 mW microwave power. The samples were reduced with NADH in the presence of redox mediators. The dotted line indicates the $g_z$ of N2. The signals are annotated to the corresponding iron-sulphur clusters. Other EPR conditions were: microwave frequency, 9.44 GHz; modulation amplitude, 6 mT; time constant, 0.064 s; scan rate, 17.9 mT/min.

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Figure 4 Reduction and reoxidation of complex I isolated from the strain rescued by wild-type cDNA (A, B) and from mutant C101S (C, D) followed by UV/visible spectroscopy

Complex I at 1 μM was reduced by 15 μM (A, C) or 0.5 μM (B, D) NADH and reoxidized by oxygen. Traces at 340 and 425 nm, are shown. Traces at 340 nm, in (A) and (C) are drawn at a third of the measured extinctions.

Figure 5 UV/visible difference spectra of complex I from the strain rescued by wild-type cDNA (traces A–D) and from mutant C101S (traces E–H)

Difference spectra A, B and E, F were obtained from experiments involving reduction by excess NADH, whereas spectra C, D and G, H were derived from experiments with sub-stoichiometric amounts of NADH. Spectra A, C, E and G were obtained by subtracting a spectrum before reduction with NADH from a spectrum after complete reoxidation by oxygen (trace A, Figure 4A, 4.5 min — 0.4 min; trace C, Figure 4B, 3 min — 0.3 min; trace E, Figure 4C, 4 min — 0.2 min; trace G, Figure 4D, 2 min — 0.3 min). Spectra B, D, F and H were obtained by subtracting a spectrum after complete reoxidation from a spectrum of partially reduced complex I after reoxidation of NADH (trace B, Figure 4A, 3 min — 4.5 min; trace D, Figure 4B, 0.5 min — 3 min; trace F, Figure 4C, 2.5 min — 4 min; trace H, Figure 4D, 0.4 min — 2 min).
yielded a high NADH:decyquinone oxidoreductase activity (30 s\(^{-1}\) at 20 \(\mu M\) NADH and 2 \(\mu M\) decyquinone), which was efficiently inhibited by rotenone (> 75\%), complex I from the mutants C101S and C102A displayed low NADH:decyquinone reductase activity (< 5 s\(^{-1}\)) that was largely insensitive to rotenone (< 30\%). Verified with different preparations of wild-type and mutant complex I, the rotenone-sensitive NADH: quinone reductase activity of both mutants was consistently below 10\% of wild-type activity.

**EPR spectroscopic analysis**

The isolated enzymes were analysed spectroscopically to characterize the effects of the mutations. EPR spectra were recorded of complex I reduced by an approx. 1000-fold excess of NADH. In preparations of wild-type complex I from \(N. crassa\) the clusters N1b, N2, N3 and N4 are readily detectable by EPR spectroscopy [8]. All of those were found in the strain rescued with wild-type cDNA. However, cluster N2 was completely missing in complex I obtained from either of the site-directed mutants, whereas clusters N1, N3 and N4 were basically unaffected by the mutations (Figure 3). Spectra recorded at 40 K revealed the presence of the binuclear cluster N1 in all complexes analysed (results not shown). At 13 K the well-known signals of N3 and N4 were readily detectable, as well as the signals of N1 (Figure 3). The relative intensities of the signals of N1, N3 and N4 were nearly identical in the preparations of complex I from the strain rescued by wild-type cDNA and in both mutant complexes (Figure 3).

**UV/visible spectroscopic characterization**

In addition to EPR spectroscopy, complex I obtained from the different strains was analysed by UV/visible spectroscopy. As the UV/visible spectra are characterized by broad overlapping absorptions not allowing a differentiation of redox groups, UV/visible redox difference spectra were taken. The redox difference spectra of complex I display absorption bands attributable to FMN and iron-sulphur clusters as well as additional absorption bands that were considered to be caused by redox group X [7,12]. To compare redox difference spectra of wild-type and mutant complex I, reduction of complexes by different amounts of NADH and reoxidation by oxygen were monitored by UV/visible spectroscopy. Analysis of complex I from the strain rescued by wild-type cDNA again revealed wild-type characteristics. Addition of NADH caused an immediate reduction of complex I, indicated by a drop in absorbance at 425 nm (Figures 4A and 4B). Oxygen present in the solution reoxidized complex I. In the presence of excess NADH, reoxidation was slow (Figure 4A). While the NADH/NAD\(^+\) ratio decreased, the redox state of flavin and the isopotential iron-sulphur clusters was gradually shifted to the oxidized state, causing an increase in the absorbance at 425 nm. After NADH had been oxidized completely, increase of the absorbance at 425 nm accelerated, indicating fast reoxidation of complex I. The redox difference spectra obtained by subtracting the spectrum of complex I before and after the accelerated reoxidation is dominated by negative absorbances at 325 and 425 nm (Figure 5, trace B). Further reoxidation of complex I involving cluster N2 and redox group X is very slow [12]. The UV/visible spectrum of partly reoxidized wild-type complex I in this state, after subtraction of a spectrum of the untreated oxidized complex I, is characterized by a positive absorption band around 300 nm, and a broad negative absorption band around 430 nm (Figure 5, trace A). If sub-stoichiometric amounts of NADH are added, complex I becomes partly reduced (Figure 4B). The more NADH that is added, the more clusters N6a and N6b are reduced. In the absence of excess NADH these clusters are reoxidized without delay. The difference spectrum remaining after the fast oxidation phase is essentially the same as obtained with complex I reduced with an excess of NADH and reoxidized by oxygen (Figure 5, trace C).

