SYNTHESIS OF A LARGER PRECURSOR FOR THE PROTEOLIPID SUBUNIT OF THE MITOCHONDRIAL ATPase COMPLEX OF *NEUROSPORA CRASSA* IN A CELL-FREE WHEAT GERM SYSTEM

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1. Introduction

The proteolipid subunit of the ATPase complex from *Neurospora crassa* [1] is synthesized on cytoplasmic ribosomes [2] and coded for by a nuclear gene [3]. This raises the question of how this extremely hydrophobic protein [4] is transported into the mitochondria and how it is assembled with the numerous other subunit polypeptides to form the functional ATPase complex located in the mitochondrial inner membrane. Translation of polyadenylated RNA from *Neurospora crassa* in a cell-free system of wheat germ resulted in the synthesis of a polypeptide with an app. mol. wt ~12 000 that was specifically immunoprecipitated by antibodies raised against the authentic 8000 mol. wt proteolipid. The in vitro synthesis of a higher molecular weight precursor of the ATPase subunit is reminiscent of the preforms identified after in vitro synthesis of hormones and other secretory proteins (see e.g., [5]). We propose that the additional amino acid sequence plays a role either during the transfer of the proteolipid into the mitochondrion or during the assembly of the mitochondrial ATPase complex.

2. Materials and methods

Hyphae of *Neurospora crassa* (wild-type 74A) were grown into late exponential phase as in [6].

This paper is dedicated to Professor Th. Bücher on the occasion of his 65th birthday

For the extraction of total RNA 25 g hyphae (wet wt) were vigorously shaken with 100 ml extraction medium (medium I/medium II 1:1 (v/v); medium I: 10 mM Tris–HCl, 50 mM KCl, 6% *p*-aminosalicyclic acid, 1% triisopropylnaphthalene sulfonic acid (pH 8.0); medium II: phenol/chloroform/isoamylalcohol 50:49:1 (v/v/v) and 75 g sterilized quartz sand for 20 min at room temperature. The resulting homogenate was ground for a further 10 min in a mortar. The slurry was centrifuged for 12 min at 27 000 × *g*. The upper liquid phase was extracted 2 times with medium II and then with ether. RNA was precipitated with 2 vol. ethanol at −20°C. The yield of total RNA was 2000–2500 A_{260}/25 g hyphae (wet wt). About 1% of the total RNA was recovered as polyadenylated RNA (poly(A)-RNA) by affinity chromatography on poly(U)–cellulose, as in [7].

Fresh commercial wheat germ was supplied by the 'Kraemer'sche Kunstmühle München KG. The 30 000 × *g* supernatant (S-30) was prepared exactly as in [8] except that the preincubation step was omitted. The composition of the standard protein synthesis mixture in [8] was used. Application of 0.3 mCi [^{3}H]leucine (New England Nuclear, Boston, MA) (50–55 Ci/mmol) and 2.5 A_{260}/ml incubation mixture resulted in the incorporation of 100–150 × 10^6 dpm into hot trichloroacetic acid-precipitable material. Incorporation in the absence of poly(A)-RNA was 10-times lower.

Antisera against the ATPase proteolipid from *Neurospora crassa* were raised in rabbits and the immunoglobulin fraction was prepared as in [1].

3. Results

Poly(A)-RNA from *Neurospora crassa* is efficiently translated by a cell-free wheat germ system into polypeptides of mol. wt 7000–60 000 (fig.1). Antibodies directed against the proteolipid subunit of the mitochondrial ATPase complex were used to investigate whether the authentic polypeptide or possibly a precursor with a higher molecular weight is synthesized in this in vitro system. At optimal concentrations of these specific immunoglobulins, 0.5% of the total translation products could be isolated as antigen–antibody complex. Gel electrophoretic analysis revealed that the immunoprecipitation is highly specific since the recovered in vitro product migrates as a single band of app. mol. wt 12 000 (fig.2). During chromatography on Biogel P-30 in the presence of 80% formic acid, the same material was eluted in one peak slightly before cytochrome *c* (data not shown). In contrast, the isolated authentic ATPase

