Cloning, *in vitro* mitochondrial import and membrane assembly of the 17.8 kDa subunit of complex I from *Neurospora crassa*

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We have cloned and sequenced a cDNA encoding a 17.8 kDa subunit of the hydrophobic fragment of complex I from *Neurospora crassa*. The deduced primary structure of this subunit was partially confirmed by automated Edman degradation of the isolated polypeptide. The sequence data obtained indicate that the 17.8 kDa subunit is made as an extended precursor of 20.8 kDa. Resistance of the polypeptide to alkaline extraction from mitochondrial membranes and the existence of a putative membrane-spanning domain suggests that the 17.8 kDa subunit

is an intrinsic (bitopic) membrane protein. The *in vitro* synthesized precursor of the 17.8 kDa subunit can be efficiently imported into isolated mitochondria, where it is cleaved to the mature species by the metal-dependent matrix-processing peptidase. The *in vitro* imported mature subunit is found mainly exposed to the mitochondrial intermembrane space. However, a significant fraction of the imported polypeptide acquires the same membrane topology as the endogenous subunit, indicating that correct assembly in the mitochondrial inner membrane did occur.

INTRODUCTION

The rotenone-sensitive NADH:ubiquinone oxidoreductase of the mitochondrial respiratory chain (complex I; EC 1.6.99.3) is a complicated assembly of about 34 nuclear-encoded subunits (at least 25 in *Neurospora crassa*), seven mitochondrially encoded subunits, several iron–sulphur clusters, one FMN and, possibly, an internal ubiquinone. The enzyme catalyses the transfer of electrons from NADH to ubiquinone and couples the energy of this redox reaction to the extrusion of protons across the mitochondrial inner membrane (for reviews see Ragan, 1987; Weiss et al., 1991; Walker, 1992).

Recently, electron microscopy studies have suggested that *Neurospora crassa* complex I has the form of an L-shaped rod with one arm embedded in the membrane (the hydrophobic arm) and the other (the peripheral arm) protruding into the mitochondrial matrix (Hofhaus et al., 1991). Interestingly, the two arms of the enzyme could be assigned to the two complementary subcomplexes previously characterized by Weiss and coworkers. Thus the hydrophobic arm seems to correspond to the hydrophobic fragment (Friedrich et al., 1989), a fraction of the enzyme obtained by chaotropic salt treatment of the isolated complex that comprises all mitochondrially encoded subunits, about 12 nuclear-encoded subunits, one iron–sulphur cluster (Weiss et al., 1991) and the ubiquinone binding site (Heinrich et al., 1992). The peripheral arm was shown to be similar to the so-called small form of complex I (Tuschen et al., 1990), an assembly of 13 nuclear-encoded subunits containing the NADH binding site and three Fe–S clusters (Wang et al., 1991), which is made in *Neurospora* cells poisoned with chloramphenicol.

Although knowledge about complex I is increasing rapidly, the mechanisms of catalysis, assembly and the fine structure of the enzyme are far from being understood. Moreover, the existence of other biochemical activities in this multimeric enzyme is plausible. Indeed, the finding of an active acyl carrier protein among complex I subunits (Runswick et al., 1991; Sackmann et al., 1991) may suggest additional, still unknown, functions.

As an attempt to elucidate some of these aspects, we have recently cloned several cDNAs coding for subunits of *Neurospora* complex I (Videira et al., 1990a,b,c, 1993; Preis et al., 1991; Azevedo et al., 1992; Heinrich et al., 1992). Here the cloning and sequencing of a 17.8 kDa subunit belonging to the hydrophobic fragment of the enzyme are described. Using an *in vitro* mitochondrial import system, the first steps of the biosynthetic pathway of this subunit could be followed and characterized.

