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Accumulation of the preassembled membrane arm of NADH:ubiquinone oxidoreductase in mitochondria of manganese-limited grown *Neurospora crassa*

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The NADH:ubiquinone oxidoreductase (complex I) of mitochondria is constructed from two arms arranged perpendicular to each other. The peripheral arm protruding into the matrix contains the proximal section of the electron pathway, and the membrane arm with all mitochondrially encoded subunits contains the distal section of the electron pathway. When *Neurospora crassa* is grown under manganese limitation the formation of the peripheral arm is disturbed, but the membrane arm containing the iron–sulfur cluster N-2, is accumulated. An extra-polypeptide, assumed to be a chaperone, is found to be associated with this pre-assembled membrane arm.

NADH:ubiquinone oxidoreductase; Complex I; Assembly; Iron–sulfur cluster; Manganese limitation; *Neurospora crassa*

1. INTRODUCTION

The NADH:ubiquinone oxidoreductase (complex I) of mitochondria transfers electrons from NADH via FMN and a series of iron–sulfur clusters to ubiquinone and links this process with the translocation of protons across the inner membrane. The enzyme is known to consist of more than 30 different subunits in fungi [1] and more than 40 subunits in mammals [2]. Seven subunits are encoded by mitochondrial genes. Electron microscopic analysis of the *Neurospora crassa* complex I has revealed an unusual L-shaped structure with two arms perpendicular to each other [3]. These two arms are assembled separately and are combined en bloc to construct the complex [4]. If protein synthesis in *N. crassa* mitochondria is inhibited by chloramphenicol, only the peripheral arm with exclusively nuclear-encoded subunits is made [5]. It contains the proximal section of the electron pathway of complex I with the FMN and the iron–sulfur clusters, N-1, N-3 and N-4 [5,6]. The independent assembly of the membrane arm which contains all mitochondrially encoded subunits was demonstrated by pulse-chase labelling of *N. crassa* with [^{35}S]methionine and by following the flux of the radioactivity through intermediates of the assembly process into the holocomplex [4]. The location of the iron–sulfur cluster, N-2, which is not found in the pre-assembled peripheral arm, could not be established in this way.

We report here that the formation of the peripheral arm of complex I in *N. crassa* can be specifically disturbed by manganese limitation, leading to the accumulation of the preassembled membrane arm containing the iron–sulfur cluster, N-2.

2. MATERIALS AND METHODS

Manganese-limited growth of *N. crassa* wild-type 74 A was obtained by using glass-destilled water for all media, cleaning all glassware with isopropanol plus 10 M KOH and with 2 mM EDTA and omitting all components with manganese and iron from the minimal medium [7]. Beyond this, 100 mg/l of the complexing agent, 3-(2-pyridyl-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid (ferrozine) was added to the medium. A low inoculum of 5×10^7 conidia per l reduced the manganese introduced by the conidia. In the control experiment the medium was supplemented with 50 mM $MnCl_2$. Dry weight was determined according to [8]. The labelling of cellular protein, immunoprecipitation of complex I, measurement of the NADH-ferricyanide reductase activity, SDS-PAGE, autoradiography and protein determination procedures were described in [4]. EPR spectroscopy was carried out according to [6].

3. RESULTS

Manganese-limited growth of *N. crassa* resulted in a pale pellet-like mycelium instead of the usual light orange-coloured filamentous mycelium. Manganese-supplemented and -limited *N. crassa* cultures showed similar growth rates and maximal cell yields (Fig. 1A). The contents of mitochondrial cytochromes were normal under manganese-limited conditions, implying an un-

