Characterization of the 9.5-kDa Ubiquinone-Binding Protein of NADH:Ubiquinone Oxidoreductase (Complex I) from *Neurospora crassa*†

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ABSTRACT: A small polypeptide subunit of the NADH:ubiquinone reductase (complex I) from *Neurospora crassa* has been identified by photoaffinity labeling to participate in the binding of ubiquinone [Heinrich, H., & Werner, S. (1992) *Biochemistry* (preceding paper in this issue)]. This polypeptide is further characterized by its primary structure and by an assessment of its localization within complex I. A λgt11 cDNA expression library was screened using a specific antibody directed against this individual subunit of complex I. Two groups of clones, coding for polypeptide subunits of the appropriate apparent molecular weight, were isolated. One group was shown to contain the relevant recombinants. The derived amino acid sequence for the 9.5-kDa ubiquinone-binding polypeptide shows a similarity with a putative ubiquinol-binding subunit (also a 9.5-kDa polypeptide) from complex III of bovine heart [Usui, S., Yu, L., & Tu, C.-A. (1990) *Biochemistry 29*, 4618–4626]. The polypeptide has a hydrophobic stretch of a sufficient length to span the membrane. It resists against extraction with NaBr or Na_2CO_3, and therefore probably is buried in the so-called hydrophobic membrane portion of complex I. This nuclearly-encoded subunit lacks a typical cleavable presequence and is imported into isolated mitochondria by a membrane potential-dependent process.

Considering the complex nature of the NADH:ubiquinone oxidoreductase (EC 1.6.5.3) (about 30 different polypeptide subunits of nuclear and mitochondrial origin, including FMN and a number of iron–sulfur clusters) and the resulting problems to investigate such a large membrane protein (750 kDa), several attempts had been undertaken to subfractionate complex I [for reviews see Weiss et al. (1991) and Ragan (1987)]. With the aid of chaotropic agents, bovine complex I has been split into three fragments, two of which are water-soluble, the so-called FP and IP fragments (consisting of flavin and iron–sulfur containing proteins, respectively), and a residual hydrophobic portion (HP fragment). When complex I of the filamentous fungus *Neurospora crassa* is treated with NaBr (Friedrich et al., 1989), it seems that all those subunits, which correspond to the above-defined water-soluble parts, are stripped off. Grown in the presence of chloramphenicol, *N. crassa* assembles a (rotenone- and piericidine-insensitive) small form of the enzyme (Friedrich et al., 1989), whose composition is almost identical with that of the soluble fraction. The remaining hydrophobic subcomplex contains all the mitochondrially encoded polypeptides and a few species encoded by the nucleus. These findings imply that the division of complex I into functional fragments not only represents topological features but also reflects its evolutionary pathway.

Recently, the primary structure of a fair number of polypeptide subunits became available. The detection of sequence homologies with other proteins [e.g., with the soluble NAD-reducing hydrogenase of *Alcaligenes eutrophus*; see Pilkington et al. (1991)] and the existence of conserved characteristic motifs permitted the relation of individual subunits to distinct redox centers and cofactors, respectively. This approach indeed confirmed a previous idea based on EPR (Ohnishi et al., 1985) and affinity labeling studies (Chen & Guillory, 1984; Deng et al., 1990) that the 51-kDa subunit of the FP fragment contains the binding sites of NAD(H), FMN, and one of the iron–sulfur clusters (Preis et al., 1991; Pilkington et al., 1991). Hence, this polypeptide serves as the electron acceptor port.

The fractionation experiments have also shown that only one of the EPR-detectable iron–sulfur clusters, the so-called N-2 cluster, resides in the membrane-embedded HP fragment (Ohnishi et al., 1985; Wang et al., 1991). This cluster is known to possess a phospholipid-dependent redox potential and represents most likely the electron donator for the substrate ubiquinone. The binding site(s) for the specific inhibitors rotenone and piericidin A, which may act at or close to the reaction center, is(are) also located in the membrane fragment. The mitochondrial ND1 product has been identified as the putative binding site for rotenone, using a photoreactive derivative (Early et al., 1987). Consequently, the hydrophobic subcomplex seems to be responsible for the inhibitor-sensitive electron output, implying that this is the physiological relevant electron transfer to the acceptor ubiquinone.

