Biochem. J. (1992) 288, 29–34 (Printed in Great Britain)

Primary structure and mitochondrial import *in vitro* of the 20.9 kDa subunit of complex I from *Neurospora crassa*

Jorge E. AZEVEDO,* Uwe NEHLS,† Christoph ECKERSKORN,‡ Helga HEINRICH,* Heide ROTHE,* Hanns WEISS† and Sigurd WERNER*§

*Institut für Physiologische Chemie, Universität München, D-8000 München 2, †Institut für Biochemie, Heinrich–Heine-Universität, Düsseldorf and ‡Max-Planck-Institut für Biochemie, Genzentrum, München, Federal Republic of Germany

The 20.9 kDa subunit of NADH:ubiquinone oxidoreductase (complex I) from *Neurospora crassa* is a nuclear-coded component of the hydrophobic arm of the enzyme. We have determined the primary structure of this subunit by sequencing a full-length cDNA and a cleavage product of the isolated polypeptide. The deduced protein sequence is 189 amino acid residues long and contains a putative membrane-spanning domain. Striking similarity over a 60 amino-acid-residue domain with the M (matrix) protein of para-influenza virus was found. No other relationship with already known sequences could be detected, leaving the function of this subunit in complex I still undefined. The biogenetic pathway of this polypeptide was studied using a mitochondrial import system *in vitro*. The 20.9 kDa subunit synthesized *in vitro* is efficiently imported into isolated mitochondria, where it obtains distinct features of the endogenous subunit. Our results suggest that the 20.9 kDa polypeptide is made on cytosolic ribosomes lacking a cleavable targeting sequence, interacts with the mitochondrial outer membrane (in a process that does not require an energized inner membrane), and is imported into mitochondria at contact sites. The 20.9 kDa subunit is then inserted into the inner membrane acquiring a topology similar to that of the already assembled subunit.

INTRODUCTION

Complex I (NADH:ubiquinone oxidoreductase, EC 1.6.99.3), the first enzyme in the mitochondrial respiratory chain, catalyses the transfer of two electrons from NADH to ubiquinone with concomitant translocation of four or five protons across the mitochondrial inner membrane (for reviews see Ragan, 1987; Weiss *et al.*, 1991). The enzyme is a complicated assembly of about 32 subunits, seven of which are encoded and synthesized in mitochondria (Chomyn *et al.*, 1985, 1986; Videira & Werner, 1989). One FMN, a still undefined number of iron–sulphur clusters (Beinert & Albracht, 1982; Ohnishi *et al.*, 1985) and probably one internal ubiquinone (Suzuki & King, 1983; Burbaev *et al.*, 1989) comprise the redox groups of complex I.

Recently, a number of primary structures of subunits from ox and *Neurospora* complex I have been determined. In a few cases, well-known functional domains (Dupuis *et al.*, 1991; Pilkington *et al.*, 1991), or interesting sequence similarities with chloroplast (Shinozaki *et al.*, 1986; Fearnley *et al.*, 1989; Videira *et al.*, 1990*a*; Dupuis *et al.*, 1991; Masui *et al.*, 1991) and bacterial proteins (Böhm *et al.*, 1990; Pilkington *et al.*, 1991; Preis *et al.*, 1991) were found, allowing new insights into the function of these particular subunits. However, it is obvious that our knowledge about structure and function of complex I is still very limited. The recent finding of an acyl-carrier protein among complex I subunits (Runswick *et al.*, 1991; Sackmann *et al.*, 1991) illustrates this point and strongly suggests the existence of biochemical activities in this enzyme other than electron transport and proton translocation.

Because of the enormous complexity of this oligomeric protein, attempts to degrade it into smaller, easier to study, fragments have been made. *Neurospora crassa* complex I can be separated into two distinct fractions (Friedrich *et al.*, 1989; Tuschen *et al.*, 1990; Wang *et al.*, 1991): the so-called small form of complex I [an assembly of 13 nuclear-encoded subunits containing the NAD(H)-binding site and three Fe–S clusters], which is made in *Neurospora* cells poisoned with chloramphenicol; and the hydrophobic fragment, a fraction obtained by chaotropic treatment of the isolated complex, which comprises all the mitochondrially encoded subunits, about 10 nuclear-coded subunits and one Fe–S cluster. Among the latter group of constituents the ubiquinone-binding polypeptide was found (H. Heinrich, J. E. Azevedo and S. Werner, unpublished work).

Electron microscopy studies revealed that *N. crassa* complex I forms 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). Most importantly, these studies showed the equivalence between the small form of the enzyme and the peripheral arm and, on the other hand, the identity of the hydrophic fraction with the hydrophobic arm. Thus, complex I seems to be constituted of two relatively independent structural units which are thought to have independent assembly pathways also (Tuschen *et al.*, 1990).

