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Role of the Conserved Cysteine Residues of the 11.5 kDa Subunit in Complex I Catalytic Properties

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Mitochondrial complex I exists as a mixture of two inter-convertible forms: active (A) and de-activated (D), the latter being sensitive to SH-modifying compounds. To investigate if the conserved cysteine-rich 11.5 kDa subunit of Neurospora crassa complex I is involved in this process, we subjected the corresponding genomic DNA to site-directed mutagenesis. The four cysteine residues of the subunit were separately substituted with serine residues and the resulting proteins were independently expressed in a null-mutant strain. All of the obtained mutant strains were able to assemble a complex I with similar kinetic properties to those observed in the wild-type enzyme, indicating that none of the cysteine residues of the 11.5 kDa protein is individually relevant for the A/D transition process. Diminished amounts of assembled complex I seem to be the major effect of these specific mutations. The cysteine residues are likely important to the acquisition and stabilization of the correct 11.5 kDa protein conformation and this is reflected in the assembly/stability of complex I.

Key words: active/de-active transition, complex I, mitochondria, *Neurospora crassa*, site-directed mutagenesis.

The mitochondrial NADH: ubiquinone oxidoreductase (complex I) couples the oxidation of NADH and the reduction of ubiquinone to the generation of a transmembrane proton gradient, which is then used for ATP synthesis. The minimal structural elements common to the mitochondrial and the bacterial complex are 14 polypeptides arranged in an L-shaped structure, one non-covalently bound FMN group and 8–9 iron–sulphur clusters (*1–3*). Mitochondrial complex I contains many additional accessory subunits for which the function is largely unknown. The bovine complex I contains 45 different polypeptides, while the enzyme from the filamentous fungus *N. crassa* is composed of at least 39 mostly conserved subunits (*4–7*).

The mammalian mitochondrial complex I exists in two distinct inter-convertible forms, the active (A) and the de-activated (D) forms (*8*, *9*). Only the A form of the enzyme is able to catalyse a rapid oxidation of NADH and the $\Delta\mu H^+$-dependent reaction of reverse electron transport. The D form can be fully reduced by NADH and oxidized by artificial electron acceptors, but is unable to transfer electrons to ubiquinone. De-activation occurs if complex I activity is limited due to the lack of one of the substrates of the enzyme. The D form can be transformed into the A form by incubation with NADH under conditions where the enzyme turnover occurs. The rate of enzyme activation decreases at alkaline pH or in the presence of bivalent cations (*10*, *11*). The A form is spontaneously converted in the D form in a reaction extremely temperature dependent. The enzyme de-activates during incubation without substrates at high temperatures. Both forms differ in their affinity for the complex I inhibitors rotenone and piericidin A and in their accessibility to thiol reagents (*12*). The active form of complex I is not sensitive to sulphydryl reagents, whereas the D form is irreversibly inhibited by *N*-ethylmaleimide (NEM) (*13*).

This process is known in mammals for a long time, and was recently described in other organisms, including the fungi *N. crassa* and *Yarrowia lipolytica* (*10*, *14*). The fungal enzymes display A/D transitions with much lower activation barrier compared to the bovine enzyme. It is believed that A/D transitions are exclusive to eucaryotes, although nothing is known about the catalytic properties of the plant enzyme so far. The molecular mechanism of the A/D transition and the complex I subunits implicated in the process are still unknown. We have recently demonstrated that a 29.9 kDa protein in *N. crassa* is involved in the active/de-active transition. A mutant lacking the 29.9 kDa subunit of complex I retains the capability to undergo A/D transitions, though with a slower de-activation process (*15*). Another small subunit of the bovine complex I, with an apparent molecular weight of 15 kDa, was specifically labelled by the fluorescent NEM analogue in the D form of the enzyme. The bovine IP-15 subunit of complex I was a likely candidate for this specific labelling, since it has around 15 kDa and contains four highly conserved cysteine residues that can possibly interact with the thiol reagent (*13*). Thus, we decided to study the catalytic

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properties of *N. crassa* mutants in the 11.5 kDa subunit, the homologue of bovine IP-15. The 11.5 kDa subunit appears to be located in the membrane arm of the *N. crassa* enzyme, and its lack leads to the accumulation of intermediates of the membrane arm and to the impairment of the peripheral arm assembly, which prevents any kinetic measurements (*7*). To circumvent this result, we performed independently site-directed mutagenesis of the four conserved cysteines of the protein, and analysed the assembly and function of complex I in the resulting Neurospora strains. Our results indicate that none of the cysteine residues of the 11.5 kDa subunit is individually important for the A/D transition, but they are important to the assembly and stability of complex I.

