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Part K

Membrane Biogenesis: Assembly and Targeting
(Prokaryotes, Mitochondria, and Chloroplasts)

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[26] Biosynthesis and Assembly of Nuclear-Coded Mitochondrial Membrane Proteins in *Neurospora crassa*

By RICHARD ZIMMERMANN and WALTER NEUPERT

The vast majority of mitochondrial proteins are coded for by nuclear genes and synthesized on cytoplasmic ribosomes. These proteins must be transported into the different mitochondrial compartments: the outer membrane, the intermembrane space, the inner membrane, and the matrix space. Three major problems are currently being investigated in order better to understand the molecular basis of this transport process: What determines the specificity of the process, i.e., how is a mitochondrial protein selectively directed into a mitochondrion? How are the newly synthesized proteins translocated across the lipid bilayer of one or two mitochondrial membranes? How are the proteins assembled, so that they assume their specific orientation in the membranes and are put together to multisubunit structures?

Experimental approaches to these problems can be made by studying the various steps *in vivo,* or *in vitro* in reconstituted systems. Both of these approaches have been demonstrated to yield useful information.[1] The transport into mitochondria has been found to be a posttranslational process involving the occurrence of extramitochondrial precursor proteins. Synthesis of precursor proteins and transfer into the mitochondria are processes that are not, at least not obligatorily, coupled.[2-7] These soluble precursors in most cases have an additional peptide sequence that is not present in the mature membrane-bound protein. Specific receptors on the mitochondrial surface appear to function in binding mitochondrial precursors to the mitochondria.[8-11] We describe here methods to analyze, *in vivo,*

[1] W. Neupert and G. Schatz, *Trends Biochem. Sci.* **6,** 1 (1981).
[2] G. Hallermayer, R. Zimmermann, and W. Neupert, *Eur. J. Biochem.* **81,** 523 (1977).
[3] M. A. Harmey, G. Hallermayer, H. Korb, and W. Neupert, *Eur. J. Biochem.* **81,** 533 (1977).
[4] H. Korb and W. Neupert, *Eur. J. Biochem.* **91,** 609 (1978).
[5] M.-L. Maccechini, Y. Rudin, G. Blobel, and G. Schatz, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 343 (1979).
[6] Y. Raymond and G. S. Shore, *J. Biol. Chem.* **254,** 9335 (1979).
[7] M. Mori, S. Miura, M. Tatibana, and P. P. Cohen, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 7044 (1980).
[8] R. Zimmermann and W. Neupert, *Eur. J. Biochem.* **109,** 217 (1980).
[9] R. Zimmermann, B. Hennig, and W. Neupert, *Eur. J. Biochem.* **116,** 455 (1981).
[10] B. Hennig and W. Neupert, *Eur. J. Biochem.* **121,** 203 (1981).
[11] M. Schleyer, B. Schmidt, and W. Neupert, *Eur. J. Biochem.* **125,** 109 (1982).

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the kinetics of transfer of newly synthesized proteins into mitochondria and to identify precursors of mitochondrial proteins after labeling of intact cells. Furthermore, methods with *in vitro* systems are described that can be employed to investigate the following problems: (*a*) structure and properties of extramitochondrial precursor proteins; (*b*) identification of ribosomes involved in the synthesis of mitochondrial precursor proteins; (*c*) transfer of precursor proteins in reconstituted systems and dissection of the process into different steps; (*d*) energy requirements of the transfer process.

For these studies it is desirable to investigate individual, well-defined mitochondrial proteins; preferentially those with known function, structure, and submitochondrial topology. Unfortunately, only a few mitochondrial proteins are currently characterized in such detail.

The methods described here deal mainly with the study of integral proteins of the inner mitochondrial membrane of *Neurospora crassa,* the ADP–ATP carrier,[12] and subunit 9 of the oligomycin-sensitive ATPase (also called DCCD-binding protein).[13] Similar methods have been used to study the various subunits of the cytochrome bc_1 complex.[14,15] Elsewhere[16] we describe methods for the study of the biogenesis of cytochrome *c*.