In the present paper we describe the cloning of a cDNA encoding a 20.9 kDa subunit of the hydrophobic arm of complex I. As an attempt to elucidate the assembly of this subunit we have studied its mitochondrial import pathway *in vitro*.

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 (Ise *et al.*, 1985; Filser & Werner, 1988); isolation of mitochondria for import *in vitro* experiments (Rassow *et al.*, 1989); SDS/PAGE (Laemmli, 1970; Videira & Werner, 1989); Western-blotting (Towbin *et al.*, 1979) and detection of horseradish peroxidase- or alkaline phosphatase-

Abbreviations used: SEM buffer (250 mM-sucrose, 1 mM-EDTA, 10 mM-Mops/KOH, pH 7.2); SEMK, SEM buffer containing 100 mM-KCl; M, matrix; cyt c, cytochrome c; α-IDH, α-isocitrate dehydrogenase.

The nucleotide sequence reported here has been submitted to the EMBL Database under the accession number X60829.

§ To whom correspondence should be addressed.

conjugated second antibodies on Western blots (Roswell & White, 1978; Blake *et al.*, 1984); synthesis of proteins in rabbit reticulocyte lysate (Amersham International, Amersham, Bucks., U.K.) (Pelham & Jackson, 1976) in the presence of [^{35}S]-methionine (specific activity 1000 Ci/mmol; Amersham) by coupled transcription/translation (Stueber *et al.*, 1984; Hartl *et al.*, 1986); preparation of reticulocyte lysate to supplement import reactions (Hartl *et al.*, 1986); determination of protein concentration (Bradford, 1976); extraction of mitochondria with carbonate buffer (Fujiki *et al.*, 1982); automated Edman degradation of a CNBr-cleavage fragment of the isolated subunit (Wachter & Werhahn, 1979; Eckerskorn *et al.*, 1988).

The strategy used for screening a λgt11 cDNA expression library, subcloning and sequencing cDNA inserts has been described previously (Videira *et al.*, 1990*b*).

The import assays were carried out as reported (Hartl *et al.*, 1986) with the following modifications: to a microtube pre-equilibrated at 0 °C the following solutions were added: 2 μl of 0.1 M-NADH, 5 μl of 0.1 M-$MgCl_2$, 10 μl of reticulocyte lysate, 70 μl of BSA buffer [250 mM-sucrose, 3 % (w/v) lipid-free BSA, 80 mM-KCl, 10 mM-Mops/KOH, pH 7.2] and either 1 μl of 100 μM-valinomycin in ethanol or 1 μl of ethanol alone (control). Finally, 10 μl of freshly prepared mitochondria (5 mg/ml) were added. The import reaction was started by addition of 1 μl of reticulocyte lysate containing the ^{35}S-labelled 20.9 kDa subunit. The suspension was incubated for 20 min at 25 °C and divided into two parts. One half received 1 μl of proteinase K at a concentration of 1 mg/ml in SEM buffer (250 mM-sucrose, 1 mM-EDTA, 10 mM-Mops/KOH, pH 7.2) and the other half SEM buffer only. Both samples were incubated for 15 min on ice and 1 μl of 0.1 M-phenylmethanesulphonyl fluoride in ethanol was added. After a further 5 min of incubation on ice, the mitochondria were pelleted by centrifugation (10 min at 15000 *g*), resuspended gently in 200 μl of SEM buffer, centrifuged again under the same conditions and then either processed for SDS/PAGE analysis, or used for the salt and alkaline extractions.

The salt extraction was performed as follows: aliquots containing 200 μg of mitochondria from an import experiment were resuspended in 2 ml of SEM buffer containing various concentrations of NaCl (0–0.5 M). After sonicating as described (Hartl *et al.*, 1986), the samples were divided into two portions. One half was kept on ice as a control (named total sample) and the other was separated into membrane pellet and supernatant by centrifugation for 1 h at 165000 *g*. The membrane fractions were resuspended in 100 μl of 2 % (w/v) Triton X-100 in water and then 1 ml of SEM buffer containing the original NaCl concentration was added. All the other samples (i.e. the supernatants and the total samples) were supplemented with the same volume of detergent solution. Finally the protein was precipitated by adding trichloroacetic acid (100 % w/v) to reach a final 10 % (w/v) concentration. More than 90 % of the radioactive material was precipitated, as judged by liquid scintillation counting.

