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Different Transport Pathways of Individual Precursor Proteins in Mitochondria

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Transport of mitochondrial precursor proteins into mitochondria of *Neurospora crassa* was studied in a cell-free reconstituted system. Precursors were synthesized in a reticulocyte lysate programmed with *Neurospora* mRNA and transported into isolated mitochondria in the absence of protein synthesis. Uptake of the following precursors was investigated: apocytochrome *c*, ADP/ATP carrier and subunit 9 of the oligomycin-sensitive ATPase.

Addition of high concentrations of unlabelled chemically prepared apocytochrome *c* (1–10 μM) inhibited the appearance in the mitochondrial of labelled cytochrome *c* synthesized *in vitro* because the unlabelled protein dilutes the labelled one and because the translocation system has a limited capacity [apparent V is 1–3 pmol $\times$ min^{-1} $\times$ (mg mitochondrial protein)$^{-1}$]. Concentrations of added apocytochrome *c* exceeding the concentrations of precursor proteins synthesized *in vitro* by a factor of about 10^4 did not inhibit the transfer of ADP/ATP carrier or ATPase subunit 9 into mitochondria. Carbonylcyanide *m*-chlorophenylhydrazone, an uncoupler of oxidative phosphorylation, inhibited transfer *in vitro* of ADP/ATP carrier and of ATPase subunit 9, but not of cytochrome *c*.

These findings suggest that cytochrome *c* and the other two proteins have different import pathways into mitochondria. It can be inferred from the data presented that different 'receptors' on the mitochondrial surface mediate the specific recognition of precursor proteins by mitochondria as a first step in the transport process.

Mitochondria acquire most of their proteins by import. By a number of criteria the import process has been shown to be a post-translational event [1–4]. The evidence to date indicates that mitochondrial proteins are synthesized on free ribosomes and released into the cytosolic space [5–7]. We have suggested that the information which directs a mitochondrial protein specifically to a mitochondrion, resides in some three-dimensional structure on the precursor protein [8]. The first step in the rather complex transport reaction may be a binding to a complementary structure on the surface of the mitochondrion. Evidence for such a 'receptor', e.g. for the ADP/ATP translocator, has been presented [8,9].

In this study we pose the question: is there a unique receptor for all the different mitochondrial proteins or is there a number of receptors specific for certain classes of precursors? A possible means of determining which of the above alternatives is applicable was to conduct competition experiments in which the binding and translocation of one precursor is studied in the presence of a large excess of another precursor. Heretofore such experiments were not possible because precursor proteins are usually not available in amounts sufficient for effective competition. However, there is the exception of cytochrome *c*, where the extramitochondrial precursor apocytochrome *c* can be derived from 'mature' holocytochrome *c* in large amounts by chemical procedures [5,10,11]. In contrast to most other mitochondrial proteins, cytochrome *c* is not synthesized as a larger precursor containing an additional sequence.

We describe competition of apocytochrome *c* with the precursors of ADP/ATP carrier and of subunit 9 of the oligomycin-sensitive ATPase complex. Apocytochrome *c* must be transported across the outer mitochondrial membrane and following covalent binding of the heme group and a conformational change is bound to the surface of the inner membrane [5,10]. The latter two precursor proteins are integrated into the inner mitochondrial membrane. Subunit 9 of ATPase, also called 'proteolipid' or 'dicyclohexylcarbodiimide-binding protein', is a nuclear-gene product in *Neurospora* and is synthesized on cytoplasmic ribosomes [12]. It should be noted, however, that in yeast this protein is coded for by a mitochondrial gene, translated on mitochondrial ribosomes and inserted into the inner membrane from the matrix side [12]. The data presented show that apocytochrome *c* does not compete with these two proteins for uptake into the mitochondria. It is therefore concluded that the respective precursors are not recognized by the same receptor but that there are at least two and perhaps more different receptors on the mitochondrial surface. Further support for different transport pathways is added by the finding that carbonylcyanide *m*-chlorophenylhydrazone blocks translocation of ADP/ATP carrier and subunit 9 across the outer membrane, but does not block translocation of apocytochrome *c*.

Abbreviation. $ClPhzC(CN)_2$, carbonylcyanide *m*-chlorophenylhydrazone.