Antibodies against Mitochondrial Membrane Proteins

Immunological procedures are necessary to identify the extremely small amounts of proteins that are being transported *in vivo* and *in vitro.* Antibodies against the purified mature protein of interest can be raised in rabbits by any of a number of established procedures. For this purpose soluble, Triton-solubilized, or SDS-solubilized protein may be used. The antibodies obtained usually precipitate not only the appropriate mature proteins, but also recognize the precursor forms. There have been exceptions, however, to this generalization. For example, antibodies prepared against holocytochrome *c* did not recognize the precursor apocytochrome *c*, and antibodies obtained against chemically prepared apocytochrome *c* recognized apocytochrome *c* synthesized *in vivo* or *in vitro,* but not holocytochrome *c*.[4,16] Antibodies against ATPase subunit 9 only weekly interacted with the precursor form.

Kinetic Analysis of Transport of Proteins into Mitochondria in Whole Cells

The rate of appearance of newly synthesized proteins in mitochondria can be measured by pulse labeling whole *Neurospora* cells with [^{3}H]leucine.

[12] H. Hackenberg, P. Riccio, and M. Klingenberg, *Eur. J. Biochem.* **88,** 373 (1978).
[13] W. Sebald, T. Graf, and H. B. Lukins, *Eur. J. Biochem.* **93,** 587 (1979).

Such labeling studies are carried out at 6–9°, since at normal growth temperature assembly is too rapid to be resolved into its various steps. Furthermore, it is useful to employ cells that are grown in the presence of [^{35}S]sulfate to have an internal measure for the recovery of mature proteins in subsequent analyses. Incorporation of added leucine at 8° is complete within about 6 min, but shorter, more defined periods of radiolabeling can be achieved by adding, after the desired labeling period, cycloheximide plus millimolar concentrations of leucine or a chase of leucine alone. In the first case, labeled nascent chains remain attached to polysomes, whereas chain completion and release occurs when labeling is stopped with leucine alone. Incorporation of [^{3}H]leucine into polypeptide chains is inhibited in both cases in less than 5 sec.

Hyphae of *Neurospora crassa* are grown in cultures of 1–2 liters in Vogel's minimal medium[17] containing 2% sucrose. Cultures are vigorously aerated for 13 hr at 25°, at which time the cells are in mid-log phase. To allow labeling with [^{35}S]sulfate, the concentration of sulfate in the medium is reduced to 80 μM (added as $MgSO_4$). Further reduction to 8 μM is possible without affecting growth of cells for 13–14 hr. Together with the inoculum of 2×10^6 conidia per milliliter of culture, sodium [^{35}S]sulfate (NEN, Boston, Massachusetts; specific radioactivity 10–1000 Ci/mol) is added at a concentration of 0.25–1 mCi/liter. After 13 hr, the culture is made 1 mM in $MgSO_4$ and incubated further for 1 hr. Then the temperature is shifted to 8° and maintained there for 1–2 hr prior to leucine labeling.

For pulse labeling, [^{3}H]leucine (NEN, Boston, Massachusetts; specific radioactivity 40–60 mCi/mol) is added at a concentration of 2 mCi/liter. After incubation for 1–4 min, cycloheximide (0.1 mg/ml) and leucine (final concentration 10 mM) are added. After various times of further incubation at 8°, aliquots of 100–200 ml are removed and poured into 2 volumes of ice water containing cycloheximide (0.1 mg/ml) and leucine (10 mM). Cells are immediately harvested by filtration, using a funnel specially constructed to keep the temperature at 0°. This apparatus is shown in Fig. 1; as can be seen, it allows the filtration to be performed at room temperature. This helps minimize the time between chilling and harvesting and between harvest and subsequent steps, since all the components of the experiment may be placed in close proximity to each other.

The mycelial pad (0.5 g wet weight) is scraped from the filter paper with a cooled spatula and transferred to a small beaker containing 5 ml of homogenization buffer [0.44 M sucrose, 5 mM NH_4Cl, 30 mM Tris-HCl,

[14] H. Weiss and H. J. Kolb, *Eur. J. Biochem.* **99**, 139 (1979).
[15] M. Teintze, M. Slaughter, H. Weiss, and W. Neupert, *J. Biol. Chem.* **257**, 10364 (1982).
[16] B. Hennig and W. Neupert, this volume [25].
[17] H. J. Vogel, *Am. Nat.* **98**, 435 (1964).