The reduction and reoxidation of clusters N6a and N6b obtained from the mutants C101S and C102A displayed no differences compared with the wild-type protein. The characteristic wavelengths of the negative absorption bands at 325 and 425 nm, in the difference spectra, the amplitude of these absorption bands and the reoxidation kinetics were essentially the same for wild-type and mutant enzymes (Figures 4C and 5, trace F). Differences were obvious, however, when comparing the spectra after reoxidation of clusters N6a and N6b. As mentioned, cluster N2 and redox group X in the wild-type enzyme were still reduced under these conditions. Although spectra determined for the mutant enzymes at this state showed the same characteristic absorption bands, the amplitudes were generally significantly smaller than those obtained with wild-type complex I (Figure 5, trace E). In addition, the amplitude was strongly dependent on the molar ratio of NADH to complex I. Detectable absorption bands in the difference spectra were obtained only with an excess of NADH added for reduction of complex I. When sub-stoichiometric amounts of NADH were added, clusters N6a and N6b were reduced, as found with wild-type complex I; however, the mutant complexes were reoxidized completely during the fast oxidation phase (Figure 4D). In these experiments no significant redox difference spectrum remained after N6a and N6b were reoxidized (Figure 5, trace G).

**DISCUSSION**

**PSST homologue is essential for assembly of the peripheral arm in \(N. crassa\)**

In the present work, we describe the inactivation of the nuclear gene encoding the 19.3 kDa subunit of complex I from \(N. crassa\). The subunit is located at the interface between the hydrophilic extramembrane portion and the hydrophobic membrane region [35]. The resulting mutant is unable to assemble a functional complex I. The peripheral arm is not detectable at all in a detergent extract of mitochondria obtained from the mutant, whereas an assembly of membrane-arm subunits in the detergent extract indicates accumulation of this subcomplex in the mitochondrial membrane. The 19.3 kDa subunit was detected in neither the isolated membrane arm nor the isolated peripheral arm of \(N. crassa\) complex I [20]. Nevertheless, this subunit is apparently essential for the assembly of the peripheral arm. In accordance with this finding, assembly of the overexpressed NADH dehydrogenase fragment of \(E. coli\) complex I requires co-expression of the \(E. coli\) PSST homologue, although this subunit is not present in the purified fragment [36].

The subunit composition of the subcomplexes can be determined only after detergent solubilization, which may induce dissociation of subunits. Therefore, it is unknown whether the 19.3 kDa subunit is part of the assembled peripheral arm as long as it is associated with the inner membrane. However, the presence of iron-sulphur clusters in complex I can be assessed while the complex is still embedded in the membrane. The absence of cluster N2, shown to be bound by the 19.3 kDa subunit in this study, in mitochondrial membranes of a mutant lacking the membrane arm [37] shows that stable integration of this cluster requires the presence of the membrane arm.
Iron-sulphur cluster N2 is bound by PSST

To gain insight into the functional role of the PSST subunit, we have complemented the N. crassa mutant with cDNA modified by site-directed mutagenesis. This subunit is considered to bind a tetranuclear cluster despite the fact that it lacks a canonical motif for a tetranuclear centre co-ordination. Two out of the four conserved cysteines (Cys-101 and Cys-102; numbering according to the N. crassa subunit) are located adjacenty, making it very unlikely that both are ligands to the same cluster, due to steric hindrance. It has been proposed that besides the conserved Cys residues, a Glu located three residues after the second conserved Cys is a possible fourth ligand to N2, forming the motif CysXXE ... Cys66 ... CysP. However, a recent site-directed mutagenesis study in Yarrowia lipolytica excluded this hypothesis [16]. In order to disrupt assembly of the iron-sulphur cluster bound to PSST, the two adjacent Cys residues Cys-101 and Cys-102; conserved in PSST homologues, were changed to Ser and Ala, respectively. Complementation of the nuo19.3 mutant with either wild-type cDNA or mutated cDNA resulted in fully assembled and stable complex I. EPR spectroscopic analysis of the iron-sulphur clusters revealed that both mutant complexes lacked specifically cluster N2. In accordance with the data obtained with the E. coli complex I [15], this is strong evidence for the binding of cluster N2 to the PSST subunit rather than in the TYKY subunit, as advocated previously [17,38,39]. As both mutations characterized in this study cause a complete lack of cluster N2, it is not possible yet to state which of the two Cys residues is the ligand for the cluster. We assume that one of the adjacent Cys residues acts as a ligand while alteration of the other interferes with the formation of a proper structure required for assembly of cluster N2. It is possible that the fourth ligand of N2 is provided by a neighbouring 49 kDa subunit, as suggested in [40].

Cluster N2 is required for proper reduction of redox group X

Lack of cluster N2 caused specific functional alterations. Oxidation of NADH in the presence of the artificial electron acceptor ferricyanide appears to be unaffected by the mutations in the 19.3 kDa protein. However, electron transfer to quinones is grossly hampered. Rotenone-sensitive NADH:ubiquinone reductase activity of the mutant complexes is less than in the TYKY subunit, as advocated previously [17,38,39].

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