For the digitonin fractionation experiment (Hartl *et al.*, 1986) a 22-fold scale-up of the import reaction described above was done (i.e. 1.1 mg of energized mitochondria were used) with the following modifications: after 20 min of incubation at 25 °C, trypsin (2 mg/ml in SEM buffer) was added to a final concentration of 30 μg/ml. The suspension was incubated for 15 min on ice and a 30-fold excess (by wt.) of soy bean trypsin inhibitor was added. After a further 5 min on ice, the mitochondria were re-isolated, washed (see above) and resuspended in SEM buffer containing 100 mM-KCl (SEMK) at a protein concentration of 5 mg/ml. Aliquots (20 μl) of this suspension were added to prechilled tubes already containing 4 μl of SEMK buffer (controls), or 4 μl of various digitonin solutions in SEMK to give the final desired detergent concentrations (0.05–0.3 %). After mixing briefly, the samples were incubated for 2 min on ice and were then immediately diluted with a 20-fold volume of SEMK. Proteinase K (1 mg/ml in SEM) was added to a final concentration of 30 μg/ml and, after 15 min on ice, phenylmethanesulphonyl fluoride (0.1 M in ethanol) was added to obtain a 2 mM solution. The samples were incubated for 5 min on ice, centrifuged for 20 min at 48000 *g*, washed with 1 ml of SEMK and subjected to SDS/PAGE.

RESULTS

Isolation and sequence analysis of cDNA clones

Phages (4×10^5) of a λgt11 cDNA expression library were screened with a monospecific antibody raised an individual subunit of complex I having an apparent molecular mass of 19 kDa (see Fig. 1). Five phages giving positive results were isolated and shown to carry inserts showing sequence identity by Southern-blot analysis (results not shown). The largest insert (about 950 bp) was subcloned in the pGEM4 transcription vector (Promega), yielding the plasmid pNUO-20.9, and was sequenced (Fig. 2). The cDNA is 994 bp long and consists of a 22 bp 5′-untranslated region, an open reading frame of 567 bp and a 405 bp 3′-untranslated region. The sequence GTCACAATGT surrounding the first ATG is nearly identical to the optimum translation initiation sequence of *N. crassa* (RTCACAATGG) (Paluh *et al.*, 1988). The open reading frame encodes a 189 amino-acid-residue protein with a calculated molecular mass of

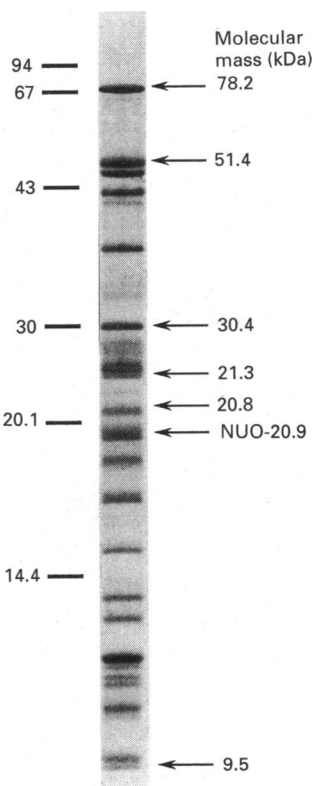

Fig. 1. SDS/PAGE of subunits of complex I from *N. crassa*

The 20.9 kDa subunit (NUO-20.9) and several subunits with already known 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 (Videira *et al.*, 1990*a*); 21.3 kDa (Videira *et al.*, 1990*c*); 20.8 kDa (Videira *et al.*, 1990*b*); and the 9.5 kDa (H. Heinrich, J. E. Azevedo & S. Werner, unpublished work)]. The numbers at the left margin indicate the molecular masses of the applied standards in kDa.

