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Cell-Free Synthesis of the Mitochondrial ADP/ATP Carrier Protein of *Neurospora crassa*

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ADP/ATP carrier protein was synthesized in heterologous cell-free systems programmed with *Neurospora* poly(A)-containing RNA and homologous cell-free systems from *Neurospora*. The apparent molecular weight of the product obtained *in vitro* was the same as that of the authentic mitochondrial protein. The primary translation product obtained in reticulocyte lysates starts with formylmethionine when formylated initiator methionyl-tRNA (fMet-tRNA$_f^{Met}$) was present. The product synthesized *in vitro* was released from the ribosomes into the postribosomal supernatant.

The evidence presented indicates that the ADP/ATP carrier is synthesized as a polypeptide with the same molecular weight as the mature monomeric protein and does not carry an additional sequence.

The ADP/ATP carrier is a major protein of the inner mitochondrial membrane, with catalyses the exchange diffusion of ADP and ATP [1,2]. It is believed to be integrated into the membrane as a dimer in a transmembraneous fashion [3]. It can be isolated after disintegrating mitochondria with detergents and is soluble only in the presence of detergents [4]. This protein is known to be translated on cytoplasmic ribosomes [5,6]. The mechanism of its transfer to the mitochondria and its integration into the functional sites in the membrane remains obscure. Kinetic studies *in vivo* and *in vitro* have led to the conclusion that extramitochondrial precursors of mitochondrial proteins exist and that mitochondrial proteins are translocated by a posttranslational mechanism [6,7]. Specifically, extramitochondrial forms of cytochrome *c* and of subunits of oligomycin-sensitive ATPase have been described [8,9].

The ADP/ATP carrier was chosen as an example of a hydrophobic integral inner membrane protein. As a first step in elucidating the transfer mechanism we have investigated the structure and properties of the original translation product of this protein. In this report it is shown that the ADP/ATP carrier of *Neurospora crassa* can be synthesized in heterologous and homologous cell-free systems. We present evidence that it is synthesized as a precursor with an apparent molecular weight indistinguishable from that of the mature protein. A preliminary account of part of these studies has been published [10].

MATERIALS AND METHODS

Organism and Cultures

Neurospora crassa (wild type 74A) was grown and labelled with [^{35}S]sulfate (NEN Chemicals, Boston, Mass., U.S.A.) as described previously [8].

Preparation of Cell-Free Homogenate and Cell Fractionation

Cell-free homogenates were prepared and isolation of cellular fractions carried out according to published procedures [8].

Protein Synthesis in the Homologous Cell-Free System

Incorporation of [^{3}H]leucine (100 Ci/mmol, NEN Chemicals, Boston, Mass., U.S.A.) was performed as described [8] with the following modifications. Chloramphenicol and mercaptoethanol were omitted; 0.03 M KCl, 0.002 M EDTA and 0.09 M triethanolamine buffer, pH 7.4, were included in the medium.

Isolation of Neurospora *Poly(A)-Containing RNA*

Neurospora cells (25 g wet weight) were frozen with liquid nitrogen immediately after harvesting. They were ground in a mortar and pestle until more than 50% of the hyphae were broken as judged from microscopic examination. For extraction of RNA,

