***In organello* assembly of respiratory-chain complex I: primary structure of the 14.8 kDa subunit of *Neurospora crassa* complex I**

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A cDNA encoding the 14.8 kDa subunit of complex I from *Neurospora crassa* was cloned and sequenced. The deduced primary structure of this subunit reveals a predominantly hydrophilic protein containing no obvious membrane-spanning domain. In agreement with this characteristic, we have localized the 14.8 kDa subunit in the peripheral arm of the enzyme. The 14.8 kDa subunit was found to be conserved in mammalian complex I. The conservation of this subunit in such distantly

related organisms suggests that the 14.8 kDa subunit is an important component of complex I. We have used an *in organello* system to study the biosynthetic pathway of this subunit. The 14.8 kDa polypeptide could be efficiently imported into isolated mitochondria. Furthermore, a fraction of the *in-vitro*-imported subunit was found to assemble in complex I. This is the first time that assembly in complex I of an *in-vitro*-synthesized subunit is demonstrated.

INTRODUCTION

Complex I (NADH:ubiquinone oxidoreductase) is the most intricate enzyme of the mitochondrial respiratory chain. It comprises some 30–40 subunits, seven of which are encoded and synthesized in mitochondria. The enzyme is known to catalyse the transfer of electrons from NADH to ubiquinone, coupling the energy of this redox reaction to the extrusion of protons across the mitochondrial inner membrane [for reviews, see Ragan (1987), Walker (1992) and Weiss et al. (1991)]. In addition, the recent finding of an acyl-carrier protein among complex I subunits (Runswick et al., 1991; Sackmann et al., 1991) suggests the existence of other, still unknown, biochemical functions in this multimeric enzyme.

Structurally, complex I from *Neurospora crassa* has the form of an L-shaped rod with one arm embedded in the mitochondrial inner membrane (the hydrophobic arm) and the other (the peripheral arm) protruding into the matrix (Friedrich et al., 1989; Hofhaus et al., 1991; Tuschen et al., 1990). In terms of polypeptide composition, the hydrophobic arm contains all the mitochondrially encoded subunits and about 15 nuclear-coded subunits; the peripheral arm comprises about 13 subunits and contains the majority of the iron–sulphur clusters of the enzyme (Wang et al., 1991).

One major goal of our work is the understanding of the biogenesis of complex I. Presently, all the information available concerning the assembly of the enzyme was obtained from pulse-labelling experiments performed in intact *Neurospora* cells. Using this method, an assembly intermediate of complex I corresponding to the membrane arm of the enzyme could be detected (Tuschen et al., 1990). Although elegant, this approach has an obvious drawback: the number of different factors affecting the assembly process that can be experimentally changed is quite limited. In an attempt to cope with this problem we have been trying to reproduce the biosynthetic pathway of some complex I subunits using an *in organello* system. Recently, we have shown that the 20.9 kDa subunit of *Neurospora* complex I could be

imported into isolated mitochondria and correctly sorted to the inner membrane (Azevedo et al., 1992). Similar results were obtained with another subunit belonging to the membrane arm of the enzyme: upon mitochondrial *in vitro* import, a fraction of the 17.8 kDa subunit was found correctly assembled in the inner membrane (Azevedo et al., 1993). Here we present further studies on the structure and biogenesis of complex I. We have cloned and sequenced a cDNA encoding the 14.8 kDa subunit of *Neurospora crassa* complex I. This subunit was synthesized *in vitro* and used in mitochondrial *in vitro* import experiments. Our results show, for the first time, full assembly of an *in-vitro*-synthesized complex I subunit using an *in organello* system.

MATERIALS AND METHODS

The following procedures were employed according to the references cited: growth of *N. crassa* (wild-type OR74A) (Werner, 1977); preparation of complex I (Wang et al., 1991); isolation of mitochondria for *in vitro* import experiments (Rassow et al., 1989); determination of protein concentration (Bradford, 1976); sucrose gradient centrifugation of detergent-solubilized mitochondrial proteins and determination of NADH:[Fe(CN)₆]³⁻ oxidoreductase activity (Tuschen et al., 1990; Friedrich et al., 1989); PAGE (Laemmli, 1970; Videira and Werner, 1989); Western-blotting (Towbin et al., 1979); synthesis of proteins in rabbit reticulocyte lysate (Amersham International, Amersham, Bucks., U.K.) (Pelham and Jackson, 1976) in the presence of [³⁵S]methionine (sp. radioactivity 1000 Ci/mmol; Amersham International) by coupled transcription/translation (Stueber et al., 1984; Hartl et al., 1986); and automated Edman degradation (Eckerskorn et al., 1988).