MATERIALS AND METHODS

Handling of Strains and Plasmids—The *N. crassa* strains were grown and handled according to the standard procedures (*16*). We used the *N. crassa* strain 74-OR23-1A and the nuo11.5 mutant strain obtained previously by repeat-induced point mutations in the *nuo*-11.5 gene. This mutant strain lacks the 11.5 kDa subunit of complex I and has no detectable enzyme assembly or activity (*7*). Spheroplasts were prepared from 7 days old conidia from the nuo11.5 strain, transformed with the recombinant plasmids derived from pMYX2 and selected on plates containing the antibiotic benomyl (0.5 μg/ml). Expression of the genomic DNA was induced by the addition of 10 mM quinic acid.

Cloning and Generation of Point Mutations—The genomic DNA coding for the 11.5 kDa subunit was amplified by PCR using the primers 5′-**CCCGGG** CAGTATGTGCGTATCATCA-3′ and 5′-**CCCGGG**GTGG TATGGTATAGGTACAA-3′ that contain the restriction site for *Sma*I (in bold). The amplified 740 bp fragment was cloned in the TOPO TA Cloning vector (Invitrogen). Then, it was excised from the recombinant vector and cloned in the *Sma*I site of the expression vector pMYX2, downstream of the *qa*-2 promoter. Site-directed mutagenesis was done using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The pMYX2 recombinant vector and pairs of synthetic complementary oligonucleotide primers containing the desired mutation were used in PCR reactions to create the mutated plasmids. The mutagenic primers were: C14S, 5′-CATCCCGTTCCTTTCCCTTTTGG-3′; C24S, 5′-TGCT CGCCTCCTACGTGGTC-3′; C40, 5′-GCAGAAAGAAGTC CTCGCCGATG-3′; C50S, 5′-ACTACGAGTCCTTGC ACCACAAGAAGG-3′. The mutagenic oligonucleotide primers were designed in order to change four independent codons in the genomic DNA (underlined nucleotides) that resulted respectively in the replacement of cysteines C14, C24, C40 and C50 by serine residues in the 11.5 kDa subunit. The mutagenesis was confirmed by complete sequencing of the PCR products.

Enzymatic Activities—Mitochondria were isolated from *N. crassa* strains (*17*) and then used for the preparation of sub-mitochondrial particles (SMP). Mitochondria were suspended in a buffer containing 50 mM Tris–HCl (pH 8.0), 0.25 M sucrose and 0.2 mM EDTA to a final protein concentration of 10 mg/ml and the pH was adjusted to 8.6 with 1 M NH_4OH. The mitochondrial suspension was then subjected to ultrasonic treatment (five 30-s pulses with 1-min intervals) and centrifuged at 30,000*g* for 15 min. The supernatant was then centrifuged at 120,000*g* for 1 h. The pellet was washed, suspended in the same buffer and stored at –80°C.

Oxidation of deamino NADH (dNADH) was measured photometrically ($\varepsilon_{340\,nm} = 6.22\,mM^{-1}\,cm^{-1}$) in a standard assay mixture containing 50 mM Tris–HCl (pH 8.0), 0.25 M sucrose, 0.2 mM EDTA and 1 mg/ml BSA. The activities were measured after addition of SMP (~50 μg/ml) to the standard medium. In the dNADH:Q_1 reductase activity assay, 2 mM KCN, 100 μM dNADH and 60–100 μM Q_1 were added to the reaction mixture. The activity was also measured in the presence of 15 μM of the complex I inhibitor rotenone.

The fully activated enzyme was obtained by pre-incubation of SMP in the reaction mixture with 10–20 μM of dNADH, until complete oxidation of the substrate, in order to eliminate the lag phase in the onset of the catalytic activity. Then, the dNADH:Q_1 reductase activity was monitored in the presence or absence of 2 mM NEM. For thermal de-activation, SMP were incubated 15 min at 30°C in the presence or absence of 2 mM NEM and their activity was determined in the same way.

NADH:hexaammineruthenium III (HAR) reductase activity was measured photometrically by the oxidation of NADH ($\varepsilon_{380\,nm} = 1.25\,mM^{-1}\,cm^{-1}$) in the presence of 2 mM KCN, 100 μM NADH, 2 mM HAR and 1 mg/ml BSA.

Protein Analysis—Blue-native polyacrylamide gel electrophoresis (BN–PAGE) was performed according to the standard procedures (*18*). One-hundred and fifty micrograms of total mitochondrial proteins were solubilized using 3.0 g of *n*-dodecyl-β-D-maltoside (DDM)/g of protein, and the resulting solubilized complexes were separated on a 3–13% gradient gel. The NADH dehydrogenase activity of complex I was detected in the gel (*19*).