In the preceding paper [see Heinrich and Werner (1992)], we have reported the identification of the ubiquinone-binding site of complex I of *N. crassa*. Using the photoreactive ubiquinone analogue ^{125}I-$Q_0C_7ArN_3$,[1] a small polypeptide subunit of about 9.5 kDa has been labeled in a significant and specific manner.

This paper presents the characterization of this putative ubiquinone-binding subunit, by its primary structure (as deduced from the cDNA sequence), the predicted properties for the secondary structure, and the rough localization of the polypeptide within complex I. Furthermore, the import of

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; $Q_0C_7ArN_3$, 2,3-dimethoxy-5-methyl-6-[7-(4-azidophenyl)heptanyl]-*p*-benzoquinone.

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the in vitro translated protein into isolated mitochondria was studied.

MATERIALS AND METHODS

Strains and Plasmids. Wild-type strain 74-OR23-1A of *N. crassa* was grown as described (Werner, 1977). *Escherichia coli* strains Y 1088, Y 1090 (Young & Davis, 1983), and DH1 (Low, 1968) were used as hosts for λgt11-derived phages and pGEM4 chimeric plasmids (Promega, Biotech, Madison, WI), respectively.

cDNA Cloning and DNA Sequencing. A sized λgt11 expression library containing cDNA inserts from *N. crassa* (300–900 base pairs) was screened with antibodies. Expression of β-galactosidase fusion proteins of the infected *E. coli* strain (Y 1090) was induced by overlaying the plates with filters impregnated with isopropyl thio-β-D-galactoside (Maniatis et al., 1982). Single positive plaques were obtained by at least two cycles of rescreening and were identified by immunodetection with an antibody. cDNA inserts were subcloned into the transcription vector pGEM4 and were sequenced by applying either the universal T7- and SP6-specific primers or other synthetic oligonucleotides. The sequence of both strands of the denatured plasmid (Chen & Seeburg, 1985) were obtained with the aid of a modified T7 DNA polymerase (Sequenase Version 2.0; U.S. Biochemical Corp.; Tabor & Richardson, 1989) according to the dideoxy-termination method (Sanger et al., 1977), using ^{35}S-labeled dATP (Du Pont–New England Nuclear; 1000 Ci/mmol).

Affinity Purification of Antibodies. The crude antisera were immunopurified against the β-galactosidase fusion proteins bound to filters (Videira et al., 1990), which were obtained by overlaying the plated phages. Therefore, the nitrocellulose filters were washed with TBS buffer (10 mM Tris, pH 7.5, 0.9% NaCl), blocked for 1 h at room temperature in 5% bovine serum albumin in the same buffer, and then incubated for 3–4 h in a 1:50 dilution of antiserum in TBS buffer, containing 0.1% bovine serum albumin. The filters were washed for at least 1 h with several changes of buffer (TBS, TBS plus 0.05% Triton X-100, and finally TBS buffer each containing 0.1% of bovine serum albumin). The immunoglobulins were eluted by immersing the filters for 90 s in 3 mL of 0.2 M glycine hydrochloride, pH 2.3. After the nitrocellulose was removed, the solution was neutralized by the addition of 2 M Tris base and finally diluted 1:15 with 0.1% of bovine serum albumin in TBS buffer. Either the obtained immunoglobulin solution was directly applied to retroblots or the immunoglobulins were collected with the aid of protein A–Sepharose (Pharmacia) and used for immunoprecipitation.

Extraction Methods. Freshly prepared mitochondria or mitochondrial membranes from *N. crassa* were utilized for the subfractionation of complex I.

Sodium bromide treatment of mitochondrial membranes (protein concentration 2 mg/mL in phosphate buffer, pH 8.0) was performed for 1 h at room temperature with different concentrations of NaBr (0–2.5 mM). The particles were pelleted by a centrifugation step (40 000 rpm, 1 h, 4 °C). The supernatants were decanted, and the membrane pellets were gently rinsed with cold phosphate buffer. The proteins of diluted supernatants were precipitated with trichloroacetic acid (10% final concentration) and then washed twice with ice-cold acetone.

Alkaline extraction of mitochondria was carried out at a protein concentration of 0.3 mg/mL according to Fujiki et al. (1982). The pellets were dissolved in Laemmli buffer, and aliquots were subjected to SDS–polyacrylamide gel electrophoresis.