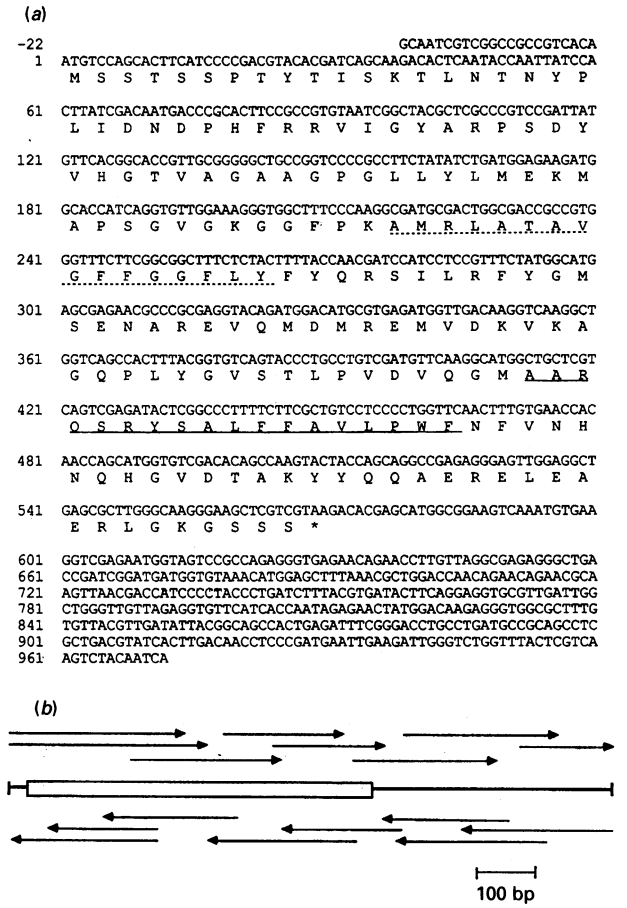

Fig. 2. cDNA sequence determination

(*a*) Nucleotide sequence and deduced protein sequence of the cDNA encoding the NUO-20.9 subunit. The amino acid sequence obtained by automated Edman degradation is underlined. A putative membrane-spanning domain is also indicated (dotted line). The stop codon is marked by an asterisk. (*b*) Sequencing strategy and structure of the cloned cDNA insert. Arrows show the direction and extent of sequence determination. The box corresponds to the coding region of the cDNA.

```
M protein (283)   SISKTLASQLVFKREICYPLMDLNPHLNLVI-WASSVEITRVDAIF
                  !***** !!         ***!* !**   ** !* ! !  !  !!
NUO-20.9 (10)     TISKTLNTN--------YPLIDNDPHFRRVIGYARPSDYVH-GTVA

M protein (328)   QPSLPGEFRYYPNIIA-KGVGKIKQWN
                   !! **   *  ! !*  ****
NUO-20.9 (47)     GAAGPG-LLYLMEKMAPSGVGKGGFPK
```

Fig. 3. Alignment of the sequences of the NUO-20.9 and the M protein of para-influenza virus

Numbers indicate the positions within the respective polypeptides of the first amino acid residue on each line. Amino acid identities (*) and conservative substitutions (!) are indicated. Hyphens were introduced to optimize the alignment.

20981 Da. One putative membrane-spanning domain (residues 73–88) was predicted using the method of Rao & Argos (1986). A search in a protein database revealed a striking similarity, within a 60 amino-acid-residue domain, with the so-called M (matrix) protein of para-influenza virus (Galinski *et al.*, 1987; Fig. 3).

Protein-sequence analysis

Automated Edman degradation of the electrophoretically isolated 20.9 kDa subunit (NUO-20.9) failed to give any results,

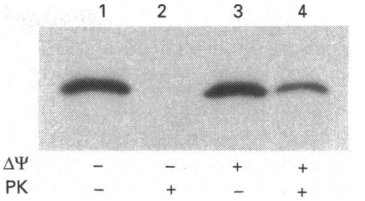

Fig. 4. Import *in vitro* of NUO-20.9 into isolated mitochondria

[^{35}S]Methionine-labelled NUO-20.9 was incubated with freshly isolated mitochondria, either in the presence (lanes 1 and 2), or absence (lanes 3 and 4) of 1 μM-valinomycin [minus and plus membrane potential ($\Delta\Psi$) respectively]. The samples in lanes 2 and 4 were treated with proteinase K (PK). After inactivation of the protease and re-isolation of the mitochondria, samples were analysed by SDS/PAGE. A fluorograph of the dried gel is shown.

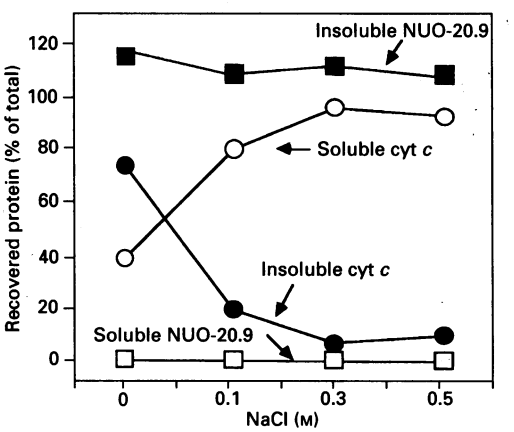

Fig. 5. NUO-20.9 imported *in vitro* is membrane bound

^{35}S-labelled NUO-20.9 was imported into mitochondria (800 μg of protein). After proteinase K treatment and re-isolation of mitochondria, aliquots containing 200 μg of mitochondrial protein were resuspended in 2 ml of SEM buffer containing the indicated NaCl concentrations. The samples were sonicated and divided into two halves. One half was kept on ice for determination of recoveries (termed 'total'). The other half was separated into a membrane fraction and a soluble fraction by centrifugation (1 h at 165000 *g*). The protein in all samples was precipitated with trichloroacetic acid (see the Materials and methods section), subjected to SDS/PAGE and blotted onto nitrocellulose. The Western-blot was first used to expose an X-ray film (to detect the subunit imported *in vitro*) and, afterwards, probed with an antiserum against cyt *c*. Membrane-associated (insoluble) NUO-20.9 and cyt *c* (■ and ● respectively), or soluble NUO-20.9 and cyt *c* (□ and ○), are expressed as a percentage of total recovered protein.

suggesting a protected *N*-terminus. Therefore, the isolated subunit was cleaved with CNBr and one fragment was partially sequenced. The sequence obtained, (M)AARQSRYSALFFAVLPWF, matches the predicted amino acid sequence of residues 137–155, hence confirming the validity of the isolated clone.

