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Molecular genetic studies of complex I in *Neurospora crassa*, *Aspergillus niger* and *Escherichia coli*

Uwe Weidner, Uwe Nehls, Regina Schneider, Wolfgang Fecke, Hans Leif, Andreas Schmiede, Thorsten Friedrich, Ralf Zensen, Ulrich Schulte, Tomoko Ohnishi and Hanns Weiss

Institut für Biochemie, Heinrich-Heine-Universität Düsseldorf, Düsseldorf (Germany) and Department of Biophysics and Biochemistry, University of Pennsylvania, Philadelphia, PA (USA)

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Introduction

The proton pumping NADH : ubiquinone oxidoreductase of mitochondria (complex I) is an assembly of some 25 different nuclear-encoded and 7 mitochondrially-encoded subunits. One FMN and 4–6 iron-sulfur (FeS) clusters have been identified as internal redox-groups [1]. Recent electron microscopic and biochemical studies have shown that the complex is made up of two arms forming an L-shaped structure. The peripheral arm which protrudes into the mitochondrial matrix contains most of the nuclear-encoded subunits, while the membrane arm contains all mitochondrially encoded subunits [2]. The two arms are assembled on separate pathways and possibly emerged independently in evolution [1,3,4]. Sequence analysis revealed homology between subunits of the peripheral arm of complex I and subunits of a soluble NAD-reducing hydrogenase of *Alcaligenes eutrophus*. Based on this relationship, binding sites for NADH, FMN and the FeS clusters N-1, N-3 and N-4 were assigned to several subunits [1,5–7]. The ubiquinone binding domain in the membrane arm of complex I was identified by sequence comparison with a bacterial glucose dehydrogenase [8]. Homology was also found between complex I subunits (essentially of the membrane arm) and subunits of a membrane bound formate hydrogen lyase of *Escherichia coli* [1,9]. This relationship has, however, not yet contributed to a better understanding of complex I, because the membrane part of the formate hydrogen lyase is itself poorly understood.

Correspondence to: H. Weiss, Institut für Biochemie, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1, 4000 Düsseldorf 1, Germany.

Here, we report two other approaches which may help to further unravel complex I. By gene disruption in the fungi *Neurospora crassa* and *Aspergillus niger*, we were able to selectively prevent constitution of the membrane arm and the peripheral arm, respectively. Sequence analysis of the *E. coli* operon coding for the proton pumping NADH : ubiquinone oxidoreductase gives us access to a related but, hopefully, simpler enzyme.

Gene disruption in fungi: a tool for studying the assembly, the molecular architecture and partial functions of complex I

Genetic inactivation of the membrane arm of complex I in N. crassa

To inactivate the 21 kDa subunit of the membrane arm of complex I, we transformed *N. crassa* with a genomic DNA-fragment including the gene of this subunit [1] interrupted by the *A. nidulans* TrpC promotor and the *E. coli* gene for hygromycin B phosphotransferase. Heterocaryotic transformants were selected and crossed with the parental strain to obtain homocaryonts. In 5 out of 11 analysed transformands the original gene was replaced by the defective gene. One transformant, the mutant *nuo*21, was further characterized.

When supplied with enough sucrose, this mutant grows almost as fast as the parental strain. Mitochondrial cytochrome contents and respiratory activities were normal. Respiration with NADH and succinate was inhibited by antimycin A or KCN, but pyruvate/malate respiration was insensitive to piericidin A. Electron transfer from NADH to ubiquinone is most likely carried out by the alternative NADH : ubiquinone oxidoreductase found in fungal mitochondria [1,10].

We analysed the FeS-clusters of complex I in the mutant by EPR-spectroscopy and found the clusters N-1, N-3, and N-4, which are known to be located in the peripheral arm [11]. The cluster N-2 supposed to lie in the membrane was absent [1]. Therefore, at least parts of complex I must be pre-assembled in the mutant.