MATERIALS AND METHODS

Growth of Neurospora *Cells and Cell Fractionation*

Neurospora crassa cells (wild type 74A) were grown on Vogel's minimal medium as described [10]. Mitochondria were isolated after preparation of spheroplasts as reported previously [8].

Protein Synthesis in Reticulocyte Lysates and Protein Transfer in vitro

Reticulocyte lysates were prepared from anaemic rabbits [13,14]. *Neurospora* poly(A)-containing RNA was isolated by chromatography on oligo(dT) cellulose [6]. Translation of *Neurospora* message in reticulocyte lysates was performed in the presence of [^{35}S]methionine (specific radioactivity 1000 Ci/mmol, NEN, Boston, MA) for 60 min [6,15]. At the end of incubation period, the lysates were cooled to 0 °C and centrifuged for 1 h at 166000 × *g* in a Beckman rotor Ti 75. The supernatant was collected; unlabelled methionine and sucrose were added to final concentrations of 50 μM and 0.3 M, respectively. Mitochondria isolated from spheroplasts were carefully resuspended (ca. 1 mg protein/ml) in a teflon/glass homogenizer. The suspension was incubated for 60 min at 25 °C, then cooled to 0 °C and centrifuged for 12 min at 17300 × *g*. The supernatant was separated from the mitochondria. Protease treatment was performed with mitochondria as described before [8].

Isolation of Proteins and Preparation of Antibodies

Holocytochrome *c* was isolated as described earlier [10]. Apocytochrome *c* was prepared from holocytochrome *c* essentially following the procedure of Ambler and Wynn [16] and Fisher et al. [17]. A solution was prepared containing 8 M urea, 100 mM NaCl, and the pH was adjusted to 2.0 by addition of 6 M HCl. 5 mg holocytochrome *c* and 20 mg $HgCl_2$ were dissolved each in 0.5 ml of this solution and mixed. The mixture was incubated at 23 °C for 16 h in the dark. Then it was passed through a column of Sephadex G-25 (90 × 0.9 cm) equilibrated with 100 mM ammonium acetate pH 6.0. The fraction containing the protein was lyophilized. The protein was taken up in 1 ml 8 M urea, 250 mM ammonium bicarbonate, 6.5 mM dithiothreitol and incubated for 30 min at 37 °C. Then it was passed through a column of Sephadex G-25 (90 × 0.9 cm) equilibrated with 250 mM ammonium bicarbonate. The protein fraction was lyophilized. The white residue was dissolved in 100 μl 8 M urea, 250 mM ammonium bicarbonate, 6.5 mM dithiothreitol and dialysed in Visking 8/32 tube (Union Carbide, Chicago, IL) twice against 100 ml 250 mM ammonium bicarbonate, 14 mM 2-mercaptoethanol and then four times against 100 ml 250 mM ammonium bicarbonate. After the last dialysis step, protein concentration was adjusted to 10 mg/ml and aliquots of 5 μl were stored at −20 °C. After thawing, samples were used immediately. Repeated freezing and thawing was avoided, since it was observed that this procedure would cause denaduration.

For preparation of antibodies against holocytochrome *c* and apocytochrome *c*, the proteins were transferred into 100 mM sodium phosphate buffer pH 7.0 by dialysis of samples containing 6 mg protein in 1 ml. Glutaraldehyde was added at a concentration of 5 mM and the mixture were incubated for 1 h at 23 °C. Solid L-lysine was added to final concentration of 100 mM and the solution was kept for further 60 min at 23 °C. Then dialysis against 100 mM sodium phosphate buffer pH 7.0 was carried out. For immunization of rabbits, 0.25 ml of the final solutions containing 1 mg of apocytochrome *c* or holocytochrome *c* were mixed with equal volumes of either complete or incomplete Freund's adjuvant. Rabbits were injected subcutaneously in the neck region, first with antigen in complete adjuvant, and then at weekly intervals thrice with antigen in incomplete adjuvant. One week after the final injection, blood was drawn from the ear vein and serum was checked for the titer on Ouchterlony plates. 200 − 300 μl serum were sufficient to quantitatively precipitate 1 nmol of holocytochrome *c* by direct immunoprecipitation.

Subunit 9 of oligomycin-sensitive ATPase was isolated from *Neurospora* according to the method described by Sebald et al. [18]. The final product obtained by precipitation with ether was taken up in H_2O and homogenized by sonication. 0.5 ml of the suspension containing 0.5 mg protein was mixed with 0.5 ml Freund's complete adjuvant and injected into a rabbit subcutaneously. The procedure was repeated four times at weekly intervals. One week after the last injection blood was drawn from the ear vein and serum obtained. 500 μl of the serum were employed to quantitatively precipitate subunit 9 from 1 mg mitochondrial protein.