100 ml of medium A was added. Medium A consisted of phenol mixture/detergent medium/chloroform/isoamyl alcohol (50/25/24.5/0.5, v/v/v/v). Phenol mixture was prepared from 1000 g phenol, 95 ml freshly distilled *m*-cresol and 1 g 8-hydroxyquinoline and saturated with 10 mM Tris-HCl, pH 8.2 [11]. Detergent medium contained 1% tris(isopropyl)naphthalene sulphonate, 6% *p*-aminosalicylate, 50 mM KCl, 10 mM Tris-HCl, pH 8.2 [11]. To the mixture of broken hyphae and medium A sodium dodecylsulfate was added to a final concentration of 1% and the mixture shaken vigorously for 15 min at room temperature. The organic phase was separated from the aqueous phase by centrifugation at $30000 \times g$ for 15 min. The aqueous phase was collected and extracted with 3 vol. medium B (phenol mixture/chloroform/isoamyl alcohol (50/24.5/0.5, v/v/v). The organic phase and the interface were re-extracted with an equal volume of medium A. The aqueous phases were pooled and reextracted with 3 vol. medium B; 2 vol. ethanol, which was cooled to $-20\,^{\circ}C$, were added and the mixture was kept at $-20\,^{\circ}C$ overnight. The precipitated nucleic acids were sedimented by centrifugation at $12000 \times g$ for 20 min at $-20\,^{\circ}C$. The pellet was washed twice with 60% ethanol (v/v) at $-20\,^{\circ}C$ and dried over $CaCl_2$ under vacuum. Poly(A)-containing RNA was isolated using chromatography on oligo(dT)-cellulose (Boehringer, Mannheim, F.R.G.) according to Aviv and Leder [12] with the following modification. Poly(A)-containing RNA was precipitated without addition of CH_3COOK, finally dissolved in H_2O at a concentration of 1 mg/ml and stored in aliquots at $-70\,^{\circ}C$. The average yield was about 100 mg of total nucleic acids and 0.2–0.4 mg of poly(A)-containing RNA.

Preparation of N-*Formyl[*^{35}*S]methionyl-tRNA*$_f^{Met}$

$tRNA_f^{Met}$ from calf liver was aminocylated and formylated using homogeneous *Escherichia coli* methionyl-tRNA synthetase [13] and *E. coli* $100000 \times g$ supernatant [14]. The reaction conditions were: 0.01 M $MgCl_2$, 7.5 mM ATP, 2.8 mM 2-mercaptoethanol, 5 µM [^{35}S]methionine (spec. act. > 500 Ci/mmol, NEN Chemicals, Boston, Mass., U.S.A.), 0.05 M Tris-HCl, 0.8 mM 10-formyltetrahydrofolate (prepared from folinic acid, obtained from Serva, Heidelberg, according to Dubnoff and Maitra [14]), 3 mg/ml bulk tRNA from calf liver (Boehringer, Mannheim, F.R.G.), 20 µl *E. coli* methionyl-tRNA synthetase (containing 40 µg protein), 100 µl *E. coli* $100000 \times g$ supernatant (containing 50 µg protein) in a final volume of 1 ml. Incubation was carried out for 20 min at 37 °C. Sodium acetate was added to a final concentration of 0.2 M, pH 4.5, and the mixture was passed through a reversed-phase chromatography column (RPC 5, Serva, Heidelberg, F.R.G.) to separate *N*-formyl[^{35}S]methionyl-$tRNA_f^{Met}$ from free [^{35}S]methionine and unformylated [^{35}S]methionyl-tRNA. Chromatography was carried out by a modification of the procedure of Kelmers and Heatherly [15], employing a linear gradient of 0.35–0.85 M NaCl with a flow rate of 2–3.5 ml/min. Fractions of 1.2 ml were collected. Fractions 5–15 contained unreacted [^{35}S]methionine, fractions 31–55 formyl[^{35}S]methionyl-tRNA. The latter fractions were pooled and desalted on a column of Biogel P4 (100 × 1 cm) (100–200 mesh, Bio-Rad Laboratories, München, F.R.G.). The eluate was dried in a rotary evaporator and the residue taken up in 350 µl H_2O. It contained 0.04 mg of the aminoacyl-tRNA and 0.5 mCi ^{35}S radioactivity. The sample was brought to pH 4.5 by addition of 1 µl of 2 M sodium acetate, pH 4.5. Aliquots of 35 µl were frozen at $-70\,^{\circ}C$. In order to confirm that the labelled product was *N*-formylmethionyl-tRNA a digestion was performed with pancreatic ribonuclease and thin-layer electrophoresis on cellulose plates was performed according to Fraser and Rich [16]. Scanning of the thin-layer plate demonstrated that only *N*-formyl-[^{35}S]methionyl-adenosine was obtained.