In order to identify the stage at which the assembly of complex I was blocked in the mutant, we labelled hyphae with [^{35}S]methionine and fractionated solubilized mitochondria by sucrose gradient centrifugation. The distribution of complex I subunits in the gradient was determined by means of radioactivity immunoprecipitated by antisera to complex I. The NADH/ferricyanide reductase activity, as marker for the peripheral arm of complex I, was also measured. In a control experiment carried out with parental mitochondria, the enzymatic activity and the immunoprecipitated radioactivity sedimented as a single peak most of the way through the gradient as expected for the approx. 700000 M_r complex I. Using the mutant mitochondria, radioactivity immunoprecipitated by an antiserum against the entire complex I sedimented in form of several overlapping peaks corresponding to proteins of 100000 to 350000 M_r (Fig 1a). An antiserum to the 49 kDa peripheral subunit of complex I

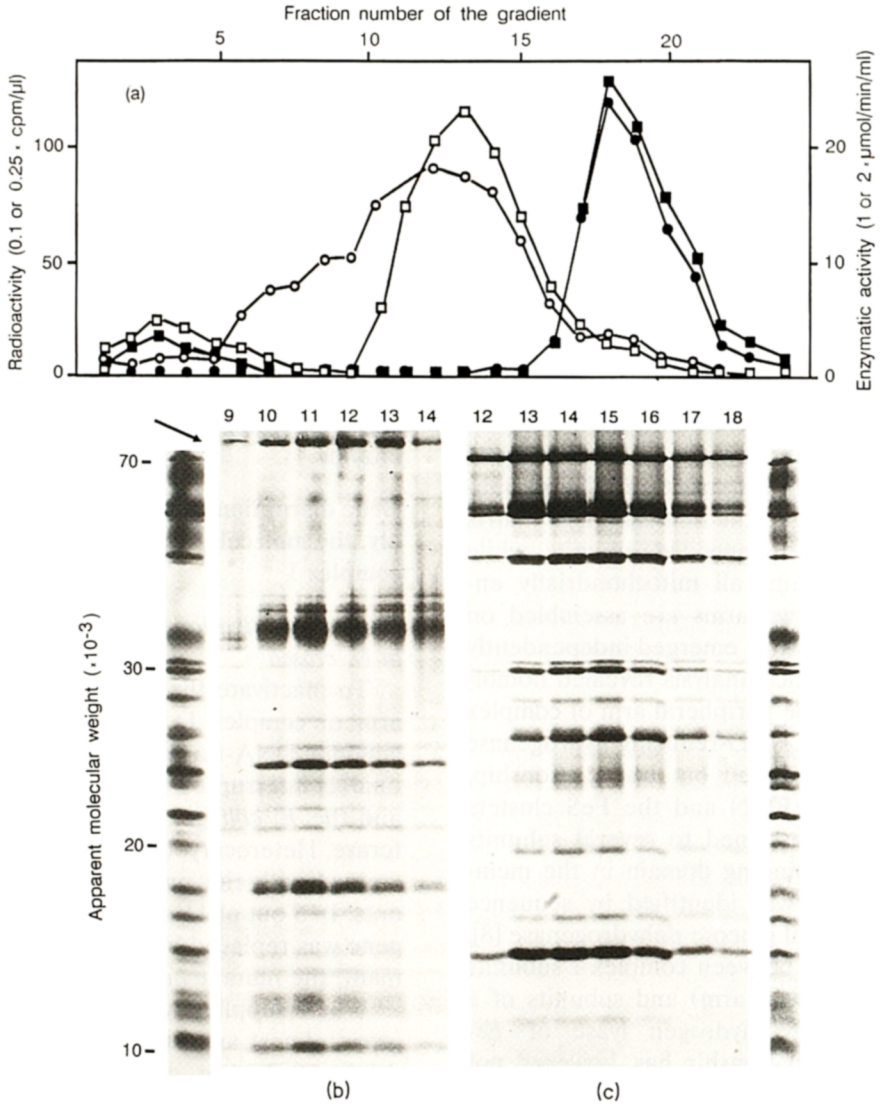

Fig. 1. Sucrose gradient centrifugation of solubilized mitochondria from [^{35}S]methionine labelled *N. crassa* parental strain and mutant *nuo*21. The profiles of NADH/ferricyanide reductase activity (■, □) and radioactivity immunoprecipitated by an antiserum against the entire complex I (●, ○) are shown in (a) as closed symbols for the parental strain and as open symbols for the mutant *nuo*21. (b) and (c) shows the patterns of radioactive polypeptides immunoprecipitated from the gradient with the mutant mitochondria using antisera against the 16 kDa subunit (b) and 49 kDa subunit (c). The single lanes at the right and left side show the polypeptide composition of complex I immunoprecipitated by the above antisera from the gradient with the parental strain mitochondria. The extra polypeptide suggested to be a chaperone is marked by an arrow. Number 1 refers to the top of the gradient and number 25 to the bottom. Note that for the mutant the scales on the ordinate are enlarged.

[1] precipitated the approx. 350000 M_r protein together with the NADH/ferricyanide reductase activity. The polypeptide pattern of this protein (Fig. 1c) clearly characterizes it as the pre-assembled peripheral arm of complex I [3,4]. An antiserum against a 16 kDa subunit of the membrane arm of complex I precipitated a approx. 