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Identification of the TYKY homologous subunit of complex I from *Neurospora crassa*

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Abstract

A polypeptide subunit of complex I from *Neurospora crassa*, homologous to bovine TYKY, was expressed in *Escherichia coli*, purified and used for the production of rabbit antiserum. The mature mitochondrial protein displays a molecular mass of 21280 Da and results from cleavage of a presequence consisting of the first 34 N-terminal amino acids of the precursor. This protein was found closely associated with the peripheral arm of complex I. © 1997 Elsevier Science B.V.

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1. Introduction

Respiratory chain NADH dehydrogenase (complex I; EC 1.6.5.3) is a multimeric enzyme of the mitochondrial inner membrane. Mitochondrial complex I contains more than 30 polypeptide subunits, seven of which are encoded by mtDNA. It transfers electrons from NADH to ubiquinone through a series of protein-linked prosthetic groups (FMN and iron–sulfur clusters) and these reactions are coupled with proton translocation across the membrane. It is accepted that complex I contains at least one binuclear and three tetranuclear iron–sulfur clusters, but probably more [1,2]. Neurospora complex I was shown to be formed from two parts, the peripheral and membrane arms, that undergo independent assembly [3]. The peripheral arm appears to contain all prosthetic groups of the enzyme, with the exception of the tetranuclear iron–sulfur cluster N-2 [4], while several largely hydrophilic subcomplexes which contain all redox centres, including cluster N-2, were isolated from bovine complex I [5,6].

The assignment of the prosthetic groups to specific subunits of the enzyme is important to understand in more detail the structure and function of complex I. Based on different approaches, most of them have been tentatively assigned to specific subunits of the enzyme. Localization of iron–sulfur cluster N-2, however, is still under debate. It has been proposed that cluster N-2 can directly reduce ubiquinone and a

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proton pumping activity is associated with this electron transfer reaction [7–9], making this prosthetic group of complex I particularly important. Two proteins, called TYKY and PSST in bovine, appear as the most likely candidates to provide its binding site [6,10–12]. Here, we report that the *N. crassa* subunit homologous to TYKY is located in the peripheral arm of complex I and, therefore, most likely does not bind cluster N-2.

2. Material and methods

We expressed the putative mature form of the *N. crassa* TYKY-homologue in *E. coli* as a fusion protein containing a His-tag. The corresponding cDNA, cloned in pGEM4 [13], was amplified by PCR using *Pfu* DNA polymerase (Stratagene), the vector-specific T7 promotor primer and the cDNA-specific primer 5′-ccatccgccatatggcaacccccgccggtc-3′ (the underlined bases were altered from the *N. crassa* sequence to create a *Nde*I site). The resulting DNA was digested with *Nde*I and *Bam*HI and cloned into the pET19b plasmid vector (Novagen). In order to avoid sequencing the entire DNA obtained by PCR, a 3′-located *Sac*II/*Bam*HI fragment was replaced with the correspondent piece from the original cDNA. Both strands of the DNA located 5′ to the *Sac*II site were completely sequenced. Bacteria carrying the recombinant plasmid were grown to exponential phase ($A_{600} = 0.6$), induced for expression of the fusion protein for 3 h at 37°C using 1 mM IPTG and collected by centrifugation. Most of the protein was present in ''inclusion bodies'' and, after purification in nickel chelation columns under denaturing conditions, was used to produce rabbit antiserum [14]. A small portion of soluble protein was also purified by affinity chromatography under native conditions and used for digestion with Enterokinase (Sigma).

Techniques such as the preparation of mitochondria [15], isolation of complex I [16] and its peripheral arm [17], the analysis of Triton X-100 solubilised mitochondrial proteins in sucrose gradients [18], protein determination [19] and sequencing [20], SDS-polyacrylamide gel electrophoresis [21], blotting [22] and immunological detection of proteins using alkaline phosphatase-conjugated secondary antibodies [23], have been published before.

3. Results and discussion

We have expressed the mature form of an iron–sulfur subunit of complex I from *N. crassa* in an heterologous system and, after purification, used it to immunise rabbits. The protein is homologous to bovine TYKY [13] and, according to the size of the mature polypeptide, was named NUO-21.3c (there are two other subunits of complex I with the same molecular mass). In agreement with this, the antiserum readily recognises a protein displaying an apparent molecular mass of 22–24 kDa from extracts of *N. crassa* mitochondria (Fig. 1, lanes 1–4). The migration of this polypeptide is similar to the migration of the fusion protein produced in *E. coli* that was incubated with enterokinase in order to remove the His-tag (compare lanes 3 and 6 of Fig. 1). It should be noticed that the latter should contain two extra amino acids. Direct determination of the N-terminus of the mature mitochondrial protein revealed the sequence ATPAG, thus confirming the cleavage site of the precursor. Interestingly, this fits into a one-step cleavage of the precursor to yield the mature protein, while it was suggested that the homologous TYKY protein of bovine results from a two-step cleavage of its precursor [24]. Although this awaits experimental evidence, it obviously represents