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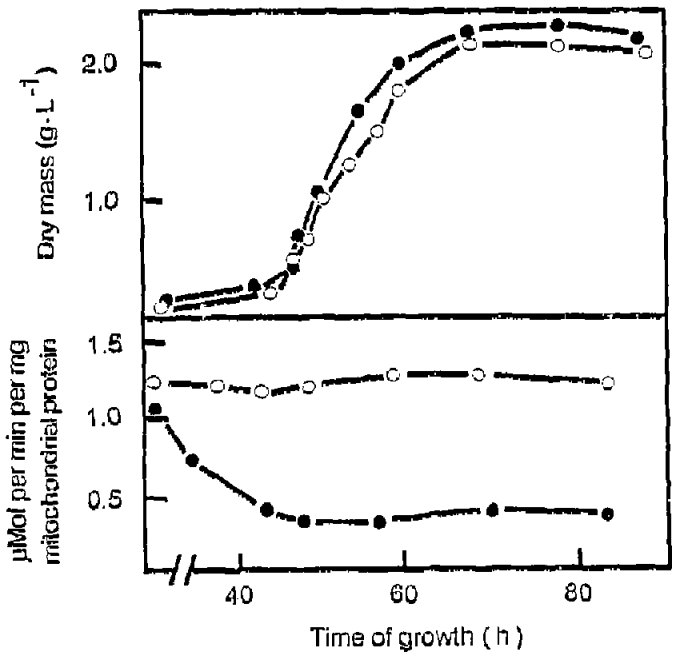

Fig. 1. Time-courses of growth and complex I activity in *N. crassa* cultivated under manganese-supplemented and limited conditions. (A) Growth curves. (B) Complex I activity determined as the high molecular weight NADH-ferricyanide reductase activity after sucrose gradient centrifugation of detergent solubilized mitochondria. (○–○) Manganese-supplemented *N. crassa*, (●–●) manganese-limited *N. crassa*.

disturbed formation of the respiratory complexes ubiquinol:cytochrome *c* oxidoreductase and cytochrome *c* oxidase. Complex I activity, however, decreased dramatically to only 25% of the control value when the culture started to grow exponentially. This complex I deficiency was prevented by supplementing the medium with 50 mM $MnCl_2$ (Fig. 1B).

To determine the step at which formation of complex I was affected by manganese limitation, total cellular protein was labelled with [^{35}S]methionine, mitochondria were isolated, solubilized with Triton X-100, and centrifuged on sucrose gradients. The distribution of complex I protein in the gradient was determined by means of the radioactivity immunoprecipitated by an antiserum against a single (22 kDa) subunit of the membrane arm of complex I and by NADH–ferricyanide reductase activity (Fig. 2).

Using mitochondria prepared from manganese-supplemented hyphae, only the completely assembled complex I could be detected, which sedimented three-quarters of the way through the gradient (Fig. 2A). Mitochondria of the manganese-limited-grown hyphae yielded about three times less completely assembled complex I. In addition complex I subunits could be found in the region of the gradient corresponding to lower molecular weight. No NADH–ferricyanide reductase activity sedimented with these subunits (Fig. 2B). A major accumulation of complex I subunits sedimented to a corresponding molecular weight of approx. 350 kDa. SDS-PAGE and autoradiography of this protein peak (Fig. 2C) showed the typical subunit pattern of the membrane arm of complex I with the characteristic broad and diffuse bands of the mitochondrially encoded subunits [4]. Most interestingly, in addition to the known subunits, this preassembled membrane arm contains an extra 73 kDa polypeptide, which is not found in the fully assembled complex I (Fig. 2B, arrow).

The iron–sulfur clusters of complex I were investigated by EPR spectroscopy of mitochondrial membranes (Fig. 3). Membranes isolated from manganese-supplemented hyphae gave an axial spectrum arising from cluster N-2 ($g_\parallel$ = 2.05 and $g_\perp$ = 1.93 with the negative peak at g = 1.916), cluster N-3 (g_x = 1.87 seen as the shoulder of the negative peak), and cluster N-4 (g_z = 2.10 and the negative peak at g_x = 1.88). Cluster N-1 was hard to identify under these EPR conditions. However, the small shoulder proximal to the g = 1.93 arrow arises mostly from cluster N-1. In mitochondrial membranes from the hyphae grown in manganese-limited medium, the cluster N-2 signal with $g_\parallel$ = 2.05 and a negative peak at g = 1.916 was observed in addition to signals arising from N-1, N-3 and N-4 clusters at much lower (3–4-times diminished) intensity. The negative peak at g = 1.916 showed the same power saturation dependence as the corresponding N-2 signal in mitochondrial membranes of wild-type *N. crassa*. Compared to the control, the intensity of the N-2 signal was lowered by a factor of about 2. The very small signal on the top of the $g_\perp$ = 1.93 resonance is from cluster N-1 of the residual complex I, which was still produced under the manganese-limited condition. Similarly, the broad negative bands around g = 1.88 most likely stem from the clusters N-3 and N-4.