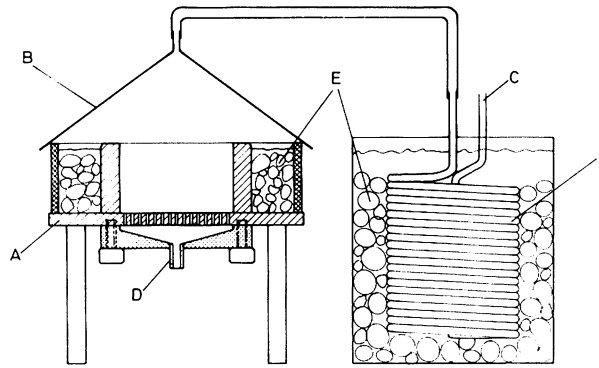

FIG. 1. Filtration apparatus to harvest *Neurospora* cells at 0°. A, aluminum filter plate; B, plastic funnel; C, air inlet; D, outlet to water aspirator; E, ice water; F, copper spiral.

pH 7.4, 1 m*M* 2-mercaptoethanol, 1 m*M* *p*-hydroxymercuribenzoate, 1 m*M* phenylmethanesulfonyl fluoride (PMSF)]. Hyphae are resuspended for 6 sec in an Ultra-Turrax homogenizer (Jahnke u. Kunkel, Stauffen, Germany) whose metal sheath was precooled to 0°. Then cells are broken in a small-scale grind mill, which is so designed that the passage of the cells occurs within about 5–10 sec.[18] The mill is kept in a freezer adjusted to −5°. The time between harvesting the cells and collection of the homogenate should not exceed 2–3 min, and care must be taken that the temperature is always close to 0°.

The crude homogenate is subfractionated by differential centrifugation according to standard procedures.[2,18] ^{3}H and ^{35}S radioactivities are determined in proteins precipitated with trichloroacetic acid. Immunoprecipitation from the various fractions lysed in Triton X-100 can be used to follow the kinetics of assembly of individual proteins.[2,9]

Comments. Kinetic analyses of this type give information on the pool sizes of extramitochondrial precursors. Figure 2A shows the labeling kinetics of various cellular fractions. Labeling of mitochondria occurs with a lag not only relative to total cellular protein, but also relative to cytosolic proteins. Various mitochondrial (mt) proteins (Fig. 2B) immunoprecipitated from isolated mitochondria show widely differing labeling kinetics with different lag periods, indicating different pool sizes of extramitochondrial precursors. Redistribution of proteins during cell fractionation, however, is a possible source of artifacts in this type of analysis and should be carefully checked.

Identification of Precursor Proteins by Labeling of Whole Cells

Many precursor proteins cannot be analyzed by the fractionation procedure described above. This can be due to a very short half-life of the

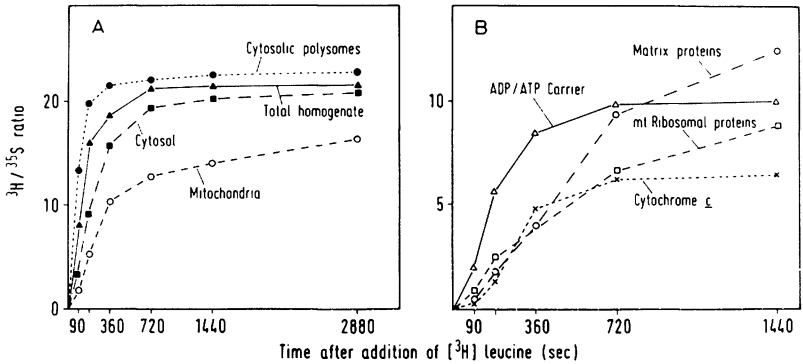

FIG. 2. Kinetics of pulse labeling of mitochondrial proteins in *Neurospora* cells. Cells were grown in the presence of [^{35}S]sulfate and pulsed with [^{3}H]leucine at 8°. Specific labeling of (A) various cellular fractions (trichloroacetic acid-precipitable protein) and (B) mitochondrial proteins immunoprecipitated from mitochondria. From Hallermayer *et al.*[2]

precursor or to the precursor being particularly sensitive to nonspecific proteolytic degration. Therefore it is necessary to stop any proteolytic reactions as rapidly and completely as possible. This can be achieved by immediately solubilizing the harvested cells in SDS followed by immunoprecipitation to detect precursor proteins.