The small form of complex I (Friedrich et al., 1989) was kindly provided by Dr. Hans Weiss, University of Dusseldorf, Dusseldorf, Germany.

The antibody against the 14.8 kDa subunit of complex I was prepared as follows: 6 mg of complex I were subjected to SDS/PAGE (a 22 cm long gel was used to achieve a good

Abbreviations used: $\Delta\Psi$, mitochondrial inner membrane potential; NUO-x, NADH:ubiquinone subunit of x kDa; PMSF, phenylmethanesulphonyl fluoride.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X76344.

separation of complex I subunits); the proteins in the gel were blotted on to nitrocellulose and stained with Ponceau S; the band corresponding to the 14.8 kDa subunit was cut, destained in deionized water, dried and dissolved in 1.5 ml of dimethyl sulphoxide (see Harlow and Lane, 1988); this solution was emulsified with an equal volume of mineral oil, and 1 ml aliquots of this emulsion were injected into rabbits as previously described (Videira and Werner, 1989). A mono-specific antiserum was obtained 40 days after the first injection.

The screening of a λ gt11 cDNA expression library, subcloning and sequencing cDNA inserts was performed essentially as described (Maniatis et al., 1982; Videira et al., 1990c; Heinrich et al., 1992). Only one positive phage (out of 2×10^5 phages screened) was obtained. After purification of this clone by two cycles of re-screening, DNA was prepared (Maniatis et al., 1982) and digested with the restriction enzyme *Eco*RI. Analysis of the restriction pattern (results not shown) indicated the existence of an *Eco*RI site within the cDNA insert. Therefore isolation of the full-length cDNA insert was accomplished by partial digestion of the phage DNA with the enzyme *Eco*RI. The full-length cDNA, as well as the two cDNA fragments obtained by complete digestion of the phage DNA with *Eco*RI, were subcloned in the plasmid pGEM4 (Promega) and sequenced.

The mitochondrial *in-vitro*-import experiments (usually 50 μ g of mitochondrial protein in a 100 μ l reaction mixture) were performed in import buffer, which contained 2 mM NADH, 5 mM $MgCl_2$, 250 mM sucrose, 2% (w/v) lipid-free BSA, 80 mM KCl, 10 mM Mops/KOH, pH 7.2, and 10% (v/v) of 'untranslated' reticulocyte lysate (see Rassow et al., 1989), which contains an ATP-regeneration system (10 mM creatine phosphate and 22 units/ml creatine kinase). Proteinase treatment of import reactions and addition of inhibitors were performed exactly as described recently (Azevedo et al., 1993).

The immunoprecipitation experiments were performed as follows. Mitochondria (generally 50 μ g of protein) were resuspended in 1 ml of Triton buffer [1% (w/v) Triton X-100/0.3 M NaCl/5 mM EDTA/20 mM Tris/HCl pH 7.0 and 1 mM phenylmethane sulphonyl fluoride (PMSF) freshly added from a 200 mM stock solution in ethanol] and incubated on ice for 10 min. The samples were subjected to a clarifying spin (15000 g, 10 min) to remove insoluble material; 5 mg of Protein A-Sepharose preincubated for 1 h with 10 μ l of the relevant antisera in 500 μ l of Triton buffer were then added to each sample. The suspension was gently rocked for 2–3 h at 4 °C. The Protein A-Sepharose beads were centrifuged (1 min at 10000 g), washed four times with 1 ml of Triton buffer and, finally, with 1 ml of 20 mM Tris/HCl, pH 7.0. The immunoprecipitated proteins were denatured in Laemmli (1970) sample buffer and subjected to SDS/PAGE.