ADP/ATP carrier was isolated according to the method of Hackenberg et al. [21]. Antibodies were prepared as described previously [1]. 100 μl of immunoglobulin solution were used to quantitatively precipitate ADP/ATP carrier from 1 mg mitochondrial protein.

Immunoprecipitation

For immunoprecipitation from mitochondria, mitochondrial pellets were resuspended in 1 ml 0.3 M KCl, 10 mM Tris/HCl pH 7.5 and 60 μl 20% Triton X-100 were added. For immunoprecipitation of ADP/ATP carrier, before addition of Triton, 0.1 ml 10 mM *p*-chloromercuribenzoate was added. A clarifying spin was carried out in a refrigerated centrifuge for 15 min at 27000 × *g*. The supernatants were collected and antibodies (immunoglobulin fractions or serum) against the various proteins were added. Precipitation was allowed to occur at 4 °C for 4 h in the case of ADP/ATP carrier and for 16 h in case of the other proteins. For immunoprecipitation of apocytochrome *c* from mitochondria, 1 nmol/ml apocytochrome *c* was added to the Triton-lysed mitochondria and then 75 μl of antiserum. After 5 min Sepharose-bound protein A (10 mg dry weight in 100 μl 10 mM Tris/HCl pH 7.5) (Pharmacia, Uppsala, Sweden) was added and the mixture incubated with shaking for a further 5 min.

Immunoprecipitation of apocytochrome *c* from the supernatant was performed after addition of 0.1 ml 3 M KCl, 100 mM Tris/HCl pH 7.5 and 60 μl 20% Triton X-100 to 1 ml of supernatant. To those samples which contained less than 1 nmol/ml apocytochrome *c*, sufficient amounts of this protein were added to obtain a final concentration of 1 nmol/ml. In cases where the concentration was above 1 nmol/ml, sufficient antibody was added to precipitate all apocytochrome *c*. Immunoprecipitation was carried out using Sepharose-bound protein A as described above. Immunoprecipitates and Sepharose beads were collected by centrifugation for 2 min in an Eppendorf centrifuge. Pellets were washed by resuspension and centrifugation twice with 1 ml of 0.3 M KCl, 0.01 M Tris/HCl, 1% Triton X-100 pH 7.5 and thrice with 1 ml 10 mM Tris/HCl pH 7.5. For electrophoresis on vertical gels [19], the final pellets were dissolved in 40 μl 2% sodium dodecylsulfate, 0.7 M 2-mercaptoethanol, 60 mM Tris/HCl pH 6.8, 10% (v/v) glycerol. Samples were boiled for 2 min. For electrophoresis on horizontal gels [10] immunoprecipitates were dissolved in 40 μl 2% sodium dodecylsulfate, 0.35 M 2-mercaptoethanol, 10 mM Tris/HCl pH 7.5 and treated for 20 min to 56 °C and 2 min to 95 °C.

Table 1. *Synthesis of various mitochondrial proteins in a reticulocyte lysate*
A reticulocyte lysate (3 ml) was incubated for 60 min with [^{35}S]-methionine (0.5 mCi/ml) and *Neurospora* mRNA. The postribosomal supernatant was prepared and immunoprecipitation was carried out with three aliquots. Antibodies against apocytochrome *c*, ADP/ATP carrier and subunit 9 of ATPase were employed; immunoprecipitates were subjected to gel electrophoresis and the radioactivity in the protein peaks was determined. From the same reticulocyte lysate, aliquots were taken and methionine was added in increasing concentrations. [^{35}S]-Methionine was incorporated into these aliquots and the total radioactivity in protein was determined. The concentration of methionine which caused a 50% reduction of the rate of incorporation was taken as the endogenous methionine concentration of the system *in vitro*. Molar rates of synthesis were calculated using this concentration. The calculation was made under the assumption that cytochrome *c* contains two methionine residues [20], ADP/ATP carrier contains eight methionine residues [21], and subunit 9 contains four methionine residues [22]. In the case of the latter protein the possible presence of methionine in the additional precursor sequence was not taken into account. The concentration of cytochrome *c* in mitochondria is taken from Jagow et al. [23], that of ADP/ATP carrier from Hackenberg et al. [21], and that of subunit 9 from Sebald et al. [18]