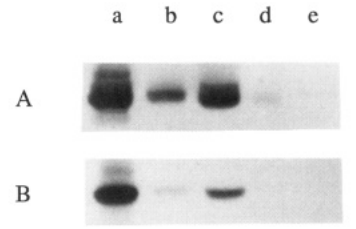

FIGURE 1: Immunoprecipitation of the photolabeled ubiquinone-binding subunit applying crude and immunopurified antibodies, respectively. Eight-hundred micrograms of isolated complex I was affinity labeled using the ^{125}I-$Q_0C_7ArN_3$ analogue of ubiquinone and was dissociated by 2% sodium dodecyl sulfate (a small sample of the preparation of about 80 μg was subjected to lane a). The solution was diluted 20 times with Triton buffer and after a clarifying spin (48000*g*; 20 min), aliquots (about 150 μg of complex I) were incubated for 2 h at 4 °C with the following antibodies bound to protein A–Sepharose: (b) crude antiserum against the 9.5-kDa polypeptide; (c) antibodies immunopurified against the fusion protein obtained from clone λ8 (group I); (d) antibodies immunopurified against the fusion protein obtained from clone λ19 (group II); (e) antibodies immunopurified against a "negative phage", as a control. The protein A–Sepharose was collected, washed with Triton buffer, and treated with 2% SDS, and the dissociated material was analyzed by SDS gel electrophoresis. The different panels represent (A) Western blot immunodecorated with the crude antiserum and (B) an autoradiograph of the blot.

Miscellaneous Techniques. Isolation of *N. crassa* mitochondria (Werner, 1974), preparation of mitochondria for in vitro import experiments (Pfanner & Neupert, 1985), mitochondrial membranes (Werner, 1977), production of specific antisera (Werner & Sebald, 1981), the technique of Western blotting (Towbin et al., 1979), and immunodetection with an alkaline phosphatase conjugated second antibody (Blake et al., 1984) have been described. A preparation of the ubiquinone-binding subunit was obtained by excising proteins of the appropriate molecular weight from nitrocellulose blots after electrophoretic separation on SDS gels. For Edman degradation the protein was eluted from nitrocellulose using 30% trifluoroacetic acid and 40% acetonitrile and for production of antibodies the membrane was solved in dimethyl sulfoxide. Protein sequence determination was carried out as reported by Wachter and Werhahn (1979). Immunoprecipitation of individual polypeptide subunits was performed according to Zauner et al. (1985), with the minor modification that Triton buffer (300 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM Tris, pH 7.5, 1 mM PMSF) was used instead of cholate/desoxycholate buffer.

In vitro transcription of full-length cDNA inserts in pGEM4 was performed with the aid of T7 RNA polymerase as described (Stueber et al., 1984). [^{35}S]Cysteine-labeled 9.5-kDa polypeptide was synthesized in rabbit reticulocyte lysate (N.150, Amersham) in the presence of an amino acid mixture (30–150 μM), containing all amino acids with the exception of cysteine, following the procedure of Pelham and Jackson (1976). Import experiments were carried out as detailed by Hartl et al. (1986). SDS gel electrophoresis was performed in 18.5% polyacrylamide gels according to Laemmli (1970). Potential secondary structures of the 9.5-kDa polypeptide subunit were predicted according to Robson and Garnier (1986). The hydrophilic profiles were determined as detailed by Hopp and Woods (1981).

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-142                                                GCCGACCACGCCGACGACGACG
-120  ATAGCGACGAGAACGAGTGTCCCTCATTCTGTTGACAGGCCACAGTCAGGGCTAGAGAAA
 -60  CGAAAACAAAACAAATACTTCAATACGCAACAACGGCAACGGACGATAGACTAGACAAAT
   1  ATGTCGGGCACAATCCCTCACTTCTGGGCCCAGCCCTTCCGCTACATTCGCTGGTCCGCC
       M S G T I P H F W A Q P F R Y I R W S A