Cells are pulse labeled, cooled to 0° by mixing with iced water (see above), and filtered on a cooled funnel. The hyphae are removed from the filter and immersed in a boiling solution containing 1 m*M* EDTA, 1 m*M* EGTA, 3% SDS with the pH adjusted to 7.5. For 0.1 g of cells (wet weight), 1 ml of this solution is used. The cells are homogenized with an Ultra-Turrax homogenizer for 10 sec. This mixture is kept in a boiling water bath for 5 min, then 9 ml of ice-cold Triton buffer (1% Triton, 0.3 *M* NaCl, 5 m*M* EDTA, 10 m*M* Tris-HCl, pH 7.5) are added, and PMSF and *o*-phenanthroline are added to a final concentration of 0.1 m*M* each. Centrifugation is carried out for 15 min at 39,000 *g*, and the supernatant is collected. This procedure was adapted for *Neurospora* by modifying a method described for yeast spheroplasts.[19] An alternative procedure is to transfer cells immediately after harvesting into a mortar containing liquid N_2 and grinding the cells under liquid N_2 for about 5 min. Then the liquid N_2 is allowed to evaporate, and a solution containing 3% SDS, 10 m*M* Tris-HCl, pH 7.5, 2 m*M* EDTA, 1 m*M* 2-mercaptoethanol, and 1 m*M* PMSF is added to the cells (5 ml/g wet weight). The frozen powder is mixed carefully with the SDS solution and allowed to thaw. The mixture is shaken at 4° for 15 min and

[18] W. Sebald, W. Neupert, and H. Weiss, this series, Vol. 55, p. 144.
[19] N. Nelson and G. Schatz, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4365 (1979).

centrifuged for 15 min at 39,000 *g*. The supernatant is then diluted with Triton buffer as described above for cells extracted with boiling SDS buffer.

Immunoprecipitation from the extracts is carried out employing *Staphylococcus aureus*.[20]

Comments. This procedure is useful to analyze the properties of precursor proteins that differ from the mature forms in apparent molecular weight, i.e., in electrophoretic mobility on SDS gels. Information can be obtained on the pool size of such precursors, on their mechanism of processing, and on the energy dependence of processing. As an example, Fig. 3 shows the synthesis and processing of cytochrome c_1. This protein is synthesized as a precursor protein with an extension of about 7000 M_r (see also the table). It is processed in two steps, as is cytochrome c_1 in yeast.[21] The intermediate form accumulates in *Neurospora* when the cells are kept at 8° and is processed when growth temperature is raised to 25°. Both processing steps are halted when cells are poisoned with the protonophore carbonyl cyanide *m*-chlorophenylhydrazone. The drawback of the method described above is that there is no way to determine the subcellular localization of precursor and mature forms. The extraction procedure involving grinding of cells under liquid N_2 is useful in those cases where boiling alters the protein in question in such a way that it cannot no longer be immunoprecipitated. One such example is apocytochrome *c*.

Analysis of Transfer of Mitochondrial Precursor Proteins *in Vitro*

Transport of proteins into mitochondria is a multistep process, and an understanding of the individual steps requires *in vitro* systems in which the complex overall reaction can be separately studied. The steps that can be resolved so far are (*a*) synthesis of precursor proteins on cytoplasmic ribosomes; (*b*) transfer through cytosol to the mitochondria; (*c*) binding to specific sites on the mitochondria; and (*d*) translocation across inner or outer membrane, or both, accompanied in most cases by proteolytic cleavage or by some other covalent modification.

Synthesis of Precursor Proteins in Cell-Free Systems

Mitochondrial precursor proteins are synthesized in reticulocyte lysates according to standard procedures.[22-24] Either total RNA from *Neurospora*

[20] S. W. Kessler, *J. Immunol.* **115,** 1617 (1975).
[21] G. Schatz and A. Ohashi, personal communication.
[22] E. H. Allen and R. W. Schweet, (1962) *J. Biol. Chem.* **237,** 760 (1962).
[23] T. Hunt and R. J. Jackson, *in* "Modern Trends in Human Leukaemia" (R. Neth, R. C. Gallo, S. Spiegelmann, and F. Stohlman, eds.), p. 300. J. F. Lehmans Verlag, Munich, 1974.
[24] H. R. B. Pelham and R. J. Jackson, *Eur. J. Biochem.* **67,** 247 (1976).