Cell-Free Protein Synthesis in Reticulocyte Lysate and Wheat Germ Extracts

Reticulocyte lysates were prepared according to Hunt and Jackson [17] from rabbits which had been made anaemic by injection of phenylhydrazine as described by Allen and Schweet [18]. The lysates were stored in aliquots of 0.8 ml at $-70\,^{\circ}C$. Cell-free protein synthesis in reticulocyte lysates was carried out according to Pelham and Jackson [19] and in wheat germ extracts according to Roberts and Paterson [20]. 1 ml of incubation mixture contained 40 µg poly(A)-containing RNA and either 500 µCi [^{35}S]-methionine (spec. act. > 500 Ci/mmol) or 50 µCi f[^{35}S]Met-tRNA. When fMet-tRNA was used unlabelled methionine was also present (12.5 µM).

Immunoprecipitation

Antibodies against purified ADP/ATP carrier were obtained as described [6]. Immunoglobulin preparations were prepared from antisera by ammonium sulfate precipitation. For immunoprecipitation, samples were made 1% in Triton X-100 and 0.3 M in KCl. The lysates were clarified by centrifugation for 30 min at $114000 \times g$ at 2 °C.

With *Neurospora* cell-free homogenates and with isolated mitochondria, precipitation was performed directly by adding appropriate amounts of immunoglobulins followed by incubation for 4 h at 4 °C. With reticulocyte lysates the following procedures were carried out: (a) direct precipitation in the presence of 10 µg purified ADP/ATP carrier; (b) addition of

reduced amounts of immunoglobulin (1/10 of the amount used in direct precipitation) followed after 5 min by addition of Sepharose-bound protein A (Pharmacia, Uppsala, Sweden) and incubation for a further 10 min; (c) addition of immunoglobulins, after 30 min addition of antibodies from sheep to rabbit immunoglobulin and incubation for 12 h at 4 °C. Immunoprecipitates were washed and dissolved in sodium-dodecylsulfate-containing buffer as described [8].

Gel Electrophoresis

Horizontal slab gel electrophoresis and slicing of gels were carried out as described [8]. Electrophoresis on phenol/formic acid gels was performed as reported by Sebald et al. [21]. Vertical slab gel electrophoresis was made as described by Laemmli [22]. The dried gels were exposed to Agfa-Gevaert Curix RP1 X-ray film. Other methods for determination of radioactivity were reported earlier [8].

Deformylation of N-*Formylmethionine-Labelled Protein and Edman Degradation*

Immunoprecipitated protein was dissolved in 100 µl 2% sodium dodecylsulfate, 2.5% 2-mercaptoethanol; 1 ml methanol/6 M HCl (10/1, v/v) was added and the sample kept for 2 h at 37 °C as described by Marahiel et al. [23]. Protein was precipitated by addition of an equal volume of ice-cold acetone. The resulting pellet was washed with acetone and dried over $CaCl_2$ under vacuum.

Immunoprecipitates were subjected before and after deformylation to Edman degradation according to the procedure described by Blombäck et al. [24]. The ethylene chloride extracts containing phenylthiohydantoin derivatives were dried under nitrogen, dissolved in sodium-dodecylsulfate-containing buffer and counted in a Packard Tricarb scintillation spectrometer.

Cyanogen Bromide Cleavage

Cyanogen bromide cleavage of immunoprecipitated ADP/ATP carrier was carried out as described [8]. Cleavage products were dissolved in phenol/formic acid and subjected to electrophoresis according to Sebald et al. [21].