The techniques used for development of the rabbit antiserum directed to the 11.5 kDa subunit of the complex I (*7*), determination of protein concentration (*20*), SDS–polyacrylamide gel electrophoresis (*21*), blotting and detection of alkaline phosphatase-conjugated secondary antibody (*22*), have been described before.

RESULTS AND DISCUSSION

Complex I Assembly in the nuo11.5 Mutants—The nuo11.5 mutant strain lacking the 11.5 kDa subunit accumulates intermediates of the membrane arm, preventing the further characterization of complex I activity. Since this subunit was a possible candidate for the SH-labelling related with the A/D transitions, we decided to change independently the four conserved cysteine amino acids for serine residues (C14S, C24S, C40S and C50S). The altered genes as well as the intact gene were expressed in the null-mutant strain nuo11.5 under the control of a heterologous promoter. The presence of the 11.5 kDa subunit on the various strains was checked by western blotting and the strains expressing the highest amount of protein were chosen for further analysis. As shown in Fig. 1, the 11.5 kDa protein is present in all

site-directed mutant strains, although in lower amounts than in wild-type. The electrophoretic migration behaviour of the 11.5 kDa protein in all the strains is the same in the presence and absence of the disulphide reducing agent mercaptoethanol (5% v/v), indicating that the polypeptide does not form intramolecular disulphide bonds (data not shown).

To characterize complex I assembly in the strains harbouring the point mutations, we solubilized the mitochondrial proteins with the non-ionic detergent DDM and separated them by BN–PAGE. As depicted in Fig. 2A, complex I is present in all of the site-directed mutant strains though in different relative amounts. It is evident that the C40S and C50S mutations have the most severe effect in complex I assembly. The in-gel NADH dehydrogenase activity revealed the presence of complex I activity in all four site-directed mutants, as well as the existence of a smaller complex with a molecular weight of ~500 kDa, likely representing the peripheral arm of complex I (Fig. 2B). These mutant strains accumulate peripheral arm, possibly due to the limiting amount of properly assembled membrane arm. This suggests that the four conserved cysteine residues are required for the acquisition and stabilization of the protein structure and consequently to the correct assembly of the membrane arm. The involvement of this subunit in the assembly of the membrane arm was already observed in the null-mutant strain (*7*). This strain also assembles tiny amounts of peripheral arm, not previously observable because it is only detectable by in-gel NADH dehydrogenase activity (Fig. 2B).

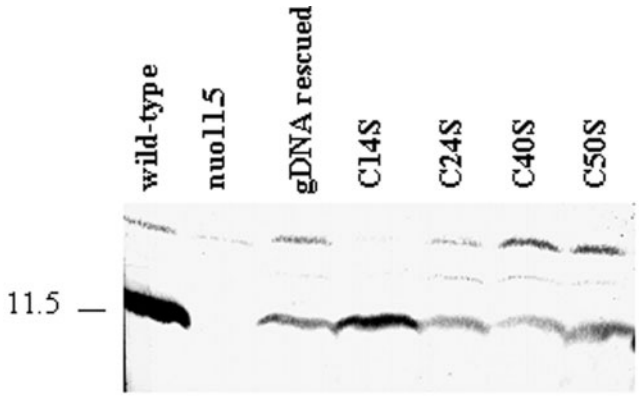

Fig. 1. **Western blotting analysis of mitochondrial proteins from the site-directed mutant strains.** Total mitochondrial proteins from wild-type and mutant strains were analysed by western blotting with an antiserum against the 11.5 kDa subunit of complex I.

Enzymatic Characteristics of the Mutants—We investigated complex I activities with different electron donors/acceptors in SMP from the wild-type and the mutant strains. The NADH:HAR reductase activity, which can be used to estimate the amount of complex I or its peripheral arm, is residual in nuo11.5 due to the small amounts of assembled peripheral arm in this strain (Fig. 2). The null-mutant rescued by wild-type genomic DNA strain has roughly 50% of the wild-type activity. In the strains harbouring the site-specific mutations C14S, C24S, C40S and C50S, the NADH:HAR activity is 52, 31, 22 and 26%, respectively, which is in agreement with the results obtained by the BN–PAGE. The mutation C14S is the less harmful for complex I assembly, resulting in a complex I activity similar to wild-type rescued enzyme.