RESULTS AND DISCUSSION

Sequence analysis and localization of the 14.8 kDa subunit of complex I

The cDNA encoding the 14.8 kDa subunit of *Neurospora* complex I (NUO-14.8) was isolated by immunoscreening of a λ gt11 expression library. The complete sequence of this clone is shown in Figure 1. The cDNA is 515 bp long and contains an open reading frame encoding a polypeptide with a calculated molecular mass of 14814 Da. Residues 2–17 of the deduced protein sequence match completely the amino acid sequence obtained by automated Edman degradation of the purified complex I subunit, indicating that indeed the correct clone was isolated.

The codon for the N-terminal residue of the mature subunit is

immediately preceded by ATG. This triplet most likely represents the initiator methionine, since the DNA sequence surrounding this ATG is in good agreement with the optimum translation initiation sequence of *N. crassa* (Paluh et al., 1988) and, most importantly, the polypeptide encoded by this cDNA has all the information necessary to be efficiently imported into isolated mitochondria (see below). These data also imply that the 14.8 kDa subunit is synthesized without a cleavable mitochondrial targeting sequence.

In an attempt to get some information concerning the function of NUO-14.8 we have compared its deduced primary structure with proteins of known sequence. A high similarity (53%) was found with a recently described subunit of bovine complex I, the B14 polypeptide (Walker et al., 1992; see Figure 2). The conservation of this subunit in such distantly related organisms suggests that NUO-14.8 is an important component of complex I, although its function in the enzyme cannot yet be ascertained. [It is worth mentioning that almost one-third of the nuclear-encoded subunits of *N. crassa* complex I sequenced to date (five subunits out of 18) do not have mammalian counterparts.] This observation, however, may not be valid for complex I from prokaryotic organisms: no homologue of NUO-14.8 has so far been detected in operons encoding complex I or related enzymes (Böhm et al., 1990; Tran-Betcke et al., 1990; Yagi et al., 1992). It is possible that NUO-14.8 is exclusively found in mitochondrial

Figure 1 Nucleotide sequence and deduced protein sequence of the cDNA encoding NUO-14.8

(a) The amino acid sequence obtained by automated Edman degradation of the isolated complex I subunit is underlined. The stop codon is marked by an asterisk. (b) Sequencing strategy and structure of the cloned cDNA. The sequence was obtained from the full-length cDNA and from two subclones derived from digests with the restriction enzyme *Eco*RI (E). Horizontal arrows show the direction and extent of sequence determination. The open reading frame is boxed. (The two arrows in the 3'–5' direction of the cDNA at the bottom left-hand side of (b) do not represent the sequencing of two identical fragments; they represent two independent sequence determinations of the same region.)

NUO-14.8 (1)	MPITPTKYAITTRQSANWSDAKRRVFALYR
	! ! ! ! ! ! * ! !*****
B14 (1)	MAASGLRQAATASTSVKPIFSRDMNEAKRRVRELYR
NUO-14.8	RWLRSTPEMQSMYSLPLPISV--IRTRIRQEFERNRF
	* * * ! ! ! * * * * ! ! * ! *
B14	AWYREVPNTVHLFQ--LDISVKQGRDKVREMFKKNAH
NUO-14.8	VNKLPPVDVLLTKGHADYQETMNFWRQTTHMSYFNE
	! ***! * ! ! ! ! ! * ! * * ! * ! *
B14	ITDPRVVDLLVIKGMEEETIKVWKQRTTHVMRFHE
NUO-14.8	ESFRGAKRLPSSFDIGFLQGRN (124)
	! * * * *
B14	TEAPRPKDFLSKFYVGHDP (128)

Figure 2 Alignment of the protein sequences of NUO-14.8 and the B14 polypeptide from bovine complex I

Amino acid identities (*) and conservative substitutions (!) are indicated. Gaps were introduced to optimize the alignment. The sequence of the B14 protein is taken from Walker et al. (1992).

complex I. The absence of a cleavable targeting sequence in NUO-14.8 (and its mammalian counterpart) could also favour this idea. According to the endosymbiotic theory for the origin of mitochondria, mitochondrial nuclear-encoded proteins originally encoded in the genome of the ancestral organelle have acquired presequences in order to enable them to be targeted back to mitochondria (for a review, see Hartl et al., 1989). Although there are some exceptions to this rule (e.g., cytochrome *c* haem lyase and cytochrome *c*), it is noteworthy that all nuclear-encoded complex I subunits having homologues in prokaryotes have a cleavable targeting sequence. Homologues of nuclear-encoded subunits lacking a presequence have never been found in prokaryotes.