MATERIALS AND METHODS

The following procedures were employed according to the references cited: growth of *Neurospora crassa* (wild-type OR74A) (Werner, 1977); preparation of complex I (Ise et al., 1985; Filser and Werner, 1988); isolation of mitochondria for *in vitro* import experiments (Rassow et al., 1989); SDS/PAGE (Laemmli, 1970; Videira and Werner, 1989); Western blotting (Towbin et al., 1979) and detection of horseradish peroxidase- or alkaline phosphatase-conjugated second antibodies on Western blots (Roswell and White, 1978; Blake et al., 1984); automated Edman degradation of proteins blotted on to glass fibre membranes (Eckerskorn et al., 1988); synthesis of proteins in rabbit reticulocyte lysate (Pelham and Jackson, 1976) in the presence of [³⁵S]methionine (specific radioactivity 1000 Ci/mmol; Amersham) by coupled transcription/translation (Stueber et al., 1984; Hartl et al., 1986); preparation of reticulocyte lysate to supplement import reactions (Rassow et al., 1989); determination of protein concentration (Bradford, 1976); alkaline extraction (Fujiki et al., 1982); and salt extraction of mitochondria (Hartl et al., 1986; Azevedo et al., 1992).

The strategy used for screening of a λ gt11 cDNA expression library, subcloning and sequencing of cDNA inserts has been described previously (Videira et al., 1990b).

The import assays were carried out essentially as outlined in a previous paper (Azevedo et al., 1992). Usually, 50–100 μ g of freshly prepared *Neurospora* mitochondria were added to 90 μ l of import buffer, consisting of 2 mM NADH, 5 mM MgCl₂, 70 %

Abbreviations used: MPP, mitochondrial matrix-processing peptidase; NUO-X, NADH:ubiquinone oxidoreductase subunit of X kDa; $\Delta\Psi$, membrane potential across the mitochondrial inner membrane; CCHL, cytochrome c haem lyase; mtHSP70, mitochondrial 70 kDa heat shock protein.

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(v/v) BSA buffer [250 mM sucrose, 3% (w/v) lipid-free BSA, 80 mM KCl, 10 mM Mops/KOH, pH 7.2] and 10% (v/v) untranslocated reticulocyte lysate (Rassow et al., 1989) which contains an ATP regeneration system (10 mM phosphocreatine and 22 units/ml creatine kinase). The import reaction was started by addition of 1 μ l of reticulocyte lysate containing the 35 S-labelled precursor of the 17.8 kDa subunit. The suspension was incubated at 25 °C for 20 min. The membrane potential ($\Delta\Psi$) was dissipated with the K⁺ ionophore valinomycin (1 μ M final concentration) added from a 100-fold concentrated stock solution in ethanol. The control reactions (in the presence of $\Delta\Psi$) received the same volume of ethanol. Proteinase treatment was carried out at 0 °C for 30 min with proteinase K (50 μ g/ml) or trypsin (30 μ g/ml). In the majority of the experiments the use of trypsin was avoided because of the fact that digestion of the precursor protein with this proteinase results in a 15.5 kDa fragment that remains associated with the mitochondrial outer membrane. The proteinases were inactivated by an incubation step at 0 °C for 5 min in the presence of 1 mM phenylmethanesulphonyl fluoride (for proteinase K), or a 30-fold excess (by wt.) of soy bean trypsin inhibitor if trypsin was used. Mitochondria were re-isolated from the import reactions by centrifugation (10 min at 15000 g), resuspended in 200 μ l of SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM Mops/KOH, pH 7.2), centrifuged again under the same conditions and then either processed for SDS/PAGE analysis or used for the salt and alkaline extractions.

The mitochondrial matrix-processing peptidase (MPP) was inhibited as reported (Hartl et al., 1986) with the following modifications: 200 μ g of *Neurospora* mitochondria were pre-incubated for 5 min at 0 °C in import buffer containing 12 mM EDTA. *o*-Phenanthroline (20 mM in ethanol) was then added to a concentration of 0.2 mM. After 2 min at 0 °C, the import reaction was started as described above and the suspension was incubated for 10 min at 25 °C. The sample was divided into halves and the mitochondria were re-isolated. One half was resuspended in import buffer containing the above-mentioned additions to maintain the inhibition of MPP. The other half was resuspended in import buffer containing 1 mM MnCl₂ and 1 μ M valinomycin in order to chase the already imported but still unprocessed 17.8 kDa subunit. Both samples were incubated for 10 min at 25 °C and treated with proteinase. The mitochondria were then re-isolated, washed and analysed by SDS/PAGE.