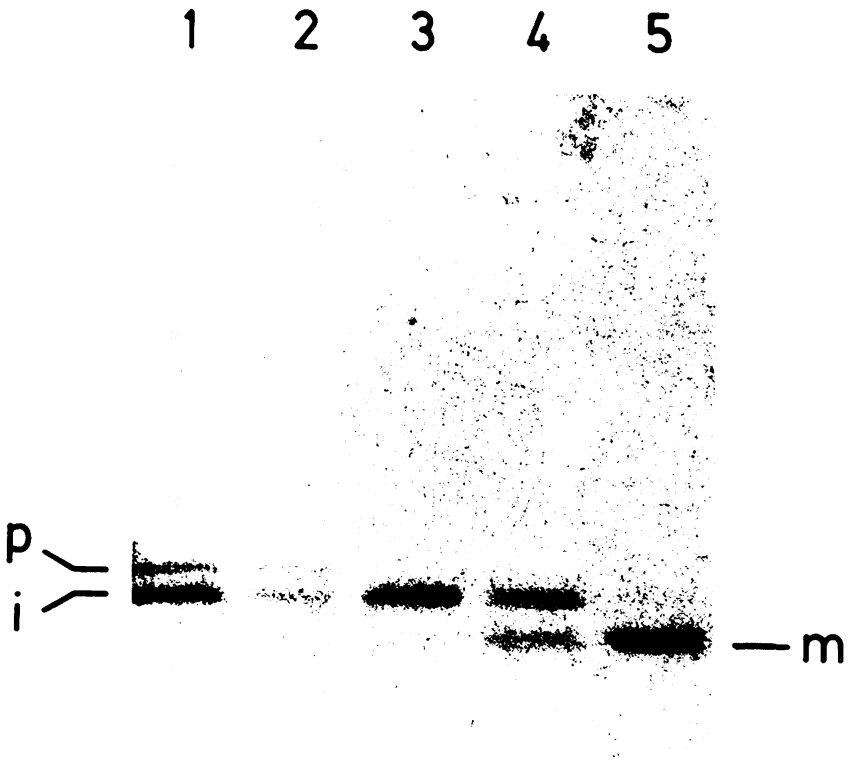

FIG. 3. Labeling of cytochrome c_1 precursor in whole *Neurospora* cells. *Neurospora* cells were pulse-labeled at 8° with [^{3}H]leucine. They were cooled to 0°, harvested, and extracted with hot SDS-containing medium. After dilution with Triton buffer, immunoprecipitation was carried out using antibodies against cytochrome c_1 and Sepharose-bound protein A. Immunoprecipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography. Lanes: 1, Pulse for 3 min, then cycloheximide (CHI) and a leucine chase were added, and the sample was immediately cooled to 0° and harvested; 2, Pulse for 3 min, then CHI and chase were added together with 12.5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and the sample was kept at 8° for 7 min, followed by cooling to 0° and harvesting; 3, as for lane 2, but without addition of CCCP; 4, pulse for 10 min at 8°, then addition of CHI, chase, and CCCP and further incubation for 5 min at 25°, then cooling to 0° and harvesting; 5, as for lane 4, but without addition of CCCP. p, Precursor; i, intermediate form; m, mature cytochrome c_1.

cells or poly(A)-containing RNA can be used to program reticulocyte lysates to synthesize mitochondrial proteins. RNA is isolated by phenol extraction by the following procedure. *Neurospora* hyphae (25 g wet weight, grown for 14 hr) are harvested by filtration and rapidly frozen with liquid nitrogen. The frozen hyphae are ground in a mortar cooled with liquid nitrogen to obtain a fine powder until at least 50% of the hyphae are broken, as determined by microscopic examination.