NUO-14.8 is a component of the so-called 'small' form of complex I (see Figure 3), a fragment of the enzyme which is made in *Neurospora* cells grown in the presence of chloramphenicol (Friedrich et al., 1989). This functional/structural unit of complex I is identical with the peripheral arm of the enzyme, a part of complex I which is constituted by a small membrane anchor and a globular domain protruding into the mitochondrial matrix (Hofhaus et al., 1991). Taking into consideration the absence of a membrane-spanning domain in NUO-14.8, it is quite likely that this subunit belongs to the globular part of the peripheral arm, and so that it exists in close proximity to components such as the 78.2, 51.4 and 30.4 kDa subunits of complex I (for the polypeptide composition of the peripheral arm see Weiss et al., 1991).

Mitochondrial import *in vitro* of NUO-14.8

The 14.8 kDa subunit was synthesized *in vitro* in the presence of [³⁵S]methionine by coupled transcription-translation. The radio-labelled polypeptide was then incubated with freshly isolated *Neurospora* mitochondria in either the presence or absence of a membrane potential ($\Delta\Psi$). After re-isolation of the organelles, aliquots from the two import reactions were treated with various amounts of proteinase K and analysed by SDS/PAGE. The integrity of the mitochondrial outer membrane was monitored by the inaccessibility to the proteinase of cytochrome *c* haem lyase: an intermembrane-space protein highly sensitive to proteinase K (Lill et al., 1992).

As shown in Figure 4(a), when the ³⁵S-labelled polypeptide is incubated with energized mitochondria, about 60% of the

Figure 3 NUO-14.8 is a component of the small form of complex I

The subunits of complex I (25 μ g/lane; lanes 1 and 3) and of the small form of complex I (15 μ g/lane; lanes 2 and 4) were resolved by SDS/PAGE and blotted on to nitrocellulose. Lanes 1 and 2 were stained with Amido Black; the nitrocellulose strip containing lanes 3 and 4 was immunodecorated with antisera directed to the 14.8 kDa subunit. The positions of some complex I subunits of known primary structure are also marked: 78.2 kDa and 51.4 kDa (Preis et al., 1991); 30.4 kDa, 21.3 kDa, 20.8 kDa and 12.3 kDa (Videira et al., 1990a,b,c, 1993); 20.9 kDa and 17.8 kDa (Azevedo et al., 1992, 1993), and the 9.3 kDa subunit (Heinrich et al., 1992).

material which co-sedimented with mitochondria (lane 1, $+\Delta\Psi$) became proteinase-protected over a wide concentration range of proteinase K (lanes 1–6, $+\Delta\Psi$). If the mitochondria were solubilized with a mild detergent before the proteinase treatment, NUO-14.8 was almost completely degraded (lane 7, $+\Delta\Psi$).

In the absence of a membrane potential the amount of radiolabelled NUO-14.8 associated with mitochondria (lane 1, $-\Delta\Psi$) is approximately the same as the one obtained in the presence of a $\Delta\Psi$. However, 80% of the bound material is readily degraded at the lowest proteinase concentration used (lane 2, $-\Delta\Psi$). The remaining proteinase-resistant subunit does not reflect imported material since it can be further degraded by increasing the proteinase concentration (lanes 3–6, $-\Delta\Psi$). It should be mentioned that the proteinase K concentrations used in this experiment exceed at least 10-fold the concentration of protease required to degrade more than 95% of the labelled polypeptide under the same experimental conditions, but in the absence of mitochondria (results not shown). Thus the observed increased resistance of NUO-14.8 to proteolysis most likely arises from a $\Delta\Psi$ -independent interaction of the subunit with the mitochondrial outer membrane.

In summary, these results indicate that the import of NUO-14.8 into mitochondria is dependent on the existence of a potential across the mitochondrial inner membrane.