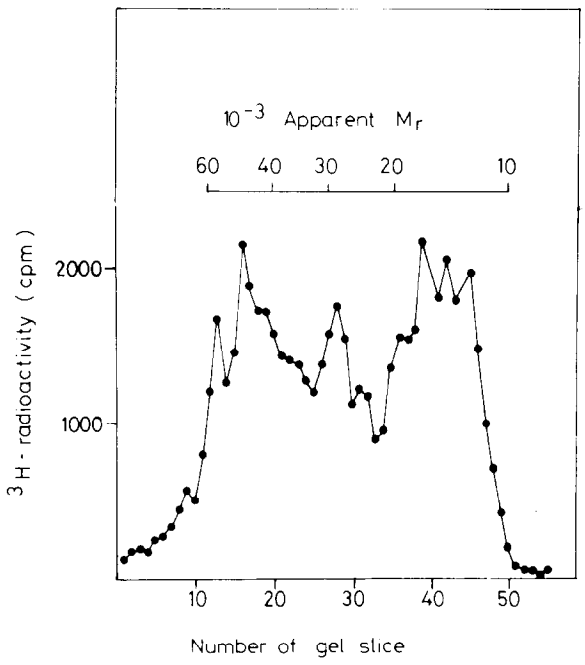

Fig.1. Total translation products of a cell-free wheat germ system in response to poly(A)-RNA from *Neurospora crassa*. The hot acid-insoluble radioactivity incorporated during a 60 min incubation period was analysed by dodecylsylfate–gel electrophoresis on 15% gels [9].

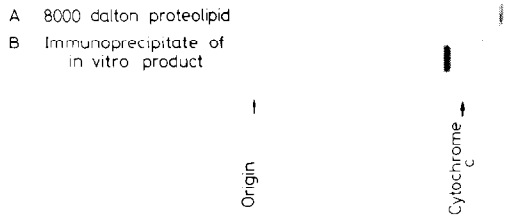

Fig.2. Coelectrophoresis of (A) the authentic ATPase proteolipid and (B) the immunoprecipitate of the in vitro product. (A) the [^{3}H]leucine-labelled proteolipid (4.5 × 10^6 dpm/mg) was isolated from *Neurospora* cells grown in the presence of [^{3}H]leucine [1]. (B) cell-free wheat germ system (5 ml) (see section 2) was incubated for 60 min with poly(A)-RNA from *Neurospora*. The whole mixture was treated with sodium dodecylsulfate and Triton X-100 as detailed in [10]. Immunoglobulins directed to the ATPase proteolipid (1.25 mg) were added and after incubation for 3 h at room temperature the antigen–antibody complexes were bound to 62.5 mg protein A–Sepharose CL-4B (Pharmacia, Uppsala) by a further incubation for 2 h. The Sepharose was washed twice with 1% Triton X-100 in 0.1 M phosphate buffer (pH 8.0), twice with phosphate buffer and finally with water. The protein was solubilized with 1 ml 80% formic acid. The recovered ^{3}H-labelled material (3.5 × 10^6 dpm) amounted to 0.49% of the total hot acid-insoluble radioactivity present in the incubation mixture. Aliquots of the proteins corresponding to 15 × 10^3 dpm were analysed by polyacrylamide gel electrophoresis in sodium dodecylsulfate [9] and fluorography of the stained gels [11].

proteolipid, which was analysed as a control on the same gel slab, exhibited a much higher electrophoretic mobility corresponding to an app. mol. wt 8000 in excellent agreement with the known amino acid sequence [4].