Mitochondrial import of NUO-20.9 synthesized *in vitro*

The 20.9 kDa subunit was synthesized *in vitro* in the presence of [^{35}S]methionine and used in a mitochondrial import experiment *in vitro*. Isolated mitochondria were incubated with the NUO-20.9 subunit synthesized *in vitro*, either in the presence (Fig. 4, lanes 3 and 4) or absence (Fig. 4, lanes 1 and 2) of a membrane potential. Import into the interior of mitochondria was monitored by treatment of the import reactions with proteinase K. Proteinase-resistant subunit is found only in the presence of a

membrane potential (Fig. 4, lane 4). When the mitochondrial membrane potential is abolished no imported NUO-20.9 is observed (Fig. 4, lane 2), although binding to mitochondria is not diminished (Fig. 4, compare lanes 1 and 3). It is also clear that the imported subunit is not processed to a mature form having an appreciably different molecular mass, suggesting that this particular complex I subunit is not synthesized with a cleavable mitochondrial targeting sequence. This observation is further supported by the fact that the NUO-20.9 synthesized *in vitro* co-migrates exactly with the assembled form of the subunit upon SDS/PAGE (results not shown).

Localization of NUO-20.9

As demonstrated in the previous section NUO-20.9 can be efficiently imported into mitochondria in the presence of a membrane potential. The proteinase K resistance of NUO-20.9 observed under these conditions indicates that NUO-20.9 has at least crossed the outer membrane of mitochondria, but no more information regarding its localization can be inferred. In a first attempt at localizing the subunit imported *in vitro*, we tried to find out whether NUO-20.9 exists as a soluble or membrane-bound species inside the mitochondria. The following experiment was performed: after a standard import reaction *in vitro* in the presence of a membrane potential, mitochondria were re-suspended in SEM buffer containing various concentrations of NaCl and were sonicated; after centrifugation for 1 h at 165000 *g*, the supernatants and the membrane pellets were analysed for the presence of labelled NUO-20.9 (Fig. 5). NUO-20.9 is resistant to NaCl extraction of membranes, even at a high salt concentration (0.5 M). The *in vitro* synthesized NUO-20.9 alone subjected to the same treatment could not be sedimented under these conditions (results not shown). Thus, this result indicates that the newly imported subunit is probably tightly bound to membranes.

The nature of this interaction with membranes was investigated further by analysing the extractability of both the form imported *in vitro* and the endogenous form of NUO-20.9 with 0.1 M-Na_2CO_3 (pH 11.5). With this alkaline extraction technique it is possible to distinguish between intrinsic and peripheral membrane proteins. Mitochondria from an import *in vitro* experiment, as described above, were incubated with the carbonate solution and separated into soluble and membrane fractions by centrifugation. These fractions were analysed by Western-blotting and fluorography to detect the endogenous and the *in vitro* imported form of NUO-20.9 respectively. As shown in Fig. 6, the endogenous form of NUO-20.9 is found associated with membranes, providing strong evidence that it is an intrinsic membrane protein. This is in sharp contrast to the behaviour of the 30.4 kDa subunit of complex I, which is completely extractable under these conditions. The majority of the newly imported NUO-20.9 is also resistant to the extraction procedure, although about 20% was consistently found in the soluble fraction. When the membranes were extracted a second time with carbonate no more labelled subunit could be recovered (results not shown). Thus, we assume that the extractable protein represents an intermediate stage in the import, where the subunit interacts with the membrane (virtually all the labelled subunit is resistant to NaCl extraction), but is not yet inserted into its lipophilic environment.

To localize more specifically the subunit imported *in vitro*, as well as the endogenous NUO-20.9, we tried to assess the membrane topology of these species by subfractionation of mitochondria with digitonin. Mitochondria re-isolated from an import reaction were subjected to increasing concentrations of digitonin (in order to open successively the intermembrane space and the matrix) and a constant amount of proteinase K was added. The protease sensitivity profile of both forms of the

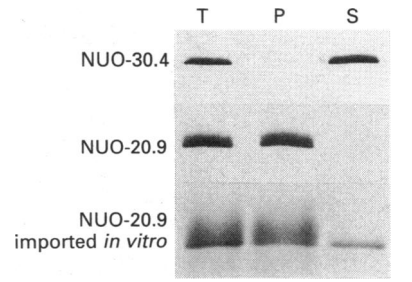

Fig. 6. Extractability of NUO-20.9 by alkaline treatment

Mitochondria (200 μg of protein) from an import *in vitro* experiment were resuspended in 2 ml of 0.1 M-Na_2CO_3 and incubated for 30 min at 0 °C. Half of the sample was kept on ice for determination of recoveries (T sample). The other half was separated into pellet (P) and supernatant (S) by centrifugation (1 h at 165000 *g*). Sample P was resuspended in 1 ml of the carbonate solution to make all solutions chemically identical. After precipitation with trichloroacetic acid [final concentration 12.5% (w/v)] the samples were subjected to SDS/PAGE. The NUO-20.9 imported *in vitro* was analysed by fluorography of a dried gel. The endogenous NUO-20.9 and the 30.4 kDa subunit were detected on Western blots with monospecific antisera.