The import of most mitochondrial precursor proteins is mediated by proteinaceous components located on the mitochondrial outer membrane [for a review, see Pfanner and Neupert (1990)]. By subjecting mitochondria to a mild proteinase treatment, part of this import machinery can be disabled, leading to a block in the import of those precursor proteins (Pfanner et al., 1988). There is, however, a class of mitochondrial proteins that

Figure 4 Mitochondrial *in vitro* import of NUO-14.8

(a) [^{35}S]Methionine-labelled NUO-14.8 was incubated with freshly isolated mitochondria (350 μg of protein) either in the presence ($-\Delta\Psi$) or absence ($+\Delta\Psi$) of 1 μM valinomycin. At the end of the incubation (20 min at 25 $^\circ\text{C}$) the sample ' $+\Delta\Psi$ ' received the same amount of valinomycin. The organelles from the two samples were re-isolated by centrifugation (10 min, 12000 g) washed with SEM buffer (250 mM sucrose/1 mM EDTA/10 mM Mops/KOH, pH 7.2) and gently resuspended in the same buffer. Aliquots (50 μg of protein) were then treated with the indicated amounts of proteinase K, but the organelles in lane 7 were first dissolved with 2% Triton X-100 (TX-100) prior to the treatment. After 25 min at 0 $^\circ\text{C}$, proteinase K was inactivated by addition of 1 mM phenylmethanesulphonyl fluoride (PMSF) and the protein in all the aliquots was precipitated with trichloroacetic acid (10% final concn.) dissolved in Laemmli sample buffer and subjected to SDS/PAGE. The protein in the gel was then blotted on to nitrocellulose and the membrane was first used to expose an X-ray film (to detect the *in-vitro*-imported subunit) and afterwards probed with an antiserum directed to cytochrome *c* haem lyase (CCHL). (b) Freshly isolated *Neurospora* mitochondria [50 μg of protein in SEM buffer (see above)] were incubated for 15 min at 0 $^\circ\text{C}$ in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 30 $\mu\text{g/ml}$ trypsin. After addition of soybean trypsin inhibitor (1 mg/ml final concentration), the organelles were reisolated, washed and resuspended in import buffer (see the Materials and methods section) containing the [^{35}S]-labelled precursors of NUO-14.8 and the β -subunit of F_1 ATPase ($F_1\beta$). Import was allowed to proceed for 6 min at 25 $^\circ\text{C}$. The import reactions were then stopped by adding valinomycin to 1 μM . The samples applied to lanes 2 and 4 were treated with proteinase K (PK; 100 $\mu\text{g/ml}$ final concn.) as described above. Finally, the samples were analysed by SDS/PAGE. A fluorograph of the dried gel is shown. P and M indicate the precursor and mature form of the β -subunit of F_1 -ATPase respectively.

apparently do not need these proteinase-accessible components for an efficient import *in vitro* (Nicholson et al., 1988; Miller and Cumsky, 1991). In order to determine to which class NUO-14.8

Figure 5 *In-vitro*-imported NUO-14.8 undergoes assembly

^{35}S -labelled NUO-14.8 was incubated with freshly isolated mitochondria (50 μg protein) either in the presence ($+\Delta\Psi$) or absence ($-\Delta\Psi$) of a membrane potential, as indicated. After 20 min at 25 $^\circ\text{C}$, the samples ' $+\Delta\Psi$ ' received valinomycin to 1 μM to stop further import. Then the samples in lanes 2, 4, 6, 8, 10 and 12 were treated with proteinase K (+PK; 100 $\mu\text{g/ml}$ final concentration). After inactivation of the proteinase, mitochondria in all the samples were re-isolated and washed with SEM buffer (for details see legend to Figure 4). The organelles were then either resuspended in 1 ml of Triton (TX-100) buffer (see the Materials and methods section) containing 0.08% SDS (lanes 5–12) or first incubated in 30 μl of a denaturing solution (2.7% SDS/300 mM NaCl/5 mM EDTA/20 mM Tris/HCl, pH 7.2) for 10 min at 50 $^\circ\text{C}$ to dissociate complex I subunits and, afterwards, diluted with 970 μl of Triton buffer (lanes 1–4). The samples were then subjected to immunoprecipitation (see the Materials and methods section for details) using either an antiserum that is able to immunoprecipitate complex I (As-22a; lanes 1–8) or a pre-immune serum (PI; lanes 9–12). A fluorograph of the dried SDS/PAGE gel is shown.

belongs, freshly isolated *Neurospora* mitochondria were pre-treated with a low concentration of trypsin (30 $\mu\text{g/ml}$) and subjected to an import experiment in the presence of a membrane potential. The import reaction was allowed to proceed for 6 min, a time point within the linear range of import of NUO-14.8 (results not shown) and the β subunit of F_1 -ATPase ($F_1\beta$; Pfanner et al., 1987), a control used in this experiment.