The precipitation of only the 12 000 mol. wt product from the wheat germ protein synthesis mixture indicates the specificity of the applied immunoglobulins raised against the authentic proteolipid. It may be mentioned that with the same immunoglobulins only the 8000 mol. wt proteolipid could be precipitated from whole mitochondria. This strongly suggests that antigenic sites of the 8000 mol. wt proteolipid are present in the 12 000 mol. wt wheat germ product. Actually, amino acid sequence studies prove that the 8000 mol. wt proteolipid is part of the 12 000 mol. wt in vitro product as shown by the experiment described in fig.3. A mixture of

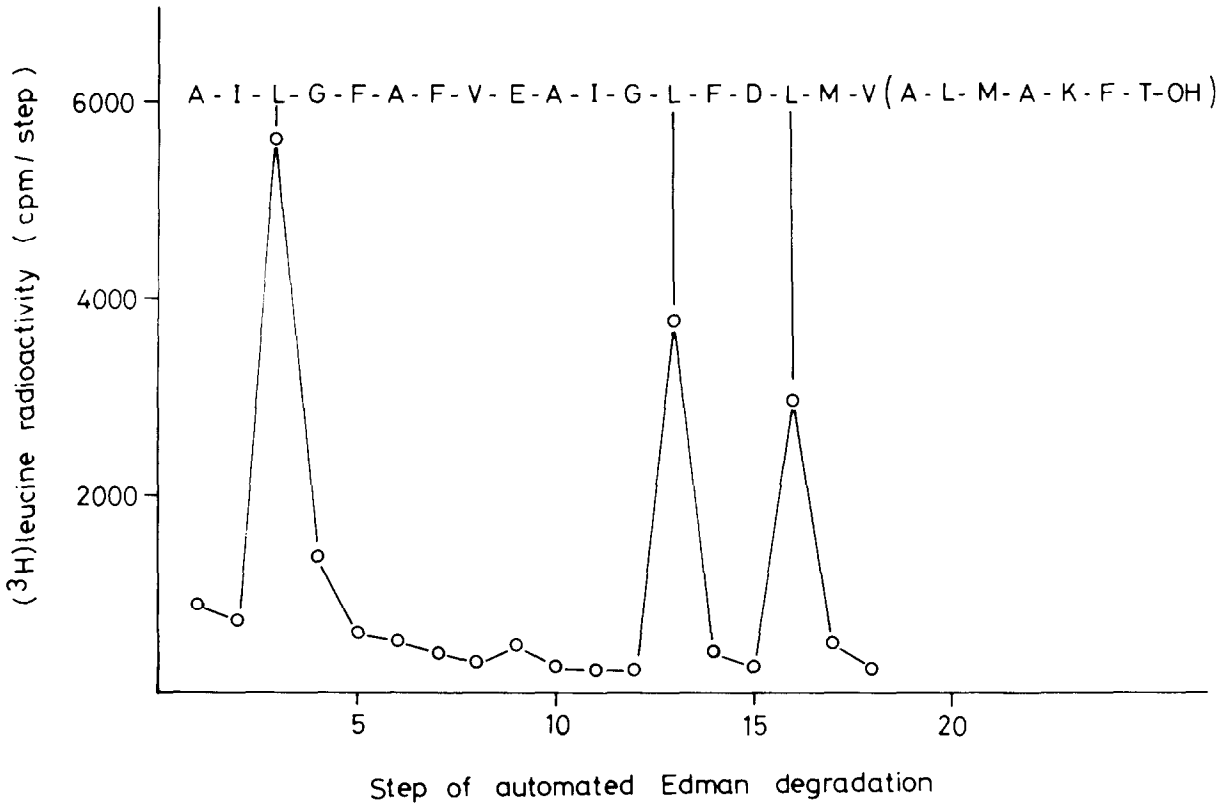

Fig.3. Sequence analysis of a 25-residue fragment obtained from the immunoprecipitated in vitro product. The immunoprecipitate described in fig.2 (1.8×10^6 dpm) was mixed with 1 mg authentic proteolipid and cleaved with *N*-bromosuccinimide. The C-terminal 25-residue fragment (positions 57–81) was isolated by chromatography on Biogel P-30 in the presence of 80% formic acid and coupled to isothiocyanate–glass and then submitted to 19 cycles of automated solid phase Edman degradation. The details of these procedures have been described [4]. Each cycle was analysed for the released amino acid as well as for [^{3}H]leucine radioactivity.