Then, we measured the dNADH:Q_1 reductase activity in order to determine more specifically the complex I activity, since *N. crassa* has multiple alternative NADH dehydrogenases capable of oxidizing NADH but not dNADH (*6*). The rotenone sensitive dNADH:Q_1 reductase activity is lower in the site-directed mutants than in the wild-type, and the nuo11.5 has no detectable rotenone sensitive dNADH:Q_1 reductase activity (Table 1). The null-mutant rescued by wild-type genomic DNA and the C14S strains display about 40% of wild-type activity and the C24S, the

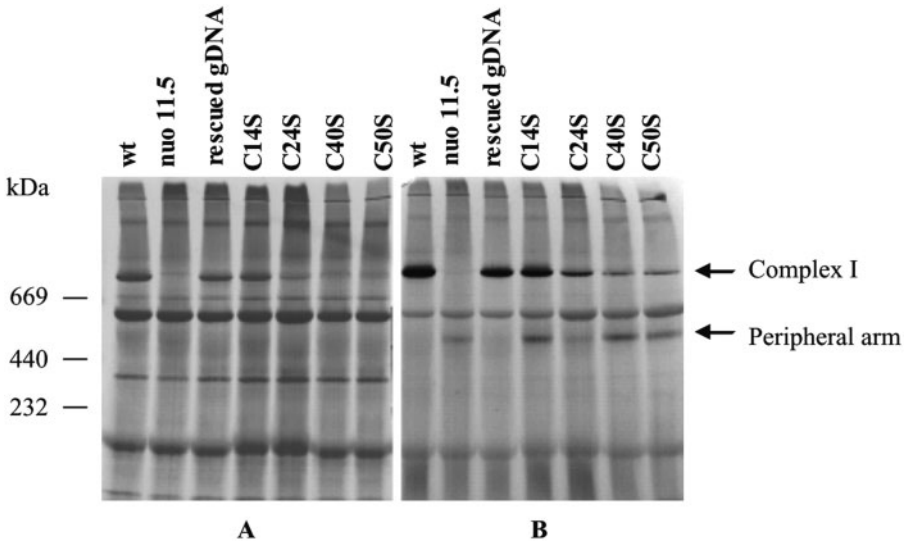

Fig. 2. **Analysis of the mitochondrial respiratory chain complexes from different *N. crassa* strains by BN–PAGE.** One-hundred and fifty micrograms of total mitochondrial proteins were solubilized with 3 g DDM/g of protein and applied on a 3–13% gradient gel. (A) Coomassie Brilliant Blue stained gel; (B) in-gel NADH dehydrogenase activity.

Table 1. **Catalytic properties of SMP from different strains at 25°C.**

N. crassa strain	Rotenone sensitive dNADH:Q_1 reductase activity[a]	NADH:HAR reductase activity
Wild-type	0.336 ± 0.020	1.093 ± 0.108
nuo11.5	0.003 ± 0.001	0.154 ± 0.012
Rescued gDNA	0.142 ± 0.016	0.524 ± 0.064
C14S	0.122 ± 0.007	0.565 ± 0.059
C24S	0.062 ± 0.003	0.335 ± 0.014
C40S	0.050 ± 0.015	0.239 ± 0.014
C50S	0.043 ± 0.002	0.283 ± 0.002

μmol (d)NADH/min per mg of protein.
[a]15 μM rotenone was added.