  61  CGCGAGAAGCCCGCCTACTTCTACTCGTGCGTCATCGCCGGACTCGGCCCCGTCTTCCTG
       R E K P A Y F Y S C V I A G L G P V F L

 121  ACGGTCGTGCCACCTGTGCGCAAGTATTTTGGGGATGTCAACCCGGCGCCGATTCCCGTT
       T V V P P V R K Y F G D V N P A P I P V

 181  ACTTATCCTATCCCCACCGGACCTAGGAAGCAACTTACGGGTTATGATGATGATACCGAG
       T Y P I P T G P R K Q L T G Y D D D T E

 241  GAGGCATAAAGGCATGATGGATGTTAAGGTATCGATTGGGCTGATGCTGGAAGAGAGAGA
       E A #

 301  AGGAGAGAAAAGAAAGACAATCACCAAGCGGAGAGACAGTACGCTGTTGTTGTGGACATG
 361  AAGGATTACAGAAACTTCCCAAACAACGTGAACGGTCGAAGTCTGGAGGAAGTATCAGAA
 421  TACAGGATGATATGTACCAGATGAAACGAGATCGATCGGTTCCCCTCTACCTTTTCATCT
 481  TCCAGTGGTTGACGCCGGGGAAACGGGGTGATAGGAAGTCTAATGTATTACGGGCTATAG
 541  TATAGAACTTGATATCTATATATTTGACCAAGGAAACCTCTAGGGCGGTGATCATAGGTA
 601  CGGCCTTGAGGGTCTGTATAATGTTATCCCGACAGATGAGATATATCTCCAAATAAAATC
 661  TTGCTCTATC
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FIGURE 2: cDNA sequence and deduced primary structure of the 9.5-kDa ubiquinone-binding subunit of *N. crassa* mitochondrial NADH: ubiquinone reductase. An in-frame TAG stop codon (9 base pairs upstream of the first ATG triplet) is indicated by a bar. The N-terminus of the mature protein is underlined. A polyadenylation signal is marked by a dashed line.

RESULTS AND DISCUSSION

Identification of the Relevant Clone Coding for the Ubiquinone-Binding Subunit of Complex I. In order to characterize the 9.5-kDa polypeptide subunit, which has been identified as ubiquinone-binding protein in complex I of *N. crassa*, antibodies were raised against subunits of the corresponding molecular weight. The obtained antisera were tested for their ability to precipitate the radioactively labeled 9.5-kDa polypeptide subunit. For this purpose, isolated complex I was specifically labeled with the photoreactive ubiquinone analogue ^{125}I-$Q_0C_7ArN_3$ (see Heinrich and Werner, preceding paper in this issue). Then, complex I was dissociated by SDS and diluted with Triton buffer and the antibodies were applied to isolate the individual subunits. One of these antisera was able to recognize the photolabeled polypeptide (see Figure 1, lane b). This particular antibody was used to screen a λgt11 cDNA expression library of *N. crassa*. Twenty positive clones were identified among 4×10^5 recombinants. After isolation of the positive clones, the crude antiserum was purified against the β-galactosidase fusion proteins of the 20 recombinants. By reprobing these immunopurified antibodies on Western blots of complex I, it was possible to divide the clones into two groups, coding for two polypeptides exhibiting slightly different mobilities on SDS–PAGE. The immunoglobulins showing the strongest reaction of each group were collected by binding to protein A–Sepharose, and then they were assayed for their ability to precipitate the photolabeled polypeptide subunit complex I as described above. The results presented in Figure 1 clearly indicate that the clones comprising group I are coding for the ubiquinone-binding subunit (lane c). This is further supported by the fact that the in vitro synthesized protein could be specifically immunoprecipitated (see below).

Primary Structure of the 9.5-kDa Polypeptide Subunit of Complex I. The nucleotide sequence obtained from group I clones (containing homologous inserts) and the deduced primary structure are depicted in Figure 2. The cDNA insert has a length of 812 base pairs and contains an open reading frame coding for a protein of 82 amino acids. The sequence shows a relative long 5′ untranslated region of 142 base pairs including an in-frame TAG stop codon, located 9 base pairs upstream of the first ATG triplet, indicating that is most probably represents the initiation codon. At its 3′ end the coding region is followed by a noncoding sequence of 424 base pairs, which contains the sequence AATAAA, a typical polyadenylation signal. A molecular mass of 9263 Da was calculated for the protein, which is in agreement with the value of the apparent molecular mass of about 9.5 kDa estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

The N-terminus of the isolated mature polypeptide was determined by Edman degradation, and the obtained amino acid sequence is underlined in Figure 2. The polypeptide is