250000 M_r subcomplex (Fig. 1b). It consists of 4 mitochondrially-encoded subunits and 6 nuclear-encoded subunits of the membrane arm of complex I [4]. In addition, this subcomplex is associated with a 73000 M_r polypeptide not found in complex I (Fig. 1b, arrow). This extra polypeptide might function as a chaperone to keep this pre-assembled moiety of the membrane arm in a competent state for further assembly.

The mutant *nuo*21 is to our knowledge the first *N. crassa* strain with a defective complex I at normal levels of the other respiratory chain complexes. The respiratory deficient *mi*-, *cni*-, and *stopper*-mutants of *N. crassa*, as well as the chloramphenicol-poisoned wild type, have reduced levels of all respiratory complexes but increased levels of the alternative respiratory enzymes [1]. The normal growth of the mutant *nuo*21 demonstrates that the obligate aerobic fungus *N. crassa* can live perfectly well without an intact complex I. This is a promising observation in view of further genetic manipulation of complex I in this microorganism.

Genetic inactivation of the peripheral arm of complex I in A. niger

For a biotechnological study reported elsewhere [12], we are also transforming *A. niger* with defective copies of complex I genes. Here, we report the effect of inactivating the peripheral 51 kDa subunit with the binding sites for NADH, FMN and one FeS-cluster [5–7]. The same selection marker as above for *N. crasssa* was used and the mutant further characterized is called *A. niger nuo*51.

This mutant grows much slower than the parental strain although mitochondrial cytochrome *a* and *b* contents and respiratory activities are normal. Again the pyruvate/malate respiration is insensitive to piericidin A, i.e., electron transfer from NADH to ubiquinone is carried out by the alternative enzyme. Immunoblotting experiments and measurement of NADH/ferricyanide reductase activity showed that the mutant contains the pre-assembled membrane arm of complex I, but is devoid of the peripheral arm. EPR spectroscopy revealed only the signal of cluster N-2, but no signals arising from the other complex I clusters (Fig. 2).

Different effects by inactivation of the peripheral arm and the membrane arm of complex I

Why does inactivation of the peripheral arm of complex I strongly reduce the vitality of fungi while inactivation of the membrane arm does not? In both