For the digitonin fractionation (Schnaitmann and Greenawald, 1968; Glick et al., 1992), 2.2 mg of mitochondria was subjected to an import experiment in the presence of a membrane potential. An aliquot corresponding to 200 μ g of mitochondrial protein was removed and mock-treated throughout the procedure. The other fraction (2.0 mg of protein) was treated with trypsin (30 μ g/ml) to remove non-imported precursor. After inactivation of the proteinase, the suspension was diluted 3-fold with SEM buffer containing 100 mM KCl (SEMK buffer) and centrifuged for 10 min at 15000 g. The mitochondria were then resuspended in SEMK buffer at a protein concentration of 9.2 mg/ml. An aliquot corresponding to 200 μ g of mitochondria was removed and mock-treated from this stage of the procedure. Proteinase K (1 mg/ml in SEMK buffer) was added to the remaining mitochondria to a final concentration of 220 μ g/ml. Immediately, aliquots (28 μ l) containing 200 μ g of mitochondrial protein were transferred to microtubes containing 12 μ l of various digitonin dilutions in SEMK buffer to give the final desired detergent concentrations (0–0.3%). After 1 min on ice, the suspensions were diluted with 160 μ l of SEMK buffer and incubated for a further 30 min on ice. The proteinase was inactivated and the protein in the samples was precipitated with trichloroacetic acid

[10% (w/v) final concentration], washed with cold acetone and processed for SDS/PAGE analysis.

RESULTS

Isolation and sequence analysis of cDNA clones

About 2×10^5 phages of a λ gt11 cDNA expression library were screened with a monospecific antibody prepared against the electrophoretically isolated 17.8 kDa subunit of complex I (Figure 1). Four positive phages were obtained and purified by two cycles of re-screening. The inserts were subcloned in the pGEM4 transcription vector (Promega) and sequenced. The complete sequence of the largest clone and the sequencing strategy are shown in Figure 2. The cDNA is 778 bp long and contains an open reading frame of 558 bp. Thus a 186-amino-acid protein with a calculated molecular mass of 20898 Da is obtained. The deduced protein sequence contains the 19 amino acid residues obtained by automated Edman degradation of the isolated subunit, indicating that the correct clone was indeed isolated.

The first 26 N-terminal residues of the open reading frame are not present in the mature protein. This particular domain has all the characteristics of a cleavable mitochondrial targeting sequence: an abundance of arginine and hydroxylated amino acids and the capability to form an amphipathic α -helix are well-known features of mitochondrial presequences (Hartl et al., 1989). Furthermore, the amino acid sequence around the cleavage site is in good agreement with the consensus sequence for the mitochondrial matrix peptidase (Hartl and Neupert, 1990), suggesting that the precursor protein is cleaved to a mature form of 17849 Da (see also below).

The hydropathy profile and the transfer energy plot of the mature protein (Kyte and Doolittle, 1982; Engelman et al., 1986; results not shown) revealed the existence of a single hydrophobic

Figure 1 SDS/PAGE of subunits of complex I from *Neurospora crassa*

NUO-17.8 and several subunits of previously deduced primary structures are indicated according to the molecular masses of their mature forms: 78.2 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 (Azevedo et al., 1992) and 9.3 kDa (Heinrich et al., 1992).

Figure 2 Nucleotide sequence and deduced protein sequence of the cDNA encoding the precursor of NUO-17.8

(a) The amino acid sequence obtained by automated Edman degradation of the isolated subunit is underlined. The putative membrane-spanning domain is also indicated (dotted line). The stop codon is marked by #. (b) Sequencing strategy and structure of the cloned cDNA. Horizontal arrows show the direction and extent of sequence determination. The open reading frame is boxed, and the presequence (+) and the putative membrane-spanning domain (▨) are indicated.

domain of sufficient length to cross a membrane in an α -helix conformation (Figure 2; residues 58–78).

No homologous proteins could be detected in the last release of the PIR database, leaving the function of this protein in complex I still undefined.