RESULTS

Synthesis of ADP/ATP Carrier in Rabbit Reticulocyte Lysates and Wheat Germ Extracts Programmed with Poly(A)-Containing RNA

Poly(A)-containing RNA from *Neurospora* stimulated the incorporation of [^{35}S]methionine into proteins in rabbit reticulocyte lysates by a factor of about 10 and in wheat germ extracts by a factor of 15. ADP/ATP carrier was immunoprecipitated from postribosomal supernatants with antibodies against the protein isolated from *Neurospora*. The immunoprecipitates were subjected to gel electrophoresis in the presence of sodium dodecylsulfate. To the same gel ADP/ATP carrier was applied which was isolated by immunoprecipitation from mitochondria prepared from cells grown in the presence of [^{35}S]sulfate. Fig. 1 shows an autoradiograph of a dried gel. The authentic protein forms a single band with an apparent molecular weight of 32000. The products synthesized *in vitro* show identical electrophoretic mobility to the product obtained *in vivo*. Experiments with eight different reticulocyte lysates gave consistent results. The same result was obtained whether immunoprecipitation was carried out with Sepharose-bound protein A, with antibodies from sheep to rabbit immunoglobulin or using the isolated protein as a

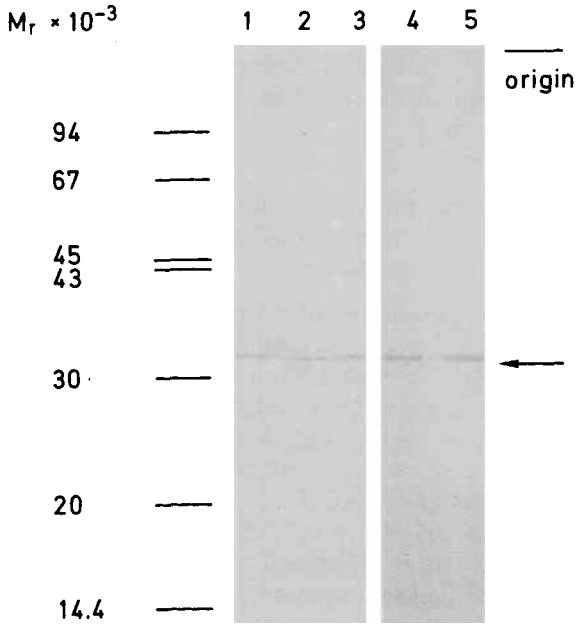

Fig. 1. *Comparison of ADP/ATP carrier protein from mitochondria and protein synthesized in reticulocyte lysate and wheat germ extract.* ADP/ATP carrier protein synthesized in a rabbit reticulocyte lysate and wheat germ extract programmed with *Neurospora* poly(A)-containing RNA in the presence of [^{35}S]methionine was immunoprecipitated after adding 10 µg of the purified protein. Authentic ADP/ATP carrier was immunoprecipitated from mitochondria isolated from *Neurospora* which had been grown in the presence of [^{35}S]sulfate. Immunoprecipitates were analysed by gel electrophoresis and autoradiography. Tracks 1 and 3, products obtained *in vitro* from two different reticulocyte lysate preparations; tracks 2 and 4, protein from mitochondria labelled *in vivo*; track 5, product obtained *in vitro* from wheat germ extract. The molecular weight scale was obtained by coelectrophoresis of marker proteins. The arrow indicates the position of protein stain of ADP/ATP carrier

carrier. These observations suggest that the ADP/ATP carrier is synthesized in a form which has the same or very similar apparent molecular weight as the mature protein and that it is released into the postribosomal supernatant.

Integrity of the Amino Terminus of the ADP/ATP Carrier Synthesized in the Reticulocyte Lysate

Since it cannot be ruled out that the ADP/ATP carrier made *in vitro* is originally made as a larger precursor but artefactually processed to the mature size in the heterologous systems, the following experiment was carried out. Protein synthesis in the reticulocyte lysate was performed in the presence of calf [^{35}S]methionyl-tRNA which was aminoacylated with methionyl-tRNA synthetase from *E. coli* and formylated with *E. coli* transformylase. This procedure leads to the selective aminocylation and successive formylation of initiator methionyl-tRNA, $tRNA_f^{Met}$ [25]. Formylmethionine is incorporated into the amino-terminal positions in reticulocyte lysates. In eurocaryotes, formylated methionine is known not to be removed by methionine aminopeptidase [26–28]. ADP/ATP carrier was immunoprecipitated and analysed by gel electrophoresis. An autoradiograph of the gel (Fig. 2) clearly shows that the protein starting with formyl[^{35}S]methionine has the same electrophoretic mobility as the authentic protein.