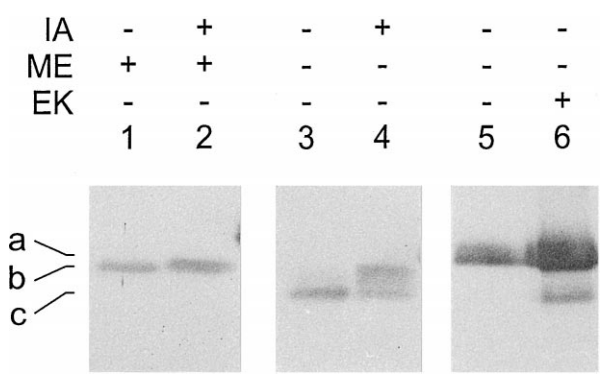

Fig. 1. Identification of the NUO-21.3c subunit of complex I. Total mitochondrial proteins (1–4) or NUO-21.3c protein that was expressed and purified from *E. coli* (5, 6) were analysed by Western blotting with antiserum against the latter polypeptide. The protein fused to a His-tag (a) as well as the mitochondrial reduced (b) and oxidised (c) forms of the protein are indicated. The experiments were performed in the presence (+) or absence (−) of iodoacetamide (IA), 2-mercaptoethanol (ME) and enterokinase (EK).

a further example where the same protein is processed in different ways in different organisms. The same has been observed with the Rieske iron–sulfur protein of complex III, which is cleaved in two steps in *N. crassa* [25], but undergoes one-step cleavage in mammals [26].

The mature NUO-21.3c protein has an electrophoretic migration that is faster in the absence than in the presence of 2-mercaptoethanol (compare lanes 1 and 3 of Fig. 1), suggesting the existence of intramolecular disulphide bridges that would prevent some of the cysteines from participating in the binding of iron–sulfur clusters. If this was true, there was not enough cysteines in the protein to bind the two iron–sulfur clusters that it is believed to contain. However, when mitochondria are dissociated with SDS in the presence of 10 mM iodoacetamide, that should prevent de novo formation of disulphide bridges, a slower migrating band can be seen in the absence of 2-mercaptoethanol (Fig. 1, lane 4). We suggest that intramolecular disulphide bridges readily form in NUO-21.3c only upon isolation of the protein from the membrane in denaturing conditions, presumably by release of the bound iron–sulfur cluster(s).

Fig. 2 shows that NUO-21.3c is associated with the peripheral arm of complex I. Using antiserum against the protein, it can be visualised in preparations of complex I or its peripheral arm alone. As controls, individual antisera against the 30.4 kDa subunit of the peripheral arm of complex I [27] and against the 20.8 kDa and 12.3 kDa subunits of the membrane arm of the enzyme [28,29] were included in this experiment. The 30.4 kDa protein can also be seen in both preparations, while the 20.8 kDa and 12.3 kDa are visible exclusively in the complex I preparation.

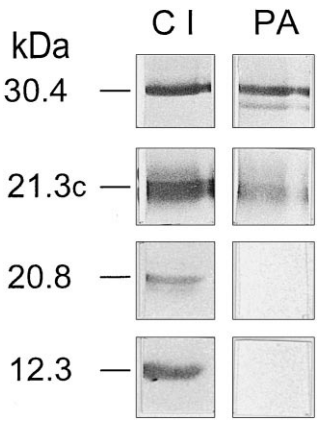

Fig. 2. Association of NUO-21.3c with the peripheral arm of complex I. Preparations of complex I (CI) or its peripheral arm (PA) were analysed by Western blotting with antisera against the subunits of complex I indicated in the left.

In order to further confirm these results, the behaviour of the NUO-21.3c protein was analysed in *N. crassa* mutants that bear different phenotypes regarding complex I. We used the stopper strain IAR 155A [30] that assembles the peripheral arm and lacks detectable membrane arm and mutant nuo30.4 that lacks the peripheral arm and accumulates the membrane arm of complex I (P.C. Alves, M. Duarte and A. Videira, unpublished observations). Mitochondrial proteins from these strains were solubilised with Triton X-100 and resolved by sucrose gradient centrifugation. Subsequently, fractions of the gradients were analysed by Western blotting with antisera against different subunits of complex I that can be used as markers for the peripheral and membrane arms of the enzyme [18,31,32]. Fig. 3 illustrates the results of this experiment. In the wild type strain, all proteins elute mostly in fractions 9–11 (panel A), the usual behaviour of complex I. In strain IAR 155A, NUO-21.3c co-elutes with the 30.4 kDa protein with a peak in fraction 8 and this material represents the peripheral arm of complex I (panel B). It can be seen that the membrane arm 12.3 kDa protein elutes mainly in fractions 2 and 3, thus representing ''free'' subunit. On the contrary, the NUO-21.3c protein behaves as ''free'' subunit in the nuo30.4 mutant, eluting in fractions 2 and 3 (panel C). In this strain, the membrane arm of complex I elutes mostly in fractions 8–10, as deduced by the behaviour of its 20.8 kDa and 12.3 kDa subunits. Furthermore, we have recently isolated a *N. crassa* mutant lacking NUO-21.3c and it appears that only the membrane arm and not the peripheral arm are assembled in this strain (details will be published elsewhere).