Mitochondrial import of *in vitro* synthesized 17.8 kDa subunit of NADH:ubiquinone oxidoreductase (NUO-17.8)

The 17.8 kDa subunit precursor (preNUO-17.8) was synthesized *in vitro* in the presence of [35 S]methionine and incubated with isolated *Neurospora* mitochondria. Import of the radiolabelled precursor was assessed by resistance of the polypeptide to externally added proteinases.

As shown in Figure 3(a), preNUO-17.8 was efficiently imported and processed to the mature form by isolated mitochondria. This process is clearly dependent on the existence of $\Delta\Psi$ (Figure 3a, lanes 1 and 2). In the absence of a $\Delta\Psi$, neither import nor processing occurred (lanes 3 and 4). However, binding of the precursor protein to the mitochondrial outer membrane was not affected (compare lane 1 with lane 3), a characteristic of the so-

Figure 3 *In vitro* mitochondrial import and processing of preNUO-17.8

(a) The 35 S-labelled precursor of NUO-17.8 subunit was incubated with freshly prepared *Neurospora* mitochondria (30 μ g of mitochondrial protein), in either the presence (lanes 1 and 2) or absence (lanes 3 and 4) of a $\Delta\Psi$. The samples in lanes 2 and 4 were treated with proteinase K (PK). After inactivation of the proteinase, the samples were analysed by SDS/PAGE and fluorography. St indicates standard, i.e. 40% of the reticulocyte lysate containing the radioactive precursor used in lanes 1–4. (b) PreNUO-17.8 is processed by MPP. 35 S-labelled preNUO-17.8 was mixed with *Neurospora* mitochondria (100 μ g of protein) in one of three different solutions: lane 1, import buffer (see the Materials and methods section) containing 1 μ M valinomycin (Valino.); lane 2, import buffer alone; lanes 3 and 4, import buffer containing 12 mM EDTA and 0.2 mM α -phenanthroline (α -Phe). After incubation for 10 min at 25 $^{\circ}$ C, the mitochondria from all samples were re-isolated by centrifugation and incubated for a further 10 min at 25 $^{\circ}$ C (second incubation) in 500 μ l of import buffer containing 1 μ M valinomycin and 1 mM MnCl $_2$ (lanes 1 and 4), import buffer alone (lane 2) or import buffer containing 12 mM EDTA and 0.2 mM α -Phe (lane 3). The samples were treated with proteinase K as specified and analysed as described above. P and M indicate precursor and mature forms respectively of the 17.8 kDa subunit.

called class I mitochondrial precursor proteins (Hartl et al., 1989).

PreNUO-17.8 is processed to the mature form by MPP. This was demonstrated by performing *in vitro* import into energized *Neurospora* mitochondria in the presence of chelating agents (Schmidt et al., 1984). Under these conditions a strong (but not complete) inhibition of the MPP was achieved. As shown in Figure 3(b), a reasonable amount (40%) of the imported (proteinase-resistant) protein remained unprocessed (Figure 3b, lane 3), although a fraction of the imported subunit had escaped the MPP blocking. [It is interesting to note the existence of a third protein species migrating slightly slower than the mature NUO-17.8. This species may represent a polypeptide chain of between one and three amino-acid residues longer than the

Figure 4 *In vitro* imported NUO-17.8 is membrane-associated

[³⁵S]Methionine-labelled preNUO-17.8 was incubated with energized mitochondria (600 µg of mitochondrial protein) at 25 °C for 20 min. After proteinase treatment and re-isolation of mitochondria, aliquots (200 µg of protein) were resuspended in SEM buffer (see the Materials and methods section) containing the indicated concentrations of NaCl. The samples were sonicated and divided into two halves; one half was kept on ice for determination of recoveries (sample T), and the other aliquot was separated into membrane (P) and soluble (S) fractions by centrifugation (1 h at 165 000 g). After trichloroacetic acid precipitation, the protein in the samples was subjected to SDS/PAGE. Cytochrome *c* (Cyt. *c*) was detected on Western blots using a monospecific antiserum. St (standard), indicates 10% of the reticulocyte lysate containing the ³⁵S-labelled precursor used for each lane.