4. DISCUSSION

In this work it is demonstrated again that the two arms of complex I are assembled separately. For the first time we could show that the preassembled membrane arm contains the iron–sulfur cluster N-2 i.e. that this cluster is added to the membrane arm before it combines with the peripheral arm to construct the holocomplex. The finding that cluster N-2 is located in the membrane arm is consistent with the assumption that this cluster is the immediate reductant of ubiquinone [1], which is supposed to be bound at the subunit encoded by the mitochondrial ND-1 gene [9]. Furthermore, it is believed that cluster N-2 takes part in proton translocation which occurs through the membrane arm [10].

It has been proposed that cluster N-2 may be located in the 23 kDa subunit of the bovine complex I (the homologous *N. crassa* subunit has not yet been identified) [11]. This nuclear gene-encoded subunit is located in the so-called hydrophobic fraction of the bovine com-

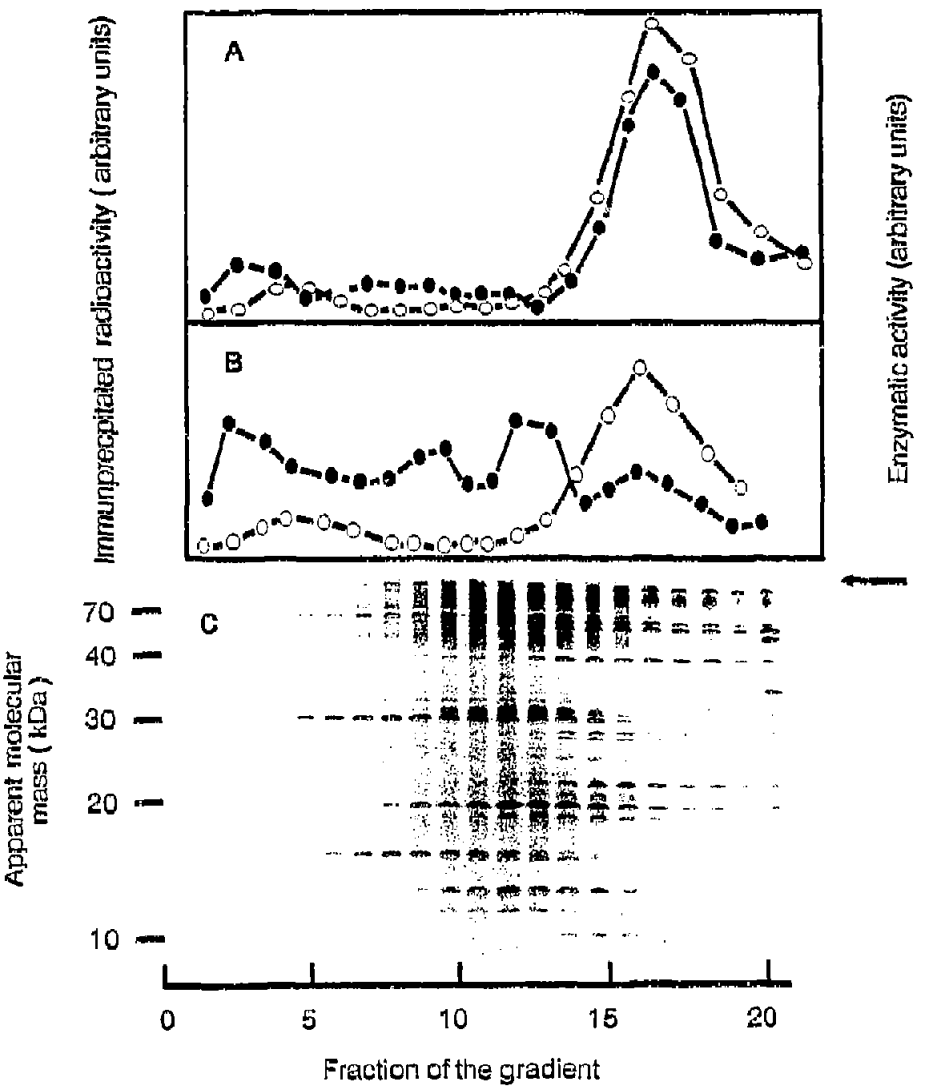

Fig. 2. Sucrose gradient centrifugation of Triton X-100 solubilized mitochondria of manganese-supplemented (○–○) and manganese-limited (●–●) *N. crassa*. (A) The distribution of NADH-ferricyanide reductase activity. (B) The radioactivity immunoprecipitated by complex I antiserum. (C) The polypeptide pattern of complex I protein immunoprecipitated from the gradient obtained from mitochondria of manganese-limited *N. crassa*. The extra 73 kDa polypeptide assumed to be a chaperone is marked with an arrow. This protein is not seen in the fully assembled enzyme [4].

plex I which roughly corresponds to the membrane arm of the *N. crassa* complex.

Another interesting new observation is that the preassembled membrane arm of complex I is associated with a 73 kDa extra-polypeptide that is not found in the fully assembled holocomplex. This polypeptide is co-precipitated together with the subunits of the membrane arm by different antisera each raised against a single subunit of the membrane arm (data not shown). In another recent study by our group [12] a *N. crassa* mutant was characterized in which the nuclear gene of a subunit of the membrane arm was disrupted by gene replacement. This mutant sub-assembles two complementary subcomplexes of the membrane arm neither of which carry an iron–sulfur cluster. One sub-assembly is also associated with this 73 kDa extra-polypeptide. We therefore believe that it is involved, as a chaperone-type protein, in the assembly of the membrane arm, keeping it in a competent state for association of other subunits and final combination with the peripheral arm.

Very little is known about the role of manganese in mitochondrial metabolism and biogenesis. A number of manganese-dependent enzymatic processes are located inside the mitochondrion [13]. Lowered oxidative phosphorylation was observed in mice fed with a manganese-free diet [14]. Our group has recently reported a correlation between manganese deficiency, decreased complex I formation, and citric acid overproduction in a mutant strain of *Aspergillus niger* [15]. We do not yet understand the effect of manganese on the formation of the peripheral arm of complex I, but we can apply manganese-limited cultivation of *N. crassa* as a new approach for studying complex I.

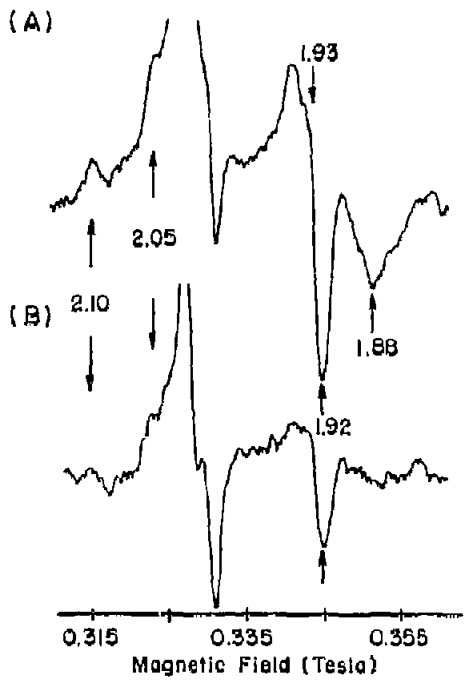

Fig. 3. EPR difference spectra of completely reduced minus succinate reduced mitochondrial membranes of manganese-supplemented (A) and manganese-limited (B). *N. crassa*. Completely reduced membranes were obtained by addition of 5 mM NADH, 10 mM succinate and 5 mM dithionite in the presence of the following redox mediators: 5 μM each of pyocyanine and methylviologen; 20 μM indigosulfonate; 30 μM safranine O. Succinate-reduced samples contain 10 mM succinate and 0.5 μM FCCP. EPR conditions: microwave frequency, 9.250 GHz; microwave power, 2mW; modulation amplitude 1.25×10^{-3} T; time constant 0.128 s; sweep rate 5×10^{-4} T/min; sample temperature, 10 K. Gain for the EPR recording was selected to compensate the difference of protein concentration in samples A and B.

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