In order to confirm that only the amino-terminal initiator methionine was labelled in the product, Edman degradation was carried out with the immunoprecipitated product before and after deformylation. Table 1 shows the radioactivities released in the first Edman cycle and retained in the protein. Before deformylation 0.8% of total radioactivity was released, after deformylation 31%. With an aliquot it was shown that the deformylation procedure left the protein virtually intact (Fig. 2). The extent of deformylation (approx. 30%) under the particular conditions is comparable to that reported for other proteins [23, 27, 28].

Synthesis of ADP/ATP Carrier in a Homologous Cell-Free Homogenate

A cell-free homogenate was prepared from ^{35}S-labelled *Neurospora* cells with a medium optimized for amino acid incorporation. This homogenate performs 'read out' of nascent chains [7]. [^{3}H]Leucine was incorporated and ADP/ATP carrier was immunoprecipitated and analysed by gel electrophoresis. The product synthesized *in vitro* and the mature protein displayed the same apparent molecular weight (Fig. 3). In a similar experiment [^{3}H]leucine was incorporated into a homogenate from unlabelled cells and ADP/ATP carrier was immunoprecipitated. An immunoprecipitate of the carrier was also obtained from mitochondria isolated from whole cells labelled with [^{14}C]leucine. The immunoprecipitates were mixed and subjected to cyanogen bromide cleavage. The fragments were only poorly soluble in dodecylsulfate-containing buffer and were therefore analysed on phenol/formic acid polyacrylamide gels (Fig. 4). ^{3}H

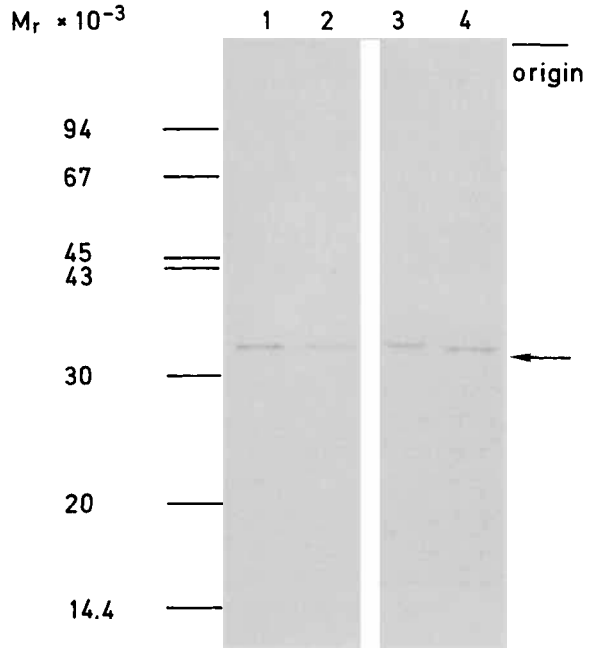

Fig. 2. *Synthesis of ADP/ATP carrier* in vitro *in the presence of* N-*formyl*[^{35}*S*]*methionyl-tRNA*$_f^{Met}$. Formyl[^{35}S]methionyl-tRNA was incorporated into protein in a reticulocyte lysate. ADP/ATP carrier was immunoprecipitated employing Sepharose-bound protein A and subjected to electrophoresis. The autoradiograph of the dried gel is shown. Tracks 1 and 4, ADP/ATP carrier precipitated from mitochondria isolated from cells grown on [^{35}S]sulfate; track 2, product synthesized *in vitro*; track 3, product synthesized *in vitro* after deformylation. The arrow indicates the position of protein stain of ADP/ATP carrier