Figure 5 Alkaline extractability of *in vitro* imported NUO-17.8

Mitochondria re-isolated from an *in vitro* import experiment (200 µg of protein) were resuspended in 1 ml of 0.1 M Na₂CO₃ and incubated for 30 min on ice. Half of the sample was kept on ice (sample T), and the other half was separated into membrane (P) and soluble (S) fractions as described in the legend to Figure 4. Endogenous NUO-17.8 and the 49 kDa subunit of complex I (NUO-49) were detected on Western blots using monospecific antisera. The *in vitro* imported subunit was detected by fluorography of a dried gel.

mature subunit, and could reflect anomalous processing activity of MPP under these sub-optimal conditions. It is striking, however, that this species is apparently chased to the mature form (see below). The significance of this finding was not investigated further.] The observed uncleaved form is not a dead-end species: after the MPP activity was restored in these mitochondria (see the Materials and methods section), the precursor protein was readily chased into the mature 17.8 kDa subunit. Furthermore, this chase could take place even if the $\Delta\Psi$ across the inner membrane had been previously dissipated with

valinomycin (Figure 3b, lane 4), suggesting that this uncleaved intermediate had already passed the $\Delta\Psi$ -dependent step of insertion into the mitochondrial inner membrane.

Localization of the *in vitro* imported NUO-17.8

The data presented in the previous section indicate that at least the N-terminal portion of the imported polypeptide reaches the mitochondrial matrix, where it is processed by MPP. However, no more conclusions regarding its localization can be reached. Is the *in vitro* imported subunit inserted into its final localization, i.e. the mitochondrial inner membrane? In order to test this possibility, we first tried to assess whether the *in vitro* imported NUO-17.8 exists as a soluble or as a membrane-associated form inside mitochondria. Thus ³⁵S-labelled preNUO-17.8 was incubated with isolated *Neurospora* mitochondria in the presence of a membrane potential. After proteinase treatment, in order to remove non-imported protein, mitochondria were re-isolated and sonicated in SEM buffer (see the Materials and methods section) containing various concentrations of NaCl (0–0.5 M). The suspensions were then separated into membrane pellet and soluble fractions by centrifugation at 165 000 g for 1 h and analysed by SDS/PAGE. Imported (mature) NUO-17.8 was exclusively found in the membrane fraction (Figure 4, lanes P), suggesting that the polypeptide is strongly attached to the mitochondrial membranes.

In order to characterize further the type of interaction that the imported subunit establishes with the mitochondrial membranes, we have analysed its extractability with 0.1 M Na₂CO₃ (Fujiki et al., 1982). This alkaline extraction method allows the discrimination of intrinsic from peripheral membrane proteins, since only the former class of proteins resists the extraction procedure. Thus mitochondria re-isolated from an *in vitro* import experiment, as described above, were incubated with the carbonate solution and separated into soluble and membrane fractions. The fractions were then subjected to SDS/PAGE and fluorography to detect the radiolabelled imported polypeptide. In parallel, the extractabilities of both the endogenous NUO-17.8 and the 49 kDa subunit of complex I (NUO-49; Preis et al., 1990) were also determined. As shown in Figure 5, NUO-49 was completely removed from the membranes by the alkaline treatment. This is in good agreement with the localization of this protein within complex I, since it is a component of the peripheral arm of the enzyme (Weiss et al., 1991). This result is in sharp contrast with the behaviour of endogenous NUO-17.8, which could not be extracted from the mitochondrial inner membrane by applying this technique. This finding, together with the existence of a putative membrane-spanning domain in the deduced protein sequence, provides strong evidence that NUO-17.8 is an intrinsic membrane protein.

The *in vitro* imported NUO-17.8, however, displayed more complex behaviour. The majority of the *in vitro* imported NUO-17.8 was found in the soluble fraction (Figure 5, lane S); however, about 10% of the ³⁵S-labelled polypeptide resisted the extraction procedure (Figure 5, lane P), suggesting that it had arrived at its final localization, namely the inner membrane. (It should be emphasized that this carbonate-resistant species is proteinase-protected in intact mitochondria. Furthermore, the polypeptide has already been cleaved in the matrix by MPP. Thus it is reasonable to assume that the observed behaviour indeed reflects the insertion of the ³⁵S-labelled polypeptide into the mitochondrial inner membrane and not into the outer membrane.)