Protein	Amount synthesized in reticulocyte lysate		Concentration in mitochondria
	dis $\times$ min^{-1} $\times$ ml^{-1}	pmol/ml	nmol/mg protein
Cytochrome *c*	55×10^3	0.28	1.0
ADP/ATP carrier	113×10^3	0.14	2.1
Subunit 9 of ATPase	22×10^3	0.055	2.5

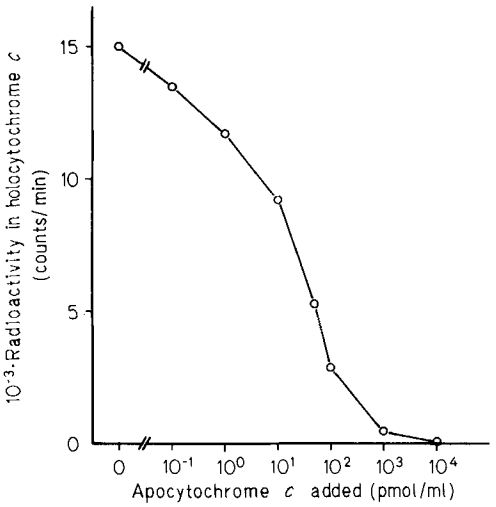

Fig. 1. *Formation of holocytochrome* c *from apocytochrome* c *by isolated mitochondria.* [^{35}S]Methionine was incorporated into a reticulocyte lysate programmed with *Neurospora* mRNA. The postribosomal supernatant was prepared. To aliquots of 0.5 ml, apocytochrome *c* was added to the final concentrations indicated. Then mitochondria prepared from *Neurospora* spheroplasts were resuspended in the supernatants (1 mg protein/ml). The suspensions were incubated for 60 min at 25 °C. Then mitochondria were sedimented by centrifugation and the supernatant removed. Immunoprecipitation was performed with antibodies against holocytochrome *c*. The washed immunoprecipitate was dissolved in dodecylsulfate containing buffer and subjected to electrophoresis in the presence of dodecylsulfate. The gels were sliced and the radioactivity in the cytochrome *c* peak was determined

Gel Electrophoresis and Autoradiography

Analysis of immunoprecipitates by gel electrophoresis in the presence of sodium dodecylsulfate was performed according to Laemmli [19] when the gels were afterwards dried and exposed to Agfa Gevaert Curix RP 1 X-ray film. When after electrophoresis gels were sliced, horizontal slab gels were used as described earlier [10].

RESULTS

Chemically Prepared Apocytochrome c *Competes with Apocytochrome* c *Synthesized* in vitro *for Transport into Mitochondria*

A rabbit reticulocyte lysate was programmed with *Neurospora* poly(A)-containing RNA and after incubation in the presence of [^{35}S]methionine for 60 min, the postribosomal supernatant was obtained. Table 1 shows the amounts of apocytochrome *c*, ADP/ATP carrier and subunit 9 of oligomycin-sensitive ATPase synthesized. The table gives also the concentrations of these proteins in the mitochondria. To aliquots of this supernatant chemically prepared apocytochrome *c* was added at various concentrations. Then the supernatants were incubated with mitochondria isolated from *Neurospora* spheroplasts. After incubation the supernatants and mitochondria were separated again. Holocytochrome *c* was immunoprecipitated from mitochondria. Fig. 1 shows the amounts of label in holocytochrome *c* depending on the apocytochrome *c* concentrations in the supernatant. Radioactivity in holocytochrome *c* decreases with increasing concentrations of apocytochrome *c*. Apparently, the endogenous apocytochrome *c* synthesized *in vitro* is effectively diluted by chemically prepared apocytochrome *c*. The particular form of the curve in Fig. 1 is consistent with the concentration of apocytochrome *c*, synthesized in the reticulocyte system, being far below the concentration necessary to saturate the system which takes up apocytochrome *c* and converts it into holocytochrome *c*. Using the specific radioactivities of apocytochrome *c* in the concentration range 1–100 nM and the radioactivities recovered in mitochondrial holocytochrome *c*, a Lineweaver-Burk plot was constructed for the reaction apocytochrome *c* in supernatant to holocytochrome *c* in mitochondria. For calculation the initial reaction velocity over the first 10 min of incubation was employed (Fig. 2). In various experiments the apparent V was 1.5–2.5 pmol $\times$ min^{-1} $\times$ (mg mitochondrial protein)$^{-1}$ and the apparent K_m was 20–40 nM. These values are only a first approximation, since a number of parameters do influence the reaction. From the growth rate of the *Neurospora* cells and from the mitochondrial cytochrome *c* content it can be estimated that *Neurospora* mitochondria import apocytochrome *c* and convert it to holocytochrome *c* at a rate of 3–4 pmol $\times$ min^{-1} $\times$ (mg mitochondrial protein)$^{-1}$. Thus it appears that the rates *in vitro* and *in vivo* are comparable if the extramitochondrial concentration of apocytochrome *c* is high enough.