Table 1. *Edman degradation of ADP/ATP carrier protein synthesized* in vitro *in the presence of* N-*formyl*[^{35}*S*]*methionyl-tRNA*$_f^{Met}$
ADP/ATP carrier was labelled *in vitro* and isolated as described in Fig. 2. The immunoprecipitate was divided into two portions. One portion was subjected to deformylation treatment, the other served as a control. Edman degradation was performed with both portions. Radioactivity which was found in the ethylene chloride extract (containing phenylthiohydantion derivatives) and radioactivity which remained with protein is shown

Fraction	^{35}S radioactivity	
	control	after deformylation
	counts × min^{-1}	
Ethylenechloride extract	34	735
Residual protein	3985	1666

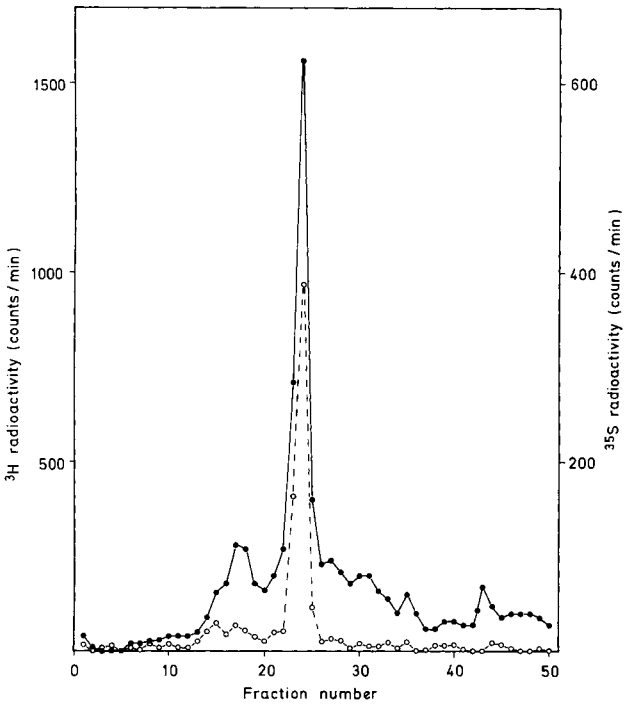

Fig. 3. *Comparison of ADP/ATP carrier synthesized in a* Neurospora *cell-free system and authentic protein.* [^{3}H]Leucine was incorporated into a cell-free homogenate obtained from cells grown on [^{35}S]sulfate. After a 5-min incubation, the homogenate was cooled to 0 °C, lysed by addition of Triton X-100, centrifuged to sediment ribosomes, and subjected to direct immunoprecipitation. The precipitate was analysed by electrophoresis in the presence of dodecylsulfate. Radioactivities were determined in gel fractions after slicing. (●——●) ^{3}H; (○----○) ^{35}S