As shown in Figure 4(b), treatment of mitochondria beforehand with trypsin results in a significant decrease of the total amount of imported (proteinase-protected) NUO-14.8. Thus these data suggest that the import of NUO-14.8 is dependent on proteinaceous components of the mitochondrial outer membrane.

Assembly of *in vitro* imported NUO-14.8

As described in the previous section, NUO-14.8 can be efficiently imported into mitochondria using an *in vitro* system. This process requires the existence of a membrane potential and seems to be mediated by proteinaceous components of the outer membrane. We then tried to determine whether the *in-vitro*-imported subunit could undergo assembly in complex I. For this purpose [^{35}S]-labelled NUO-14.8 was incubated with *Neurospora* mitochondria either in the presence or absence of a membrane potential. After protease treatment, as specified (see Figure 5), the organelles were reisolated and solubilized with Triton buffer (see the legend to Figure 5 for details). Aliquots of this material were then mixed with Protein A-Sepharose beads containing an antibody (As-22a; Videira and Werner, 1989) which is able to immunoprecipitate whole complex I but which does not cross-react with the 14.8 kDa subunit. As shown in Figure 5, *in-vitro*-synthesized NUO-14.8 can be co-immunoprecipitated by As-22a only when energized mitochondria are used (Figure 5, lanes 5 and 6). In the absence of a $\Delta\Psi$ (lanes 7 and 8), no radiolabelled subunit is detected, indicating that the antibody used does indeed not recognize NUO-14.8 *per se*. [This conclusion is further supported by the

Figure 6 *In-vitro*-imported NUO-14.8 is found in fully assembled complex I

A 2 mg portion of *Neurospora* mitochondria were incubated with ^{35}S -labelled NUO-14.8 for 20 min at 25 °C either in the presence (+ $\Delta\Psi$) or absence ($-\Delta\Psi$) of a membrane potential, as indicated. At the end of the incubation the sample '+ $\Delta\Psi$ ' received valinomycin (1 μM final concentration) and was subjected to proteinase K treatment (100 $\mu\text{g}/\text{ml}$ final concentration) to remove non-imported NUO-14.8. The organelles from both samples were then re-isolated, washed with SEM buffer (as detailed in the legend to Figure 4) and resuspended in 500 μl of 2% Triton X-100 in 0.1 M sodium phosphate (pH 7.0)/1 mM PMSF (see the Materials and methods section). After a centrifugation step (15 000 g ; 15 min) to remove insoluble material, the samples were applied to the top of sucrose gradients (7.5–25% sucrose/0.05% Triton X-100/50 mM Tris/acetic acid, pH 7.5) and centrifuged at 36 000 g for 16 h at 2 °C. The gradients were fractionated into 12 fractions (approx. 900 μl each). The distribution of the radioactive material in the gradients (●) was assessed by liquid-scintillation counting of 10 μl aliquots from each fraction. The $\text{NADH}:[\text{Fe}(\text{CN})_6]^{3-}$ oxidoreductase activity (indicated by open circles) was measured on 50 μl aliquots from the fractions. The remaining portions of the fractions were subjected to immunoprecipitation using the antiserum As-22a. The immunoprecipitated proteins were then resuspended in Laemmli sample buffer and analysed by SDS/PAGE. The gel was blotted on to nitrocellulose and the membrane was first used to expose an X-ray film (to detect the *in-vitro*-imported subunit) and, afterwards, probed with monospecific antisera directed to the 14.8 and 20.8 kDa subunits of complex I.

observation that no ^{35}S -labelled material is precipitated when the mitochondrial proteins are incubated in the presence of 2% SDS (to dissociate complex I subunits) prior to the immunoprecipitation procedure (lanes 1–4.) The fact that no signal is detected in the absence of a $\Delta\Psi$ also indicates that the observed co-immunoprecipitation arises from an authentic assembly of NUO-14.8 during the import reaction and not from an association of the subunit with complex I (or some of its components) after the solubilization step of mitochondria.