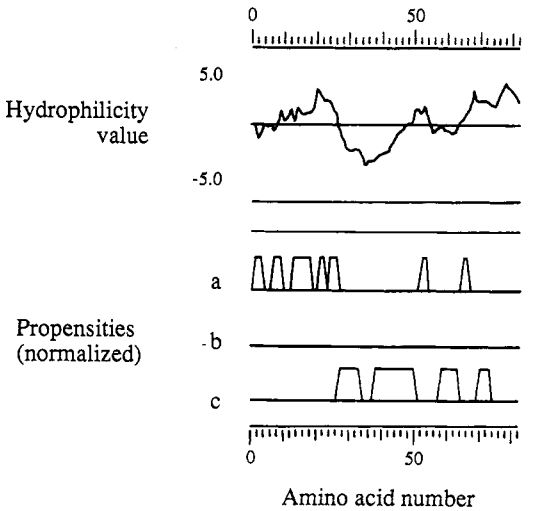

FIGURE 3: Predicted structural features of the 9.5-kDa polypeptide. Upper panel: polarity profile predicted according to Hopp and Woods (1981). Lower panel: secondary structure propensities for β-turns (a), α-helices (b), and β-sheets (c), referring to the approach of Robson and Garnier (1986).

FIGURE 4: Alignment of the amino acid sequence of the *N. crassa* (*N.c.*) 9.5-kDa polypeptide of complex I, the bovine heart (b.h.) 9.5-kDa subunit of complex III (Borchart et al., 1986), and in addition parts of the homologous 11-kDa subunit of yeast (*S.c.*) complex III [see Maarse and Grivell (1987)]. Identical residues in the aligned sequences are shaded. Similar amino acids are indicated by two dots; similarity between the *N.c.* 9.5 kDa and the *S.c.* 11 kDa is marked by only one dot. The underlined region represents the N-terminal sequence of the cleavage peptide involved in the ubiquinone binding (Usui et al., 1990).

composed of two comparatively hydrophilic regions at the N- and C-terminal segments (amino acid residues 6–26 and 64–82) and of a rather hydrophobic stretch in the middle portion of the sequence (Figure 3).

A comparison between the protein sequence of the 9.5-kDa subunit of *N. crassa* complex I and the sequences contained in the latest version of EMBL and SWISS-PROT protein data libraries exhibits a discrete similarity to an equally-sized subunit (Borchart et al., 1986) of beef heart ubiquinol: cytochrome *c* reductase (Figure 4). This similarity is of particular interest because a small polypeptide subunit had been labeled in bovine complex III (additionally to cytochrome *b*) by an ubiquinone analogue (Yu et al., 1985). Recently, Usui et al. (1990) isolated this low-molecular-weight protein and showed its identity with the 9.5-kDa subunit of complex III (subunit VII) by sequencing its N-terminal portion. These investigators also prepared a cleavage peptide of the radioactively labeled ubiquinone-binding protein by trypsin digestion. In Figure 4, the resolved N-terminal sequence of the peptide obtained is underlined.

Similarities are found predominantly at the mainly hydrophobic stretch and in addition at the charged C-terminal portion. However, an alignment with the yeast subunit VIII (11 kDa) of complex III, which was identified as a potential homologue to the beef heart complex III subunit (Maarse & Grivell, 1987), did not support a general homology among these polypeptides. Only a minor portion of amino acid residues is common to all of the three sequences. It is also not clear whether or not this 11-kDa subunit of yeast is identical with a small ubiquinone-binding protein (apparent molecular mass of 14 kDa) which had been affinity labeled in yeast mitochondria (Yu et al., 1986).

A comparison of the amino acid sequences of putative reaction centers from different proteins interacting with quinones shows the conservation of distinct amino acids within a given species (e.g., the invariant P-E-W triplet in the sequences of cytochromes *b* and b_6) but exhibits no explicit sequence motif. It has been proposed by Friedrich and coworkers (1990) that the sequence of the product, encoded by the mitochondrial ND1 gene of complex I, is related to that of the bacterial glucose dehydrogenase. The alignment, presented to conclude a ubiquinone-binding site, is mainly based on a comparison of hydrophobicity profiles of these

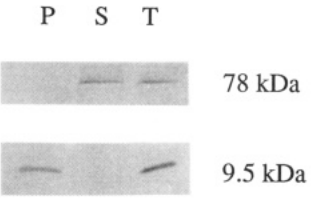

FIGURE 5: Alkaline extraction of *N. crassa* mitochondria. Mitochondria were treated with a 0.1 M sodium carbonate solution, pH 11.5, as detailed by Fujiki et al. (1982). The obtained pellet (P), the proteins precipitated from the supernatant (S), and total mitochondria (T) were subjected to SDS–polyacrylamide gel electrophoresis. The distribution was analyzed on a Western blot with purified antibodies and visualized by a second antibody conjugated with alkaline phosphatase.

proteins. It is a small wonder that chemically similar amino acids are found within hydrophobic domains and predicted membrane-spanning helices. Beyond this coincidence we were not able to detect a significant relationship between the ND1 gene product and the glucose dehydrogenase or even a conserved "ubiquinone-binding domain motif". Moreover, the selected amino acid section of the glucose dehydrogenase is located out of the sequence region, which is highly conserved in these bacterial quinoproteins.