It can be concluded from the data in Fig. 1 and 2 that chemically prepared apocytochrome *c* is transported into mitochondria and converted to holocytochrome *c*. When apocytochrome *c* is present in very high concentrations far above the apparent K_m, the apocytochrome *c* which is not converted to holocytochrome *c* remains in the supernatant and does not accumulate in the mitochondria. e.g. at concentrations of 0.1 and of 1 μM, more than 96% of the residual

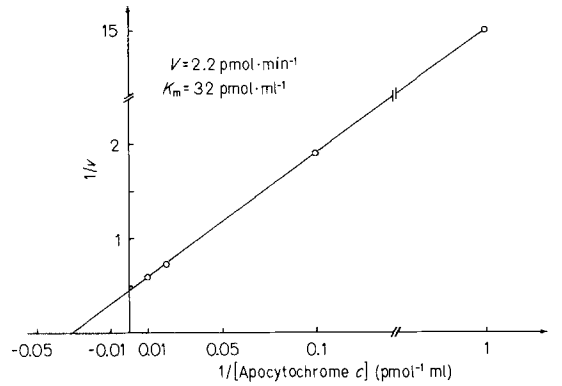

Fig. 2. *Lineweaver-Burk plot for the conversion of extramitochondrial apocytochrome* c *to mitochondrial holocytochrome* c. The amounts of holocytochrome *c* formed were calculated from the specific radioactivities of apocytochrome *c* and the radioactivities present in holocytochrome *c* which was immunoprecipitated from mitochondria after incubation of the reconstituted system. The specific radioactivities of apocytochrome *c* were derived from the total radioactivity in apocytochrome *c* of 1 ml reticulocyte supernatant and from the amount of apocytochrome *c* added to 1 ml of the reconstituted system plus the amount synthesized in the reticulocyte lysate. For calculation of *v* the initial rates of the reaction were employed, and *v* is expressed as pmol holocytochrome *c* formed $\times \min^{-1} \times$ (mg mitochondrial protein)$^{-1}$