For extraction of nucleic acids, the powder is transferred to a bottle containing 100 ml of extraction medium A [phenol medium[25] equilibrated with 10 m*M* Tris-HCl, pH 8.2; detergent medium according to Leaver and Ingle[26]; chloroform medium: 49 ml of chloroform, 1 ml of isoamylalcohol; phenol medium, detergent medium, and chloroform medium are mixed in a ratio of 2:1:1 (v/v/v)]. Solid sodium dodecyl sulfate is added to a final concentration of 1% (w/v), and the mixture is shaken vigorously for 15 min at room temperature. The organic phase is then separated from the aqueous phase by centrifugation at 4° in polypropylene tubes for 15 min at 30,000 *g*, and the aqueous phase is saved. The organic phase including the white precipitate at the interface is extracted once more with 100 ml of medium A for 15 min. Again the phases are separated by centrifugation, the organic phase and interface being discarded.

The aqueous phases are combined and 3 volumes of extraction medium B [phenol medium and chloroform medium mixed in a ratio of 2:1 (v/v)] are added. After shaking for 15 min at room temperature, centrifugation is performed as described above. The aqueous phase is collected, and 3 volumes of chloroform medium are added. After shaking for 10 min and centrifugation, the organic phase is discarded. To precipitate nucleic acids from the aqueous phase, two volumes of absolute ethanol are added and the mixture is held overnight at −20°. Precipitated nucleic acids are collected by centrifugation at −20° for 20 min at 12,000 *g*. The pellet is washed twice with 66% ethanol (v/v) at −20° and dried over $CaCl_2$ *in vacuo*. The yield is about 1000 OD_{260} units per 10 g of hyphae. Separation of poly(A)-containing RNA is carried out according to Aviv and Leder.[27] The yield is about 5 OD_{260} units per 10 g of hyphae.

Comments. The table lists the precursors of *Neurospora* mitochondrial proteins so far identified.[1,4,8,9,11,15,28-34] Four proteins have been found to

[25] J. H. Parish and K. S. Kirby, *Biochim. Biophys. Acta* **129,** 554 (1966).
[26] C. J. Leaver and J. Ingle, *Biochem. J.* **123,** 235 (1971).
[27] H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69,** 1408 (1972).
[28] H. Freitag, W. Neupert, and R. Benz, *Eur. J. Biochem.* **123,** 629 (1982).
[29] H. Freitag, M. Janes, and W. Neupert, *Eur. J. Biochem.* **126,** 197 (1982).
[30] R. Zimmermann, U. Paluch, and W. Neupert, *FEBS Lett.* **108,** 141 (1979).
[31] R. Zimmermann, U. Paluch, M. Sprinzl, and W. Neupert, *Eur. J. Biochem.* **99,** 247 (1979).
[32] B. Schmidt and W. Neupert, unpublished.

MITOCHONDRIAL PRECURSOR PROTEINS IN *Neurospora crassa*

Sample	Location	Apparent molecular weight × 10^{-3}		Reference
		Pre-cursor[a]	Mature protein	
Mitochondrial porin	Outer membrane	31	31	28,29
Cytochrome *c*	Inter-membrane space	11.9*	11.9[b]	4,30
ADT-ATP carrier	Inner membrane	32	32	8,31
Cytochrome bc_1 complex				
Subunit I (core I)		51.5	50	15
II (core II)		47.5	45	15
IV (cytochrome c_1)		38*	31	1,15
V (Fe/S protein)		28	25	15
VI		14	14	15
VII		12	11.5	15
VIII		11.6	11.2	15
ATPase complex				
α-Subunit		62	58	32
Subunit 9		15.4*	9.5	9,11,33
Citrate synthase	Matrix	47.5*	45	34

[a] Precursor proteins indicated by an asterisk have also been detected after labeling of whole cells.

[b] Molecular weight of holocytochrome *c* without the heme group.

have no detectable additional sequence. The additional sequences of the other proteins varied between 0.4×10^3 and 7×10^3 apparent molecular weight.

Transfer of Precursors Synthesized in Reticulocyte Lysates into Isolated *Neurospora* Mitochondria

Postribosomal supernatants of reticulocyte lysates after synthesis of precursor proteins are prepared by centrifuging the cooled lysates at 0° for 1 hr at 160,000 *g*. This is conveniently done in Eppendorf tubes that are placed in water-filled adaptors (made from polystyrol) in a 50 Ti Beckman rotor. The supernatants are made 0.3 *M* in sucrose and 0.05 m*M* in the amino acid used for radioactive labeling.