In an attempt to obtain more information concerning the localization of the *in vitro* imported NUO-17.8 (which, as shown

Figure 6 Membrane topology of NUO-17.8

[³⁵S]Methionine-labelled preNUO-17.8 was imported into isolated mitochondria. After trypsin treatment to remove non-imported polypeptide, mitochondria were re-isolated and resuspended in SEMK buffer (see the Materials and methods section). Aliquots containing 50 µg of protein were then treated with the indicated concentrations of digitonin in the presence of proteinase K (PK), as specified. The samples were diluted 5-fold with SEMK buffer and incubated on ice for 30 min. After inactivation of the proteinase, the protein in the samples was precipitated with trichloroacetic acid and subjected to SDS/PAGE. The gel was blotted on to nitrocellulose and the membrane was used to expose an X-ray film (to detect the *in vitro* imported subunit), and then probed with antisera directed to mtHSP70 (matrix marker), CCHL (intermembrane space marker) and NUO-17.8. P and M mark the precursor and mature forms respectively of the NUO-17.8 kDa.

above, may actually consist of at least two different subpopulations), we performed fractionation of mitochondria using digitonin (Schnaitmann and Greenawalt, 1968). By titrating intact mitochondria with this detergent in the presence of a proteinase, it is possible to cleave proteins with domains exposed in the intermembrane space before degrading proteins with domains exposed only to the mitochondrial matrix. Therefore freshly isolated *Neurospora* mitochondria were subjected to an import experiment in the presence of a membrane potential. After trypsin treatment in order to remove non-imported NUO17.8, mitochondria were re-isolated and resuspended in SEMK buffer (see the Materials and methods section). Aliquots of the suspension were then treated with several concentrations of digitonin in the presence of proteinase K. After inactivation of the proteinase, the protein in the samples was precipitated with trichloroacetic acid and analysed by SDS/PAGE. As markers for the intermembrane space and matrix, we used cytochrome *c* haem lyase (CCHL; Drygas et al., 1989; Lill et al., 1992) and the mitochondrial 70 kDa heat shock protein (mtHSP70; Kang et al., 1990) respectively. As shown in Figure 6, the endogenous/assembled NUO-17.8 is partially accessible to proteinase K only when the matrix component is open (i.e. mtHSP70 is degraded). Under these conditions, a considerable fraction of the subunit was digested to a faster-migrating species with an apparent molecular mass of 15 kDa. This fragment is still membrane-associated: when mitochondria were re-isolated by centrifugation after the digitonin treatment, the 15 kDa fragment was found in the pellet (results not shown).

As expected, the *in vitro* imported subunit displayed dual behaviour. The majority of the ³⁵S-labelled polypeptide was

degraded by proteinase K as soon as the intermembrane space was opened (at a concentration of 0.075 % digitonin). However, a fraction of the subunit remained undegraded at this detergent concentration. Only when the matrix compartment was open did this species become partially proteinase-sensitive. Furthermore, the observed proteolysis resulted in a 15 kDa fragment that co-migrated exactly with the one described for the endogenous subunit. These data taken together strongly indicate that the *in vitro* imported NUO-17.8 has acquired the same membrane topology as the endogenous/assembled subunit.

DISCUSSION

We have cloned and sequenced a cDNA insert encoding the 17.8 kDa subunit of complex I from *Neurospora crassa*. The deduced primary structure, together with automated Edman degradation of the isolated subunit, revealed that this polypeptide is produced as an extended precursor of 20.8 kDa which is then processed to a 17.8 kDa mature form. Interestingly, the N-terminal part of the mature protein has a rather high content of histidine residues (seven out of a total of 13 in the whole polypeptide), but the significance of this clustering is not known. No similarities with proteins of known primary structure were found, leaving the function of this subunit in complex I still undefined.