the [^{3}H]leucine labelled 12 000 mol. wt product and unlabelled authentic proteolipid were incubated with *N*-bromosuccinimide. The proteolipid contains two tyrosine residues in positions 1 and 56 of the polypeptide chain and can be specifically cleaved after this residues with *N*-bromosuccinimide, since no tryptophan and histidine are present [4]. The resulting fragments were submitted to gel chromatography. The C-terminal fragment of the proteolipid was eluted as well as separated peak together with a fragment of the in vitro product (data not shown), and this fraction was analysed by automated solid-phase Edman degradation [4]. The amino acids identified in steps 1–18 were in agreement with the known sequence of this fragment. The [^{3}H]leucine of the in vitro product was cleaved off in steps 3, 13, 16. The three leucine residues of the proteolipid fragment occur at the same positions. The coincidence of the sequence of the leucine residues as well as the same gel chromatographic behaviour indicate that both fragments are identical.

4. Discussion

In many instances, precursors of secreted proteins in eukaryotic (see, e.g. [5] and procaryotic (see, e.g. [12]) cells could be identified in cell-free protein synthesizing systems. In the case of organelle proteins, however, only marginal information on primary

translation products is available. Both types of proteins have to be transported through membranes. Thus, their biogenetic pathways may share common mechanisms for the specific recognition of, and the translocation through, biological membranes. Studies on the in vitro synthesis of a soluble chloroplast protein, the small subunit of ribulose-1,5-biphosphate-carboxylase, resulted in the identification of a putative precursor with mol. wt 4000 larger than the authentic subunit [13]. Remarkably, in the case of catalase and uricase, two soluble peroxisomal enzymes, similar studies failed to establish any differences in size of the in vitro and in vivo products [10,14]. No information is available at present on primary translation products and post-translational modification of mitochondrial proteins and especially of intrinsic membrane proteins.

The present study on the synthesis of the proteolipid subunit of the mitochondrial ATPase complex in a cell-free wheat germ system resulted in the identification of a 12 000 mol. wt translation product, which is recognized by antibodies directed specifically to the isolated proteolipid. Furthermore, common amino acid sequences could be established in the wheat germ product and the authentic proteolipid. In many instances it has been documented that poly(A)-RNA is correctly translated in the heterologous cell-free wheat germ system. Thus, it can be concluded that the 12 000 mol. wt polypeptide represents a primary translation product and consequently the precursor of the proteolipid. At present the biological significance of the additional amino acid sequence of ~35 residues remains unknown. Firstly, the precursor may represent a water-soluble form of the proteolipid which is possibly necessary for the transport of this extremely hydrophobic protein through the cytosol. It may be significant in this respect that the precursor can be quantitatively recovered in the post-ribosomal supernatant of the cell-free wheat germ system. Secondly, the extra sequence could exert a functional role during the assembly of the ATPase complex in analogy to the assembly of viral proteins (formative proteolysis) [15]. This appears to be unlikely, however, since the homologous proteolipid of the yeast ATPase has no precursor. In this microorganism, the proteolipid is synthesized inside the mitochondria and accordingly it has formylmethionine as N-terminal amino acid [4]. Furthermore, the DNA sequence of the structural gene contains only the codons of the authentic proteolipid [16]. Thirdly, our result may be interpreted in terms of the 'signal hypothesis' [17] postulated as a mechanism for the transport of hormones and other secretory proteins. It is possible that the transport of the cytoplasmically synthesized proteolipid of *Neurospora crassa* across the mitochondrial outer membrane involves presequences similar to those found for excreted proteins.

Acknowledgements

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