[33] R. Michel, E. Wachter, and W. Sebald, *FEBS Lett.* **101**, 373 (1979).

[34] M. A. Harmey and W. Neupert, *FEBS Lett.* **108**, 385 (1979).

Mitochondria are isolated from *Neurospora* hyphae converted to spheroplasts. To prepare spheroplasts, 10 g of hyphae (wet weight) are resuspended in 50 ml of conversion medium (1 *M* sorbitol, 4.5 m*M* sucrose in Vogel's minimal medium[17]). Then 1 ml of a solution containing 5.2 units of β-glucuronidase and 4.5 units of arylsulfatase from *Helix pomatia* (Boehringer, Mannheim) are added. The mixture is shaken gently for 30 min at 25° then cooled to 0° and centrifuged for 10 min at 5000 *g* in a Sorvall refrigerated centrifuge. The pellet is washed once with conversion medium by gentle resuspension and by centrifugation. The final pellet is transferred to a Dounce homogenizer with 10 ml of the following medium: 0.3 *M* sucrose, 20 m*M* KH_2PO_4, 10 m*M* $MgCl_2$, 2 m*M* EDTA, 30 m*M* KCl, 30 m*M* triethanolamine, 60 m*M* triethanolamine-HCl; the pH is adjusted with 5 *M* KOH to 7.4. Homogenization is carried out by 10 strokes with a loosely fitted pestle. The homogenate is centrifuged twice for 5 min at 4000 *g*, then mitochondria are pelleted by centrifugation for 12 min at 17,300 *g* and held at 0° as a pellet until use.

Mitochondria are gently resuspended in the postribosomal supernatant of the reticulocyte lysate at a concentration of 0.5–1 mg of mitochondrial protein per milliliter. After incubation at 25°, aliquots are removed, cooled to 0°, and analyzed for transfer of proteins (Fig. 4).

For analysis of transfer efficiency, mitochondria and supernatant are separated again by centrifugation for 12 min at 17,300 *g*. The mitochondrial pellet is washed once with sucrose buffer (0.3 *M* sucrose, 2 m*M* EDTA, 10 m*M* Tris-HCl, pH 7.8), resuspended in the same buffer, and treated with proteinase K for 60 min at 4°. The concentration of proteinase K required to digest precursor, but not the mature assembled protein, should be determined for each different protein. In the case of the ADP–ATP carrier, for example, a concentration of 10 μg/ml is sufficient to degrade ADP–ATP carrier that is not transferred, whereas ADP–ATP carrier transferred *in vitro* or the mature preexisting form are largely resistant to concentrations up to 260 μg/ml. Discrimination of extramitochondrial and transferred form can also be made by incubating the whole transfer mixture with proteinase K.

Comments. Isolated mitochondria show the ability to bind precursor proteins in a saturable fashion. Binding can be studied as a separate step when the mitochondrial membrane potential is dissipated by protonophores such as CCCP, FCCP, or dinitrophenol, by ionophores such as valinomycin/K^+ or by a combination of oligomycin and the respiration inhibitors antimycin A or KCN (Fig. 5). The electrical membrane potential has been identified as the primary energy form to drive import of most precursor proteins in *Neurospora*.[11]

Correct insertion of precursors in cell-free systems is difficult to demonstrate owing to the very small amounts of protein transferred. Nevertheless,