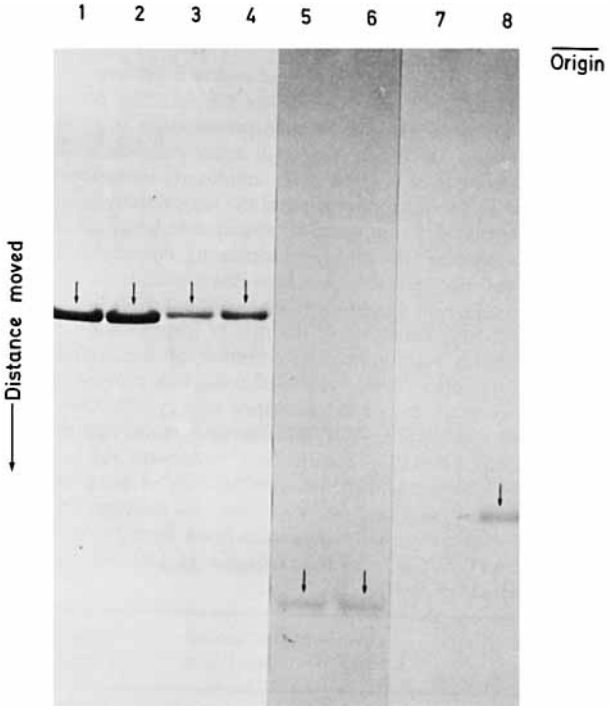

Fig. 3. *Effect of excess unlabelled apocytochrome* c *on the transport into mitochondria of precursors of ADP/ATP carrier and ATPase subunit 9* in vitro. Protein synthesis was carried out in a reticulocyte lysate. The post-ribosomal supernatant was prepared. One half of it remained as a control, to the other half apocytochrome *c* was added to a final concentration of 1 nmol/ml. Both halves were incubated with mitochondria isolated from *Neurospora* spheroplasts for 60 min. Mitochondria were isolated again and from aliquots holocytochrome *c*, ADP/ATP carrier and subunit 9 were immunoprecipitated. Immunoprecipitates were analysed by gel electrophoresis in the presence of dodecylsulfate. The dried gels were subjected to autoradiography. Lanes 1, 3, 5, 7: with added apocytochrome *c*; lanes 2, 4, 6, 8: without added apocytochrome *c*. Lanes 1, 2: ADP/ATP carrier before protease treatment; lanes 3, 4: ADP/ATP carrier after protease treatment; lanes 5, 6: subunit 9 of ATPase; lanes 7, 8: holocytochrome *c*

apocytochrome *c* was detected in the supernatant after sedimenting the mitochondria. The rest could be removed from mitochondria by washing with sucrose buffer. In contrast, no significant amounts of holocytochrome *c* were found in the supernatant. Holocytochrome *c* was exclusively associated with mitochondria.

Similar experiments as described above were made with reconstituted systems, in which the supernatant from a homologous cell-free system was employed. Also in this case added apocytochrome *c* competes with apocytochrome *c* synthesized *in vitro* [10]. Calculations were not made in this case, since the endogenous concentration of apocytochrome *c* is not known. The postmitochondrial supernatant not only contains apocytochrome *c* synthesized during the incubation period but also the apocytochrome *c* present in the precursor pool of the intact cell [9]. In agreement with the findings using the reconstituted system involving reticulocyte lysate supernatants, holocytochrome *c* was only detected in mitochondria; apocytochrome *c* which was not converted to holocytochrome *c* at higher concentrations of added apocytochrome *c*, is essentially found in the supernatant (not shown).

Apocytochrome c *Does Not Compete for Import with Precursors of the ADP/ATP Carrier of Subunit 9 of ATPase*

Protein synthesis in the presence of [^{35}S]methionine was carried out in a reticulocyte lysate programmed with *Neurospora* poly(A)-containing RNA. The postribosomal supernatant was halved; to one half apocytochrome *c* was added to a final concentration of 1 nmol/ml, the other half served as a control. Equal amounts of mitochondria isolated from spheroplasts were then resuspended in both halves. After incubation for 60 min, mitochondria were reisolated and holocytochrome *c*, ADP/ATP carrier and subunit 9 of ATPase were immunoprecipitated. Fig. 3 shows the autoradiographs of the immunoprecipitates after gel electrophoresis. Clearly, excess unlabelled apocytochrome *c* competes with the labelled apocytochrome *c* but not with the precursors of ADP/ATP carrier or subunit 9 of ATPase for uptake into the mitochondria. The ADP/ATP carrier is transferred into a protease-resistant form. The precursor of subunit 9 is transferred into mitochondria and processed to the size of the mature subunit. The transferred and processed subunit 9 shares two properties with the mature assembled protein: it is partly resistant to added protease (see below) and it is soluble in chloroform/methanol, in contrast to the extramitochondrial precursor form (Zimmermann and Neupert, unpublished results). The indicates that the precursor is actually translocated across the outer membrane into the inner membrane.

Carbonylcyanide m-*Chlorophenylhydrazone Inhibits Import* in vitro *of the ADP/ATP Carrier and of Subunit 9 of ATPase but Not of Apocytochrome* c

Mitochondria were resuspended in a supernatant from a reticulocyte lysate which had been incubated with [^{35}S]-methionine and poly(A)-containing RNA. The suspension was halved; one half served as a control, the second one received 10 μM $ClPhzC(CN)_2$. Both portions were incubated for 60 min, mitochondria were reisolated and holocyto-