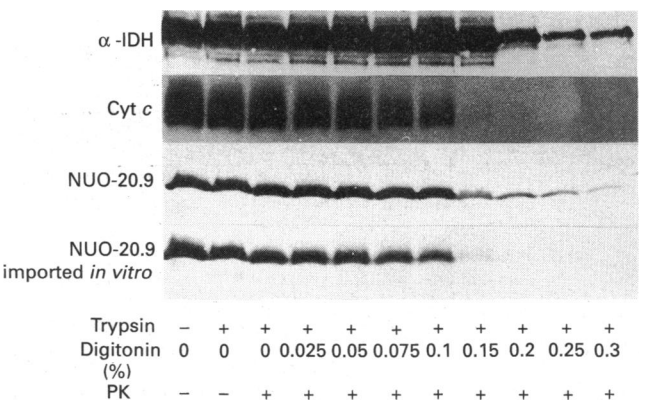

Fig. 7. Membrane topology of both the *in vitro* imported and endogenous NUO-20.9

[^{35}S]Methionine-labelled NUO-20.9 was imported into isolated mitochondria (1.1 mg of protein). An aliquot containing 100 μg of protein was kept on ice as a control (first column). The remaining mitochondria were treated with trypsin at 30 μg/ml to remove any non-imported subunit. After inactivation of the protease, mitochondria from the samples were re-isolated, washed and resuspended in SEMK buffer (see the Materials and methods section) at a protein concentration of 0.1 mg/ml. Aliquots containing 100 μg of protein were then treated for 2 min at 0 °C with the indicated amounts of digitonin and were immediately diluted 20-fold with buffer. After treatment with proteinase K (PK), as specified, mitochondria were re-isolated, washed and subjected to SDS/PAGE. The gel was blotted onto nitrocellulose and the membrane was first used to expose an X-ray film (to detect the subunit imported *in vitro*) and, afterwards, probed with antisera against cyt *c* (intermembrane space marker), α-IDH (matrix marker), and NUO-20.9.

subunit was compared with those of polypeptides of known mitochondrial localization: cytochrome *c* (cyt *c*) and α-isocitrate dehydrogenase (α-IDH) were used as markers for the intermembrane space and the matrix respectively. As shown in Fig. 7, both forms of the subunit were grossly accessible to proteinase K at a concentration of 0.15% digitonin. The same is true for cyt *c*, indicating that the intermembrane space was completely opened under these conditions. In contrast, α-IDH was not degraded at this digitonin concentration, implying that the structure of the inner membrane was still intact. However, it

should be noted that the endogenous subunit is somehow more resistant to the proteolytic action of proteinase K than the newly imported one (a fraction of the subunit remains undigested even after opening the matrix compartment). This phenomenon could reflect a more protected environment and/or a different structure of the assembled subunit. On the other hand, the existence of different subpopulations of NUO-20.9 in complex I cannot be excluded. Indeed, the stoichiometry of NUO-20.9 in relation to the 78.2 kDa and 30.4 kDa subunits is 3:1:1, as judged by laser densitometry of SDS/PAGE-resolved subunits obtained by immunoprecipitation of complex I from ^{35}S-labelled mitochondria (J. E. Azevedo & S. Werner, unpublished work).

In summary, these results strongly suggest that both the polypeptide imported *in vitro* and a considerable fraction of the endogenous form of NUO-20.9, if not all, are exposed to the mitochondrial intermembrane space.

DISCUSSION

We have cloned and sequenced a cDNA insert which encodes the 20.9 kDa subunit of complex I from *N. crassa*. In an attempt to obtain some clues about the function of this subunit we compared its deduced primary structure with the sequences compiled in the latest versions of the PIR and NBRF databases. A striking similarity over a domain of 60 amino acid residues between NUO-20.9 and the M protein of para-influenza virus (Galinski *et al.*, 1987) was found. The similarity observed is quite impressive (62%), and it is therefore possible that both regions of the two proteins may mediate the same function. However, it will be necessary to collect additional sequence data on NUO-20.9 homologues from evolutionary remote organisms to clarify this point.