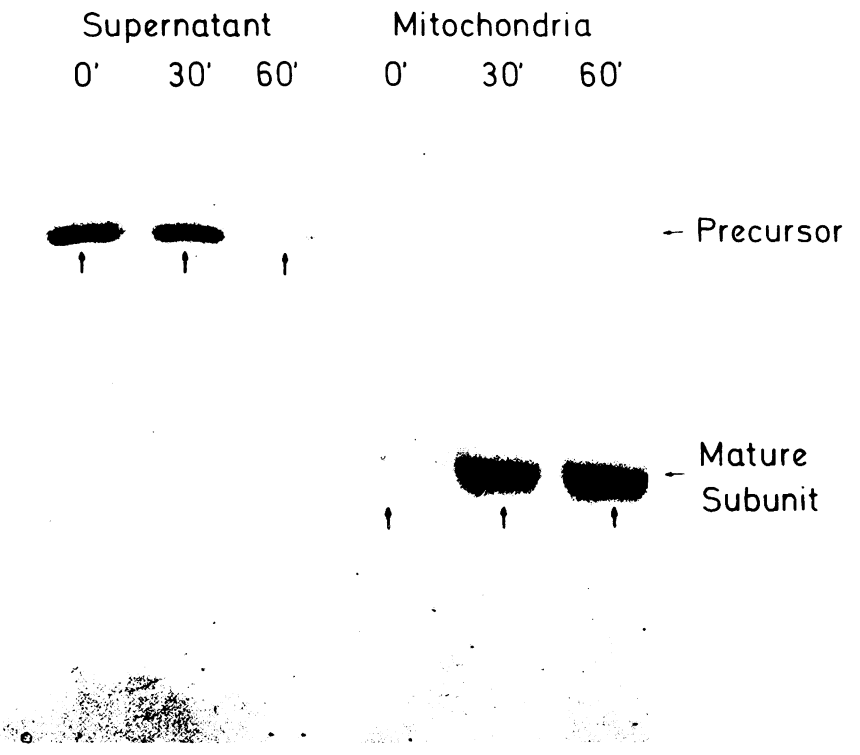

FIG. 4. Transfer and processing *in vitro* of ATPase subunit 9. A reticulocyte lysate was programmed with *Neurospora* RNA in the presence of [^{35}S]methionine. After incubation for 60 min, the postribosomal supernatant was prepared and incubated with mitochondria isolated from *Neurospora* spheroplasts. After various times of incubation, mitochondria and supernatant were reisolated. Subunit 9 was immunoprecipitated from mitochondria by direct immunoprecipitation and from the supernatant employing Sepharose-bound protein A. Immunoprecipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography.

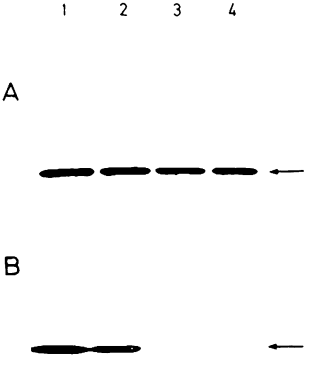

FIG. 5. Energy dependence of the transfer of ADP–ATP carrier into mitochondria *in vitro*. Transfer *in vitro* was performed as described in the legend to Fig. 4. After a 60-min transfer, one half of each sample was treated with 0.1 mg proteinase K per milliliter at 4° for 60 min; the other half was kept for 60 min at 0°. Then, from both halves, mitochondria were reisolated, and after lysis with Triton the ADP–ATP carrier was immunoprecipitated using *Staphylococcus aureus* cells. Immunoprecipitates were analyzed by SDS-PAGE and fluorography (A) without proteinase treatment, (B) with proteinase treatment; 1, control incubation; 2, plus oligomycin and rotenone; 3, plus oligomycin and antimycin A; 4 plus oligomycin and KCN. For details, see Schleyer *et al.*[11]

for a number of proteins it is possible to demonstrate properties specific for the assembled proteins. The ADP–ATP carrier is known to bind carboxyatractyloside only in the membrane-integrated dimeric form. Binding of this inhibitor protects the carrier form proteolytic fragmentation.[35] This was also demonstrated with the carrier transferred *in vitro,* indicating its assembly into a functional position.[36] For subunit 9 of ATPase, it has been found that, unlike the precursor form, the transferred and processed protein acquired the property of being soluble in chloroform–methanol and becomes precipitable with an antibody against F_1-ATPase.

The *in vitro* system can be used to determine how universal the transport mechanism is, i.e., whether species well separated in evolution are capable of taking up and processing each others precursor proteins. *Neurospora* precursors have been found to be imported and processed by yeast and rat liver mitochondria.[11,15] This indicates a degree of universality of the components of the import machinery.

[35] M. Klingenberg, H. Aquila, and P. Riccio, this series, Vol. 56, p. 407.
[36] M. Schleyer and W. Neupert, unpublished.

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