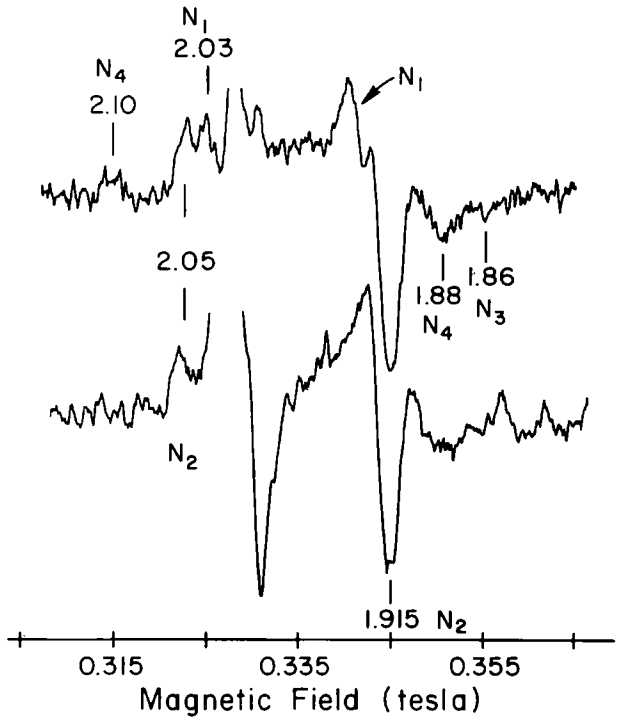

Fig. 2. EPR difference spectra (NADH/dithionite reduced minus succinate reduced) of mitochondrial membranes from the parental *A. niger* strain (upper spectrum) and the *A. niger* mutant *nuo*51 (lower spectrum). Cluster N-2 has a positive peak at $g = 2.05$ and and a negative peak at $g = 1.915$. the signal of cluster N-4 is at $g_z = 2.10$ and that of cluster N-1 at $g = 2.03$. Due to a strong signal overlapping arising from FeS-clusters of other proteins we cannot interprete the region of g_x absorptions of clusters N-3 and N-4.

cases the alternative NADH : ubiquinone oxidoreductase takes charge of the defective electron transfer function of complex I. A recent observation might give the answer. The peripheral arm of complex I, independent of its association with the membrane arm, bears an acyl-carrier protein with a bound phosphopantetheine group. This mitochondrial ACP is probably a component of a not yet defined mitochondrial synthetic pathway possibly providing special kinds of mitochondrial lipids. The peripheral arm of complex I is, therefore, bifunctional, participating in electron transfer and in this mitochondrial biosynthetic pathway [13].

The *E. coli* operon encoding the NADH : ubiquinone oxidoreductase

E. coli contains a proton-pumping NADH : ubiquinone oxidoreductase, called NDH I, and a non proton-pumping alternative enzyme called NDH II. NDH I contains FMN and at least 3 FeS-clusters and is supposed to be related to the mitochondrial complex I [14]. NDH II is a single polypeptide FAD-enzyme related to that of fungi [1,11,15]. Unlike the complex I homologue in *Paracoccus denitrificans* [16], the *E. coli*

NDH I appears to have no high potential tetranuclear FeS-cluster like the mitochondrial N-2. This could indicate a lower complexity of the membrane part of the *E. coli* NDH I compared to the membrane arm of complex I. Unfortunately, owing to its fragile nature, the *E. coli* NDH I has not yet been isolated.

We are currently analysing the genes of the *E. coli* NDH I operon. The operon was identified with *E. coli*-specific DNA probes made by PCR. The degenerative primers were derived from consensus sequences of the NAD(H) binding subunit of complex I and the *A. eutrophus* NAD-reducing hydrogenase [5–7]. We have so far identified genes of 6 proteins related to the following complex I subunits: the 51 kDa subunit with its characteristic binding motives for NADH, FMN and one FeS-cluster [5–7], the 75 kDa subunit with the binding sites for two FeS clusters [5–7], the 24 kDa subunit and the 49 kDa subunit, which are all four peripheral subunits, and two subunits of the membrane part of complex I, a nuclear-encoded 23 kDa subunit and the subunit encoded by the mitochondrial ND2 gene [1] (Fig. 3).

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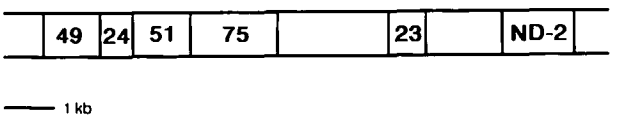

Fig. 3. Gene organisation of the the *E. coli* NDH I operon (see text).

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