To characterize the biogenetic pathway of this complex I subunit we have performed mitochondrial import experiments *in vitro*. Our results indicate that NUO-20.9 does not possess a cleavable targeting sequence. Obviously, the information to address NUO-20.9 to mitrochondria resides in the mature protein. Mitochondrial targeting sequences are generally positively charged, rich in hydroxylated residues, devoid of acidic amino acids and located at the *N*-terminus of the precursor proteins (Hartl *et al.*, 1989). The *N*-terminal region of NUO-20.9 exhibits clearly these characteristics and could therefore fulfil this function.

Furthermore we have shown that binding of the precursor protein to mitochondria is independent of a membrane potential ($\Delta\Psi$), a property of the so-called class I precursors (Hartl *et al.*, 1989). However, translocation across the outer membrane (a process that is thought to be $\Delta\Psi$-independent) is only accomplished in the presence of an energized inner membrane. This suggests that translocation across both the outer and inner membrane is a coupled event and, based on the current knowledge of import mechanisms, probably occurs at contact sites (for a review see Pfanner & Neupert, 1990).

It cannot be inferred from the import experiments *in vitro*, whether NUO-20.9 is inserted into the inner membrane at this stage (i.e. translocation at contact sites is arrested at the inner membrane), or whether the polypeptide crosses both membranes, reaches the matrix and is then redirected to the inner membrane [a mechanism that would be in agreement with the so-called conservative sorting (Pfanner & Neupert, 1990)]. The fact that we could not detect a soluble (salt-extractable) species during the import into mitochondria *in vitro* is consistent with the first pathway. However, a transient, very fast passage of NUO-20.9 through the matrix cannot be excluded. Nevertheless, it should be kept in mind that all precursor proteins sorted by the conservative pathway possess a cleavable mitochondrial targeting sequence (see Hartl *et al.*, 1989 and papers cited therein). Since this is not the case with NUO-20.9, we speculate that this subunit does not follow the conservative sorting.

Once imported, the synthesized NUO-20.9 *in vitro* mimics, in at least two aspects, the endogenous subunit: (1) it is found mainly as an intrinsic membrane protein; and (2) it acquires a membrane topology which cannot be discriminated from that of the endogenous subunit, as judged by the accessibility to proteinase K from the mitochondrial intermembrane space. Thus, these results suggest that the subunit imported *in vitro* is on its correct assembly pathway.

Pulse-labelling experiments with *N. crassa* cells (Tuschen *et al.*, 1990) demonstrated the existence of an assembly intermediate of complex I having a molecular mass of about 350 kDa. This intermediate comprises not only all the mitochondrially encoded subunits, but also a subset of nuclear-coded subunits to which the NUO-20.9 polypeptide belongs. These observations, together with our results, suggest the following biogenetic pathway for the NUO-20.9 subunit: (i) synthesis on cytosolic ribosomes of the precursor protein lacking a cleavable mitochondrial targeting sequence; (ii) interaction with the mitochondrial outer membrane in a $\Delta\Psi$-independent process; (iii) import into mitochondria, probably at contact sites; (iv) insertion into the inner membrane of the precursor protein, which already displays distinct features of the assembled form; (v) assembly into the 350 kDa intermediate; and finally, (vi) association of this intermediate with remaining nuclear-encoded subunits that, as proposed (Tuschen *et al.*, 1990) may already exist in a pre-assembled subcomplex.

We thank Dr. Christoph Hergersberg for the kind gift of the cyt *c* and α-IDH antibodies. This work was supported by the Deutsche Forschungsgemeinschaft, the Bundesministerium für Wissenschaft und Technik and the Fonds der Chemischen Industrie. J. E. A. would like to thank Junta Nacional de Investigacao Cientifica e Tecnologica, Portugal, for financial support.