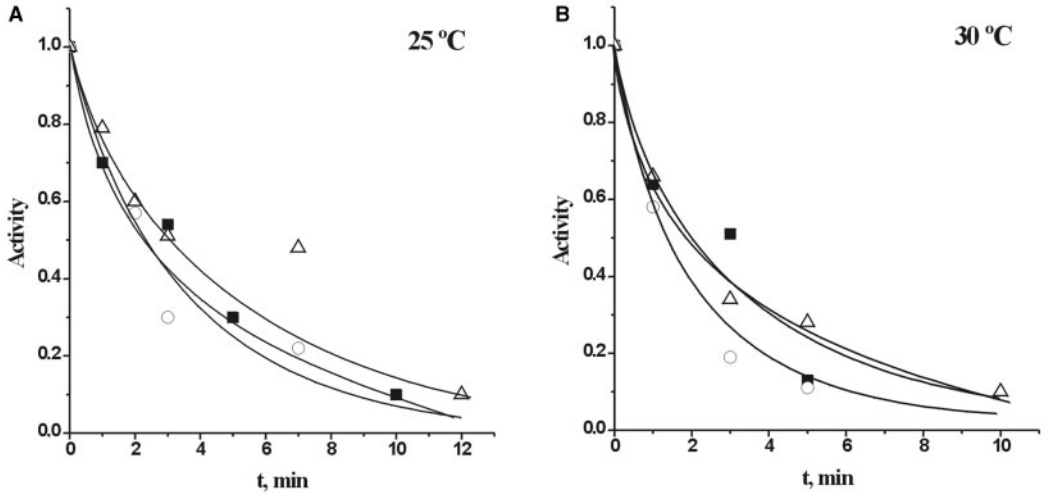

Fig. 3. **De-activation of complex I in *N. crassa* strains at two different temperatures.** Fifty micrograms per millilitre of SMP from wild-type (filled square), C14S (open circle) and C40S (open triangle) were pre-pulsed aerobically with 10–20 μM dNADH in a spectrophotometric cuvette at 25°C (A) and 30°C (B). After complete depletion of dNADH the incubation was continued for the times indicated on the abscissa and the initial rates of rotenone sensitive dNADH:Q_1 reductase activity were determined in the presence of 2 mM $MgCl_2$ at 25 or 30°C. Two millimoles of KCN and 100 μM Q_1 were added and the reaction was started by the addition of 100 μM dNADH. The initial activities for wild-type, C14S and C40S were 0.1503, 0.0774 and 0.0368 μmol dNADH/min per mg of protein at 25°C; and 0.1594, 0.0964 and 0.0398 μmol dNADH/min per mg of protein at 30°C, respectively.

C40S and the C50S strains only around 15%. The lower activities of the C24S, C40S and C50S strains reflect the small amount of assembled complex I. Moreover, the ratios between the quinone and HAR activities show that the complex I present in the mutant strains has no enzyme activity alteration, indicating that the mutations interfere with the assembly but not with the complex I activity.

A/D Transitions in the Mutant Strains—The rates of complex I de-activation of the cysteine mutants were analysed at two different temperatures. This was checked by measuring the initial dNADH:Q_1 reductase rates catalysed by SMP, in the presence of 2 mM $MgCl_2$, after their incubation for different times at a given temperature. Complex I de-activates faster at higher temperatures and thus the $t_{1/2}$ at 30°C was approximately half of the $t_{1/2}$ at 25°C. No significant differences were observed between the mutants and the wild-type enzyme concerning the kinetic parameters of complex I de-activation. The de-activation curves obtained for wild-type, C14S and C40S strains are shown in Fig. 3.

The de-activated form of complex I from different organisms is irreversibly inhibited by SH-modifying reagents such as NEM, whereas the active form is not sensitive to these inhibitors (*13*). This is a powerful diagnostic tool to study the amount of de-activated complex I in an enzymatic preparation. Inhibition by NEM was seen for the thermally de-activated SMP from all different preparations. As shown in Table 2, the dNADH:Q_1 reductase activities of all strains were not altered by the addition of 2 mM NEM when SMP were pre-pulsed with dNADH before the addition of the inhibitor, demonstrating that the active form is resistant to NEM inhibition. The dNADH:Q_1 reductase activity from all strains was inhibited around 60% by NEM, when SMP were deactivated by incubation at 30°C for 15 min. Thus, complex I from all mutant strains is able to bind NEM similarly to the wild-type enzyme, indicating that none of the cysteine residues of the 11.5 kDa protein is involved in the NEM binding and inhibition process.

It is not clear whether the complex I subunit labelled with NEM is also involved in the structural changes of the A/D transition phenomenon. However, it was

Table 2. **Effect of NEM on active and thermally de-activated SMP from different strains at 30°C.**

N. crassa strain	dNADH:Q1 reductase activity			
	Active SMP		De-activated SMP	
NEM treatment	−	+	−	+
Wild-type	0.524	0.502 (96%)	0.467 (89%)	0.240 (46%)
Rescued gDNA	0.193	0.171 (88%)	0.146 (76%)	0.180 (47%)
C14S	0.186	0.168 (91%)	0.162 (87%)	0.079 (42%)
C24S	0.115	0.113 (98%)	0.115 (100%)	0.051 (44%)
C40S	0.065	0.059 (90%)	0.050 (77%)	0.025 (39%)
C50S	0.083	0.078 (94%)	0.069 (83%)	0.036 (44%)

μmol dNADH/min per mg of protein.
In brackets % of activity related to the activity of active SMP without NEM.

recently suggested that the sulphydryl group of complex I sensitive to inhibition by NEM, and possibly involved in the active/de-active process, is located in the inner membrane facing the matrix interface (*23*). Our results suggest that the 11.5 kDa subunit of *N. crassa* is neither involved in the NEM inhibition nor in the A/D transition process, but it is important for an efficient assembly and stability of complex I.

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