We have shown that the precursor of NUO-17.8 can be efficiently imported into isolated mitochondria, where it is cleaved to the mature form by MPP. Furthermore, localization experiments indicate that a fraction of the *in vitro* imported NUO-17.8 is inserted into the inner membrane, where it acquires the membrane topology of the endogenous/assembled subunit. However, this process is relatively inefficient: only about 10 % of the *in vitro* imported subunit reaches this stage. Interestingly, the remaining portion of the subunit, presumably accumulated at some stage of the import pathway, is completely degraded by proteinase K under conditions whereby the outer membrane (but not the inner membrane) is disrupted. This species apparently becomes attached to the mitochondrial inner membrane in such a way that the presequence has already been cleaved in the matrix by MPP, but the C-terminal part is still exposed to the intermembrane space. The fact that 90 % of the *in vitro* imported subunit is carbonate-extractable suggests that this intermediate is bound to the inner membrane through protein-protein interactions.

The subunit described here is a component of the so-called hydrophobic arm of complex I, a part of the enzyme that is believed to be embedded in the mitochondrial inner membrane. In agreement with this, the assembled polypeptide cannot be extracted from the membrane by alkaline treatment. The question arises of how NUO-17.8 is inserted in the mitochondrial inner membrane. The deduced primary structure of the polypeptide reveals the existence of a strongly hydrophobic domain that is long enough to cross a membrane in an α -helix conformation (no other stretch comprising more than nine hydrophobic amino acid residues is found in the mature subunit). This fact strongly suggests that NUO17.8 is a bitopic transmembrane protein. However, the transmembrane nature of NUO-17.8 could not be demonstrated by proteinase accessibility studies. It is possible that the hydrophilic domain facing the intermembrane space has intrinsic proteinase resistance and/or is protected from externally added proteinases by other components of complex I.

The hydrophobic arm of *Neurospora* complex I contains, as well as the seven mitochondrially synthesized subunits, about 12 nuclear-encoded components. Of the latter group, the primary structure is known for the following subunits: 21.3 kDa (Nehls

et al., 1990), 20.9 kDa (Azevedo et al., 1992), 20.8 kDa (Videira et al., 1990b), 12.3 kDa (Videira et al., 1993) and 9.3 kDa (Heinrich et al., 1992). All these subunits have in common the absence of a cleavable mitochondrial targeting sequence and, at least in the case of the 20.9 kDa component, insertion into the inner membrane seems to take place from the cytosolic side (Azevedo et al., 1992). The fact that NUO-17.8 has a matrix targeting sequence could suggest that this polypeptide follows a different biosynthetic pathway. Obviously, this subunit requires an additional step before being assembled in complex I (cleavage of the presequence by MPP). However, it is unclear whether NUO-17.8 really does follow a different import pathway. In particular, is the polypeptide completely translocated into the matrix before undergoing insertion into the inner membrane, a mechanism that would be in agreement with the so-called conservative sorting (for a review see Hartl et al., 1989)? The data presented here provide no evidence for such an intra-mitochondrial sorting pathway. For instance, no salt-extractable species could be detected after *in vitro* import experiments, suggesting that the *in vitro* imported NUO-17.8 does not exist in a soluble form in the matrix. In addition, it is quite striking that the majority of the imported (mature) subunit is found exposed to the intermembrane space. Import intermediates displaying these characteristics have been recently described for proteins *en route* to the matrix (Hwang et al., 1991). However, in all the documented examples, these intermediates were obtained by performing import under sub-optimal conditions (i.e. ATP depletion in the mitochondrial matrix). It is unlikely that this is the reason why the majority of the *in vitro* imported NUO-17.8 is found attached to the mitochondrial inner membrane ('standard' import conditions were used in these experiments; see the Materials and methods section). Rather, we think that the existence of this species reflects the specific features of the import pathway of NUO-17.8. Our results could favour a non-conservative sorting mechanism in which insertion of NUO-17.8 into the lipophilic environment of the inner membrane occurs through the inner membrane itself (probably by lateral movement of the polypeptide away from the inner membrane translocation machinery directly into the lipid bilayer). It is clear, however, that more data are needed in order to confirm the existence of such an intramitochondrial sorting pathway. In this context, it will be of major importance to determine whether or not the assembly of NUO-17.8 in the inner membrane requires the action of the matrix ATP-dependent chaperonins (Cheng et al., 1989; Kang et al., 1990). We are currently pursuing this aim.

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