REFERENCES

Beinert, H. & Albracht, S. P. J. (1982) Biochim. Biophys. Acta **683**, 245–277
Blake, M. S., Johurta, K. H., Russel-Jones, G. J. & Gotschlick, E. C. (1984) Anal. Biochem. **136**, 175–179
Böhm, R., Sauter, M. & Böck, A. (1990) Mol. Microbiol. **4**, 231–243
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Burbaev, D. Sh., Moroz, I. A., Kotlyar, A. B., Sled, V. D. & Vinogradov, A. D. (1989) FEBS Lett. **254**, 47–51
Chomyn, A., Mariottini, P., Cleeter, M. W. J., Ragan, C. I., Matsuno-Yagi, A., Hatefi, Y., Doolittle, R. F. & Attardi, G. (1985) Nature (London) **314**, 592–597
Chomyn, A., Cleeter, M. W., Ragan, C. I., Riley, M., Doolittle, R. F. & Attardi, G. (1986) Science **234**, 614–618
Dupuis, A., Skehel, J. M. & Walker, J. E. (1991) Biochemistry **30**, 2954–2960
Eckerskorn, C., Mewes, W., Goretzki, H. & Lottspeich, F. (1988) Eur. J. Biochem. **176**, 509–519
Fearnley, I. M., Runswick, M. J. & Walker, J. E. (1989) EMBO J. **8**, 665–672
Filser, M. & Werner, S. (1988) Biochem. Pharmacol. **37**, 2551–2558
Friedrich, T., Hofhaus, G., Ise, W., Nehls, U., Schmitz, B. & Weiss, H. (1989) Eur. J. Biochem. **180**, 173–180
Fujiki, Y., Hubbard, A. L., Fowler, S. & Lazarow, P. B. (1982) J. Cell Biol. **93**, 97–102
Galinski, M. S., Mink, M. A., Lambert, D. M., Wechsler, S. L. & Pons, M. W. (1987) Virology **157**, 24–30
Hartl, F.-U., Schmidt, B., Wachter, E., Weiss, H. & Neupert, W. (1986) Cell (Cambridge, Mass.) **47**, 939–951
Hartl, F.-U., Pfanner, N., Nicholson, D. W. & Neupert, W. (1989) Biochim. Biophys. Acta **988**, 1–45
Hofhaus, G., Weiss, H. & Leonard, K. (1991) J. Mol. Biol. **221**, 1027–1043
Ise, W., Haiker, H. & Weiss, H. (1985) EMBO J. **4**, 2075–2080
Laemmli, U.K. (1970) Nature (London) **227**, 680–685

Masui, R., Wakabayashi, S., Matsubara, H. & Hatefi, Y. (1991) J. Biochem. **110**, 575–582
Ohnishi, T., Ragan, C. I. & Hatefi, Y. (1985) J. Biol. Chem. **260**, 2782–2788
Paluh, J. L., Orbach, M. J., Legerton, T. L. & Yanofsky, C. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3728–3732
Pelham, H. R. B. & Jackson, R. J. (1976) Eur. J. Biochem. **67**, 247–256
Pfanner, N. & Neupert, W.(1990) Annu. Rev. Biochem. **59**, 331–353
Pilkington, S. J., Skehel, J. M., Gennis, R. B. & Walker, J. E. (1991) Biochemistry **30**, 2166–2175
Preis, D., Weidner, U., Conzen, C., Azevedo, J. E., Nehls, U., Röhlen, D., Van der Pas, J., Sackmann, U., Schneider, R., Werner, S. & Weiss, H. (1991) Biochim. Biophys. Acta **1090**, 133–138
Ragan, C. I. (1987) Curr. Topics Bioenerg. **15**, 1–36
Rao, J. K. M. & Argos, P. (1986) Biochem. Biophys. Res. Commun. **869**, 197–214
Rassow, J., Guiard, B., Weinhues, U., Herzog, V., Hartl, F.-U. & Neupert, W. (1989) J. Cell Biol. **109**, 1421–1428
Roswell, D. F. & White, E. H. (1978) Methods Enzymol. **57**, 409–423
Runswick, M. J., Fearnley, I. M., Skehel, J. M. & Walker, J. E. (1991) FEBS Lett. **286**, 121–124
Sackmann, U., Zensen, R., Röhlen, D., Jahnke, U. & Weiss, H. (1991) Eur. J. Biochem. **200**, 463–469
Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K. *et al.* (1986) EMBO J. **5**, 2043–2049
Stueber, D., Ibrahimi, I., Cutler, D., Dobberstein, B. & Bujard, H. (1984) EMBO J. **3**, 3143–3148
Suzuki, H. & King, T. E. (1983) J. Biol. Chem. **258**, 352–358
Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
Tuschen, G., Sackmann, U., Nehls, U., Haiker, H., Buse, G. & Weiss, H. (1990) J. Mol. Biol. **213**, 845–857
Videira, A. & Werner, S. (1989) Eur. J. Biochem. **181**, 493–502
Videira, A., Tropschug, M. & Werner, S. (1990*a*) Biochem. Biophys. Res. Commun. **171**, 1168–1174
Videira, A., Tropschug, M., Wachter, E., Schneider, H. & Werner, S. (1990*b*) J. Biol. Chem. **265**, 13060–13065
Videira, A., Tropschug, M. & Werner, S. (1990*c*) Biochem. Biophys. Res. Commun. **166**, 280–285
Wachter, E. & Werhahn, R. (1979) Anal. Biochem. **97**, 56–64
Wang, D., Meinhardt, S. W., Sackmann, U., Weiss, H. & Ohnishi, T. (1991) Eur. J. Biochem. **197**, 257–264
Weiss, H., Friedrich, T., Hofhaus, G. & Preis, D. (1991) Eur. J. Biochem. **197**, 563–576
Werner, S. (1977) Eur. J. Biochem. **79**, 103–110

Received 29 April 1992; accepted 22 May 1992