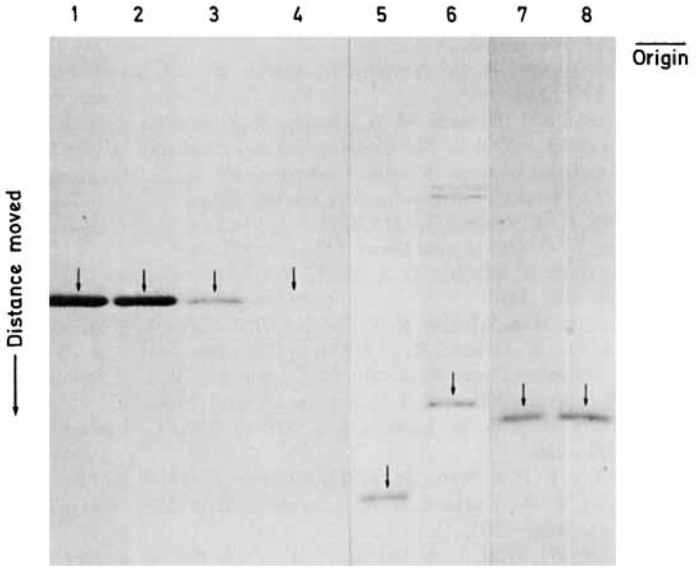

Fig. 4. *Effect of carbonylcyanide* m-*chlorophenylhydrazone [ClPhzC(CN)$_2$] on transport of various precursors into mitochondria* in vitro. Transport of apocytochrome *c*, ADP/ATP carrier and subunit 9 of ATPase was studied *in vitro* as described for Fig. 3. In this case instead of apocytochrome *c*, ClPhzC(CN)$_2$ was added to the reticulocyte lysate supernatant before resuspending the mitochondria. A control sample was run in parallel. The concentration of ClPhzC(CN)$_6$ was 10 nmol/ml. Lanes 1, 3, 5, 7: without ClPhzC(CN)$_2$; lanes 2, 4, 6, 8: with ClPhzC(CN)$_2$. Lanes 1, 2: ADP/ATP carrier before protease treatment; lanes 3, 4: ADP/ATP carrier after protease treatment; lanes 5, 6: subunit 9 of ATPase; lanes 7, 8: holocytochrome *c*

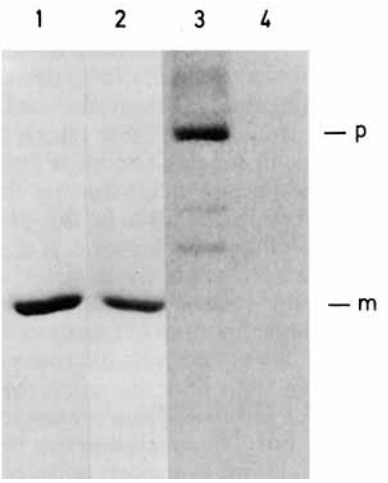

Fig. 5. *Sensitivity to added protease of ATPase subunit 9 transferred into mitochondria* in vitro. Transfer *in vitro* of subunit 9 of ATPase in the absence and presence of ClPhzC(CN)$_2$ was performed as described for Fig. 4. After incubation of mitochondria with the supernatant from the reticulocyte lysate for 60 min, the mitochondria were reisolated. The two samples were halved; one half of each sample was treated with proteinase K (100 μg/ml) for 60 min at 4 °C; then phenylmethylsulfonylfluoride was added to all samples in a final concentration of 0.6 mM. After solubilisation with Triton, subunit 9 was immunoprecipitated. The washed immunoprecipitates were subjected to gel electrophoresis and autoradiography. Lanes 1, 2: transfer in the absence of ClPhzC(CN)$_2$; lanes 3, 4: transfer in the presence of ClPhzC(CN)$_2$; lanes 1, 3: without protease treatment; lanes 2, 4: with protease treatment. p, precursor of subunit 9; m, mature form of subunit 9

chrome *c*, ADP/ATP carrier and subunit 9 were immunoprecipitated. Fig. 4 shows that after ClPhzC(CN)$_2$ poisoning practically no ADP/ATP carrier was found in protease resistant form. No mature subunit 9 appeared in the mitochondria; instead some unprocessed precursor was found associated with the mitochondria. In contrast, import and conversion of apocytochrome *c* was not affected by ClPhzC(CN)$_2$. In order to exclude that ClPhzC(CN)$_2$ might act by a different mechanism than depleting mitochondria of energy, these experiments were also performed employing 0.5 μM valinomycin. Exactly the same effect as with ClPhzC(CN)$_2$ was observed. After incubation in the presence of ClPhzC(CN)$_2$ or valinomycin the bound precursor of subunit 9 was completely sensitive to added protease (Fig. 5). In contrast, the mature form found in the absence of ClPhzC(CN)$_2$ was partly protease-resistant (Fig. 5). When cells were labelled *in vivo* by growth in the presence of [^{35}S]sulfate, the assembled subunit 9 was found to be degraded to a similar extent by proteinase K added to isolated mitochondria. This indicates that the protease-sensitive portion of the mature form of subunit 9 transferred *in vitro* may also represent protein being correctly processed and transferred. It can be inferred from these observations that the uncouplers allow association of the precursor of subunit 9 with a binding site on the outer mitochondrial membrane but inhibit processing and translocation across the outer membrane.