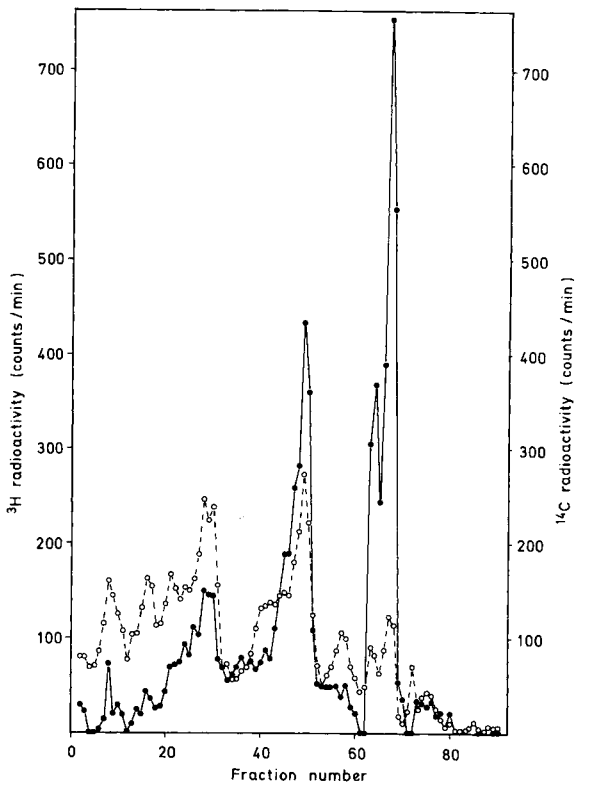

Fig. 4. *Electrophoretic separation of cyanogen bromide cleavage products of ADP/ATP carrier protein synthesized* in vitro *and* in vivo. Cells were grown with [^{14}C]leucine to obtain homogeneous labelling of ADP/ATP carrier; mitochondria were isolated and immunoprecipitation was carried out. An immunoprecipitate was also obtained from ADP/ATP carrier synthesized in the presence of [^{3}H]leucine in a *Neurospora* cell-free homogenate. The combined immunoprecipitates were subjected to cyanogen bromide cleavage. Fragments were separated on phenol/formic acid gels and radioactivities determined in gel slices. (●——●) ^{3}H; (○----○) ^{14}C

and ^{14}C radioactivity patterns showed coincidence. The quantitative relations of the bands were different. This is presumably due to the difference in labelling. Whereas the [^{14}C]leucine is homogeneously distributed over the whole sequence, a preferential ^{3}H-labelling of peptides derived from the regions closer to the carboxy terminus must be assumed, since only read out occurs. These results confirm that the homologous system synthesizes ADP/ATP carrier protein.

Fractionation of the cell-free homogenate after 15-min incorporation of [^{3}H]leucine shows that some 80–90% of the ^{3}H-labelled ADP/ATP carrier is found in the postribosomal supernatant, whereas 10–20% is associated with the mitochondrial fraction. Obviously, the protein synthesized *in vitro* is released from the ribosomes. Also, when various fractions of ribosomes were allowed to synthesize proteins in cell-free systems including the ribosome fractions and postribosomal supernatant, the ADP/ATP carrier was found almost exclusively in the postribosomal supernatant. These various ribosomes fractions were: ribosomes associated with mitochondria which were isolated in the presence of Mg^{2+} ions [29]; microsome-associated ribosomes and free ribosomes, which were separated by isopycnic sucrose density gradient centrifugation [30].

DISCUSSION

Experiments described here show that ADP/ATP carrier is synthesized in heterologous and homologous cell-free systems. In all cases the apparent molecular weight of the product synthesized *in vitro* was the same as that of the authentic protein. The integrity of the amino terminus was demonstrated by using formylated initiator methionine-tRNA fMet-tRNA$_f^{Met}$ as a probe. The presence of an additional sequence of the type of secretory proteins would have been revealed by the gel electrophoresis system employed. Accordingly it must be assumed that this protein is synthesized with no additional N-terminal sequence. Similarly it was found that apocytochrome *c* from *Neurospora* is synthesized with no additional sequence

(unpublished results). This is in agreement with findings with yeast isocytochrome *c*-1 [31–33]. Apparently, an additional signal-type sequence is not necessary to translocate proteins across the outer mitochondrial membrane and for insertion into the inner membrane. An additional sequence for translocation of proteins across the inner mitochondrial membrane may, however, be necessary. Precursors of subunits of oligomycin-sensitive ATPase were found to be synthesized *in vitro* and *in vivo* as larger precursors [9]. Similarly, recent evidence suggests the existence of a precursor form for citrate synthase with a higher apparent molecular weight (unpublished results).

The occurrence of precursors of mitochondrial proteins in the extramitochondrial space has been suggested by kinetic data for a number of proteins such as mitochondrial matrix proteins, mitochondrial ribosomal proteins, cytochrome *c* and ATP/ADP carrier [6]. In this context, the occurrence of apocytochrome *c* in the postribosomal supernatant and its transfer *in vitro* to the mitochondria has been demonstrated [8]. Similarly extramitochondrial precursors and posttranslational transfer was shown for subunits of the mitochondrial ATPase [9]. By analogy, the extramitochondrial form of ADP/ATP carrier in the cell-free homogenate from *Neurospora* could presumably represent a naturally occurring precursor form. Experiments are in progress to find conditions for its efficient transfer into mitochondria.

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