Secondary Structure of the 9.5-kDa Subunit and a Topological Approach in Complex I. Predominantly β-sheets and β-turns, but no α-helical region were predicted by secondary structure analysis for the 9.5-kDa subunit (Figure 3). The middle portion of the protein reveals a hydrophobic segment of sufficient length for a membrane-spanning domain.

Thus, the following question is raised: where is the 9.5-kDa ubiquinone-binding subunit located within complex I? According to the results obtained by fractionation of both the isolated enzyme (Friedrich et al., 1989) and membrane crystals of complex I (Hofhaus et al., 1991) by NaBr treatment, we applied this chaotropic agent to mitochondrial membranes. It turned out that at an applied concentration of 2 M NaBr the 9.5-kDa subunit remains in the membrane fraction. The 78-kDa subunit (Preis et al., 1991), which is known to reside in the "soluble part" of the complex, was used as a control. Indeed, the major amount of the 78-kDa component was found in the supernatant. An increase of the concentration to 2.5 M NaBr resulted in a complete extraction of the 78 kDa, yet it simultaneously resulted in a partial removal of the 9.5-kDa

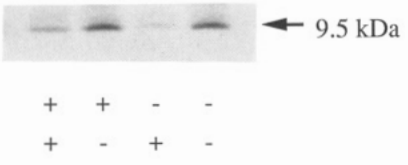

FIGURE 6: Import of in vitro synthesized ubiquinone-binding protein into isolated *N. crassa* mitochondria. Freshly prepared mitochondria were incubated with the ^{35}S-labeled 9.5-kDa polypeptide in the presence or absence of a membrane potential ($\Delta\psi$), i.e., without or with addition of valinomycin. After proteinase K (PK) treatment (200 μg/mL), the pelleted mitochondria were dissociated by SDS and the 9.5-kDa subunit was precipitated by specific immunoglobulins. The material was resolved on a SDS–PAGE and autoradiographed.

subunit (data not shown). An alternative method to discriminate the peripheral from the intrinsic membrane proteins is the alkaline extraction by carbonate solution, established by Fujiki and co-workers (1982). Figure 5 shows the results of the alkaline treatment applying whole mitochondria of *Neurospora*. Under these conditions a clear-cut result is obtained. The 9.5-kDa ubiquinone-binding subunit resists against this treatment, whereas the 78-kDa "control polypeptide" is completely stripped off from the enzyme. These findings lead to the conclusion that the 9.5-kDa subunit is indeed an intrinsic membrane protein. However, the question remains open as to how this small polypeptide is embedded in the hydrophobic membrane portion and which subunits are located in the direct neighborhood.

Import of the 9.5-kDa Subunit into Isolated Mitochondria of N. crassa. Radioactively labeled 9.5-kDa protein was synthesized by in vitro expression in a reticulocyte lysate using ^{35}S-cysteine. The ^{35}S-labeled polypeptide could be specifically precipitated with the purified (monospecific) antibody. Isolated mitochondria were incubated with the translation assay in the presence or absence of a membrane potential ($\Delta\psi$). The import efficiency of the 9.5-kDa polypeptide was demonstrated by resistance of the subunit (protected inside the mitochondria) against proteinase K digestion.

The ubiquinone-binding protein is synthesized without a typical presequence (see Figure 2) and shows no processing during the import, as judged by its electrophoretic behavior (Figure 6). The import into isolate mitochondria is enhanced by a membrane potential. Whether the small amount of the polypeptide, which resists proteinase K treatment (Figure 6, lane 3), reflects a low sensitivity to proteases or can be attributed to a partial or complete translocation through the outer membrane remains unclear. Analysis of immunoprecipitates obtained by applying an antiserum against whole complex I indicates that a minor portion of the in vitro imported subunit becomes as assembled (data not shown).

ADDED IN PROOF

The sequence of the 9.5-kDa subunit of *N. crassa* complex I is related to that of a 9-kDa subunit (named B9) from bovine heart complex I published recently [see Walker et al. (1992)].

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