DISCUSSION

The data presented here corroborate our earlier finding based on studies *in vivo* and *in vitro* that transport of proteins into mitochondria is a posttranslational process [1, 2, 5, 8, 10]. The mechanism of posttranslational transfer is still rather obscure. Most proteins are synthesized as larger precursors which are proteolytically processed to the 'mature' size, a few do not have additional sequences [4, 24–34]. Subunit 9 of ATPase belongs to the first class [35], cytochrome *c* [5] and the ADP/ATP carrier [6] to the second class. A salient feature of the transport mechanism seems to be that the precursor forms possess a structure which has the information to direct them to a complementary structure on the mitochondrion. The first of several possible steps in translocation appears to be the binding to such a 'receptor'. For the ADP/ATP carrier, specific binding to the mitochondrial surface has been demonstrated [8]. Corresponding binding of the precursor of subunit 9 has been observed (cf. Fig. 4). There are also indications that there are specific binding sites on the mitochondria for apocytochrome *c* which can be demonstrated when conversion of apocytochrome *c* to holocytochrome *c* is blocked (Hennig and Neupert, unpublished).

Is there a unique receptor which serves as a port of entry for all proteins or is there a set of different receptors? An experimental approach to this question is possible for two reasons: (a) in the case of cytochrome *c* the precursor can be prepared in large amounts from the functional protein and this precursor can be employed in competition experiments; (b) the transfer system which translocates apocytochrome *c* across the outer mitochondrial membrane has a limited capacity. Therefore it is possible to establish high concentrations of apocytochrome *c* in the extramitochondrial space (about a 5000–15000-fold molar excess over the other precursor proteins).

It has been recently reported by Basile et al. [36] that isolated apocytochrome *c* labelled with radioactive iodine is

transferred into isolated yeast mitochondria and converted to holocytochrome *c*. The concentrations applied and the amounts transferred were quite similar to those in our studies, in which a rather different approach was used.

The results presented here show clearly that apocytochrome *c* does not compete with two other proteins imported into the inner mitochondrial membrane, i.e. these precursors are transferred into mitochondria in the presence of very high concentrations of apocytochrome *c*. It can be concluded that apocytochrome *c* on the one hand, and ADP/ATP carrier and subunit 9 on the other hand, do not have the same receptor involved in the first step of transport. The possibility must be considered that yet different pathways exist for ADP/ATP carrier and subunit 9, since one enters the mitochondria as a larger precursor form requiring proteolytic maturation, while the other does not. The conclusion that there are at least two and perhaps more different ports of entry for precursor proteins is supported by the observation that the protonophor $ClPhzC(CN)_2$ blocks import of ADP/ATP carrier and subunit 9, but not of cytochrome *c*. We have previously shown that $ClPhzC(CN)_2$ inhibits transport of the carrier *in vivo* during a chase period in the presence of cycloheximide [37]. Experiments *in vitro* have further established that $ClPhzC(CN)_2$ does not inhibit the specific binding of the precursor protein to the putative receptor but does inhibit its integration into a protease resistant position [8]. In agreement with this, $ClPhzC(CN)_2$ does not inhibit the binding of the 14000-M_r precursor of subunit 9, but inhibits its processing. The inhibition of processing *in vitro* corresponds to the findings with intact yeast cells that several inner membrane proteins are not processed to the mature form when yeast cells are poisoned with $ClPhzC(CN)_2$ [38]. This uncoupler of oxidative phosphorylation probably leads to a breakdown of the proton gradient and a lowering of the intramitochondrial ATP level. Energisation of mitochondria is apparently not necessary for the import of apocytochrome *c* into the intermembrane space.

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