Our results clearly show that subunit NUO-21.3c is part of the peripheral arm of complex I from *N. crassa*. This domain holds most of the prosthetic groups of the enzyme, but has been shown to lack iron–sulfur cluster N-2 [4]. This is not due to a loss during the preparation of this subcomplex, since EPR analysis of membranes of mutant nuo21 did not detect cluster N-2 in the peripheral arm even in its native membrane surrounding [33]. Furthermore,

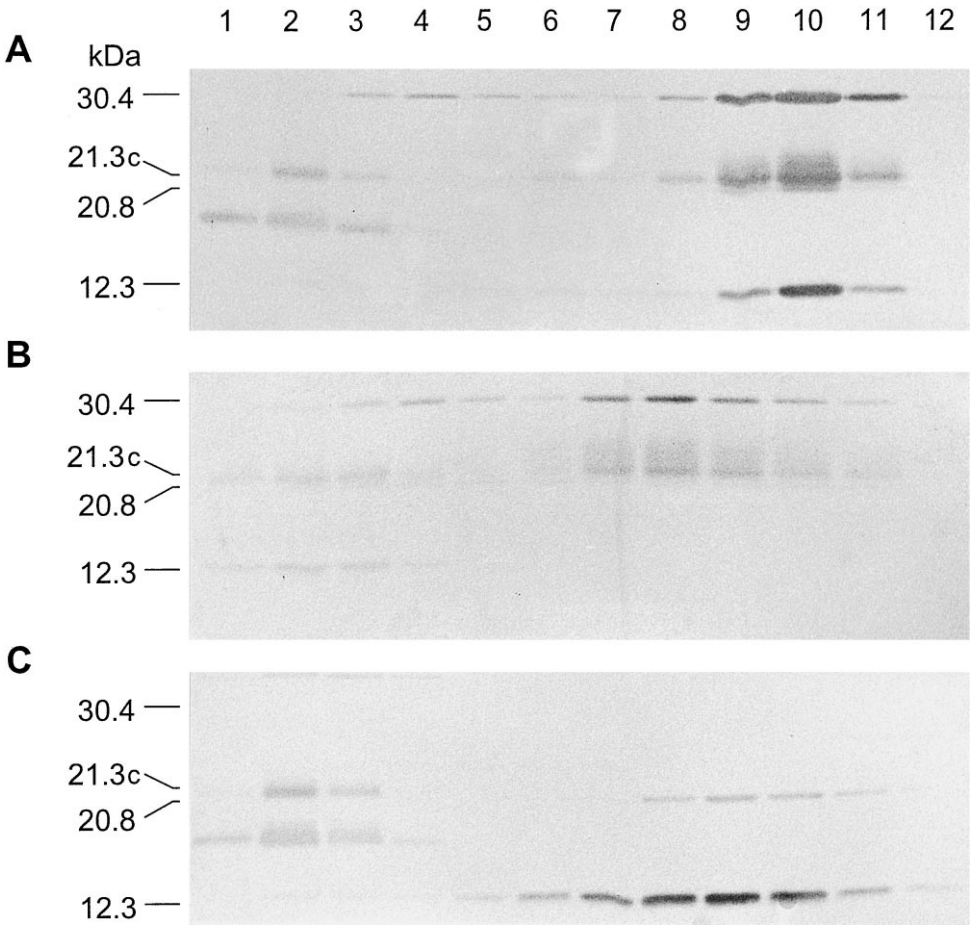

Fig. 3. Analysis of NUO-21.3c in different strains of *N. crassa*. Mitochondria from wild type (A), the stopper strain IAR 155A (B) and mutant nuo30.4 (C) were isolated and centrifuged in sucrose gradients in the presence of Triton X-100. Fractions of the gradients (labelled 1–12 from top to bottom) were collected and analysed by Western blotting with a mixture of antisera against the subunits of complex I indicated in the left.

cluster N-2 has been found in excess over the other clusters in mitochondrial membrane fragments of *N. crassa* grown under manganese depletion. Since the membrane arm is accumulated in these membranes, cluster N-2 was attributed to the membrane part of complex I [34,35]. Since all EPR-visible iron–sulfur clusters present in the peripheral arm have been attributed to the 78 kDa, 51 kDa and 24 kDa subunits, NUO-21.3c most likely binds iron–sulfur cluster(s), which have so far escaped detection by EPR. The conservation of cysteine motifs in this protein from a variety of organisms [10,13], strongly suggests that it binds two iron–sulfur clusters. Excluding TYKY and its homologues from providing the binding site for cluster N-2, leaves PSST [36] as the most likely candidate for this function. Its unusual binding motif, probably involving a glutamate residue [37], correlates to the particular features of cluster N-2 as its high pH-dependent midpoint-potential.

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