The antibody used in this experiment recognizes two subunits of complex I: the 20.8 kDa (Videira et al., 1990c) and the 29.9 kDa subunits (Van der Pas et al., 1991). Thus it cannot be inferred from the results presented above at what stage(s) of assembly is the *in-vitro*-imported subunit (full assembly of NUO-14.8 into complex I or association of the radiolabelled polypeptide with just one of the subunits recognized by the antibody would lead to the same experimental result). In order to clarify this point, the following experiment was performed. ^{35}S -labelled NUO-14.8 was incubated with *Neurospora* mitochondria in the presence or absence (as a negative control) of a $\Delta\Psi$. After re-isolation of the mitochondria, the samples were solubilized with Triton X-100 and subjected to sucrose-gradient centrifugation. The conditions of the centrifugation were such that complex I sedimented through two thirds of the gradient, well separated from proteins with a molecular mass lower than 50 kDa, which remained at the top of the gradient (Tuschen et al., 1990; results not shown). Aliquots of the gradients were then analysed for complex I activity (measured as $\text{NADH}:[\text{Fe}(\text{CN})_6]^{3-}$ oxidoreductase activity) and the distribution of ^{35}S -labelled material was monitored. As shown in the plot of Figure 6, complex I activity is almost completely recovered in fraction 10 of the gradients. As expected, in the absence of a $\Delta\Psi$ all the ^{35}S -labelled material is found at the top of the gradients (fractions 1–3),

representing non-assembled NUO-14.8. In the presence of a $\Delta\Psi$ the majority of the *in-vitro*-imported subunit is also detected in this region of the gradient. However, a small amount of radioactive material is visible in the fraction enriched in complex I activity (fraction 10; + $\Delta\Psi$), suggesting that assembly of NUO-14.8 into complex I has occurred.

In order to prove unambiguously that the observed radio-labelled material co-sedimenting with complex I represents NUO-14.8 fully assembled in the enzyme (and not just some fortuitous $\Delta\Psi$ -dependent aggregation of the radioactively labelled subunit), all the fractions from the two gradients were subjected to immunoprecipitation using the antibody mentioned above. The proteins in the immunoprecipitates were then analysed by SDS/PAGE, fluorography and probed with monospecific antibodies directed to the 14.8 and 20.8 kDa subunits of complex I. As shown in Figure 6, both the distribution of the enzymic activity and the profile of the radioactively labelled protein in the gradients correlate well with the pattern of 'bands' obtained in the Western blot and fluorograph. This result indicates that the *in-vitro*-imported NUO-14.8 co-sedimenting with complex I is indeed assembled in the enzyme.

Finally, it is worth mentioning that no intermediates of assembly containing the radiolabelled subunit are detected in this experiment: the *in-vitro*-imported NUO-14.8 could only be found in the low-molecular-mass region of the gradients and in the fractions containing fully assembled complex I. There may be two reasons for this behaviour: either the biosynthetic pathway of this subunit is too fast to allow the detection of assembly intermediates under the experimental conditions applied (20 min of import at 25 °C) or NUO-14.8 may be one of the last components to be assembled into the enzyme. This and other questions can now be easily addressed, since an appropriate *in organello* system has been developed.

In the present paper we have described and characterized in terms of localization and biosynthetic pathway the 14.8 kDa subunit of *N. crassa* complex I. Our data suggest that NUO-14.8, the fungal counterpart of the bovine B14 subunit (Walker et al., 1992), is a component of the peripheral arm of the enzyme (Hofhaus et al., 1991). This complex I subunit is synthesized without a cleavable targeting sequence and requires a $\Delta\Psi$ to be imported into mitochondria. In addition, import of NUO-14.8 seems to be mediated by proteinaceous components located in the outer membrane. However, the most important aspect of the work presented here arises from the co-immunoprecipitation and sedimentation experiments. For the first time it has been shown that the assembly process of a complex I subunit can be reproduced using an *in organello* system, opening new perspectives for the study of complex I biogenesis.

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