Mobility and Recognition in Cell Biology

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RECOGNITION AND SPECIFICITY IN THE INTRACELLULAR TRANSFER OF MITOCHONDRIAL PROTEINS

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Introduction

Most proteins of organelles of eukaryotic cells are encoded on nuclear genes and translated by cytoplasmic ribosomes. Only in the case of mitochondria and chloroplasts, a few proteins are specified by genes located within the organelle and synthesized within the organelle itself (1). This means that the cell must have mechanisms to specifically direct a certain protein to its pertinent organelle and to selectively translocate it across the membrane which confines this organelle. Both processes are only poorly understood at present; the components and reactions involved in specific recognition of organellar proteins and the mechanisms of transmembrane translocation are still major riddles in cell biology. Transfer of proteins into mitochondria, on the other hand, involves even more complicated reactions. The mitochondrion is constructed of two very different membranes: the outer mitochondrial membrane which encloses the intermembrane space and the inner membrane which encloses the matrix space. Proteins from the cytoplasm must be imported into all these different compartments, some of them across both membranes, others across only the outer membrane. Some of the proteins end up in a hydrophilic, soluble compartment, others are integrated into the lipid phase of the outer or inner membrane. In most cases import is then followed by association into functional multi-subunit complexes.

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Methods to Investigate Transfer of Proteins into Mitochondria

Basically two approaches have been used to follow the intracellular pathway of a mitochondrial protein. The first one is an *in vivo* approach. Whole cells are pulse and pulse/chase labelled with radioactive amino acids, then cells are broken and measures are taken to inhibit any further reaction in the transfer process. Individual defined proteins are then immunoprecipitated from either whole cell extracts or from subcellular fractions. The immunoprecipitates are analysed by gel electrophoresis and autoradiography. This approach has given important information on the post-translational nature of the transport process (2). If the mature functional protein differs from the precursor form (see below) or intermediate sized forms occur, or when precursor forms can be differentiated from the mature forms by specific antibodies, conclusions can be drawn on precursor pool sizes, processing pathways, energy dependence etc. (3, 4).

The second approach to studying protein transport involves experiments *in vitro*. Mitochondrial precursor proteins can be synthesized in cell free homologous or heterologous mRNA dependent systems (5-9). Protein synthesis can be separated not only in time but also space from the transfer process. Transfer into isolated mitochondria can be investigated and the overall process can be subdivided into a number of steps: a) binding to recognition sites on the mitochondrial surface, b) membrane potential dependent translocation across or into the inner mitochondrial membrane, c) processing of precursor proteins by proteolysis or by covalent modification, and d) assembly to functional oligomeric molecules. The species specificity of the process can be investigated by such an *in vitro* approach as well as the problem whether all proteins follow the same transport pathway or whether there are diversified mechanisms. Clearly also in these experiments, specific antibodies must be available as well as techniques to follow the extremely small

amounts of proteins which are in the process of being transported.

Mitochondrial Proteins are Synthesized as Precursors which Differ in Structure and/or Conformation from the Mature Forms

When the synthesis of mitochondrial proteins was analysed *in vitro*, e.g. in mRNA-programmed reticulocyte lysates, most of the proteins analysed so far were found to be made as larger precursorswith additional sequences (3,7-17) (see Fig. 1). The lengthsof these presequences fall into in wide range between 400 and 10,000 daltons. As far as they have been studied, they are located at the amino terminus and are hydrophilic in nature. The exact function of these additional sequences is not known at present. They may play a role in the recognition of mitochondrial precursors, they may serve to alter the conformation of a protein in such a way that it is not active or that it cannot form active oligomers, or they may function in conferring a conformation on the precursor in which a recognition site is exposed. Proteins synthesized as larger precursors are transferred into the intermembrane space (e.g. cytochrome c peroxidase), the inner membrane (e.g. subunits of F_1F_o ATPase, cytochrome oxidase, cytochrome bc_1 complex) and the matrix space (e.g. TCA cycle and urea cycle enzymes). There are a few proteins whose precursor forms do not differ in apparent molecular weight from the mature counterpart. One example is cytochrome c, which is synthesized as apocytochrome c, i.e. without the covalently bound heme group (5,6,18,19). In this case it has been shown that the precursor protein does not differ in a single amino acid from the mature protein. Cytochrome c is a peripheral membrane protein attached to the cytoplasmic face of the inner membrane. The ADP/ATP carrier, a hydrophobic transmembrane protein (19)which is the most abundant protein of the inner membrane of most mitochondria is also synthesized without an additional sequence (20). Another

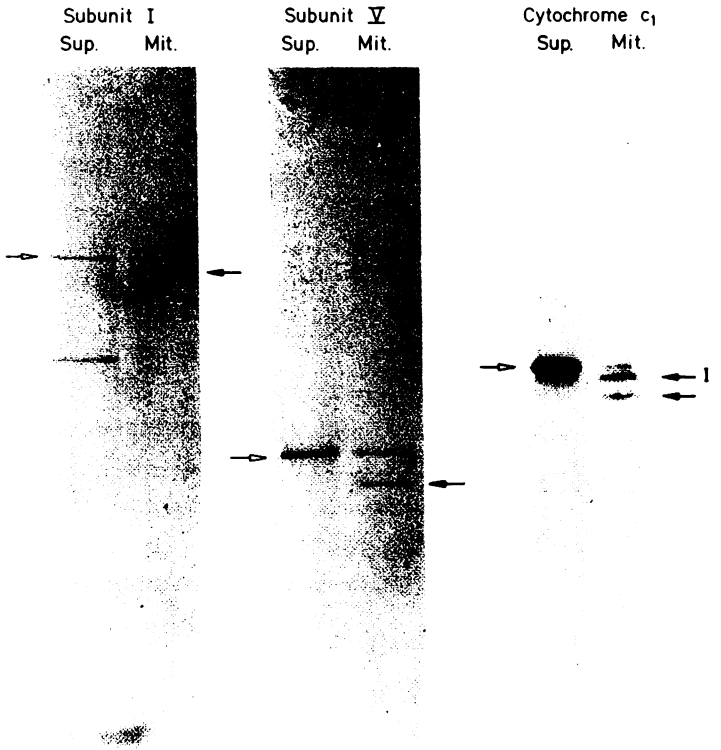

Fig. 1. Transfer *in vitro* of subunits of complex III (Ubiquinol:cytochrome c reductase). *Neurospora* RNA was used to program a reticulocyte lysate in the presence of [^{35}S]methionine. A postribosomal supernatant was prepared and incubated with mitochondria isolated from *Neurospora* spheroplasts for 60 min at 25°C. Then mitochondria and supernatant were separated again by centrifugation. Immunoprecipitation from both fractions was performed in the presence of 1 % Triton, 0.3 M NaCl employing antibodies against subunit I (core protein I), subunit V (Fe/S protein) and cytochrome c_1. The immunoprecipitated subunits were analysed by SDS gel electrophoresis and autoradiography. From the supernatants, the antibodies precipitated residual precursors not transferred into mitochondria. The mitochondria contained both precursor as well as processed subunits. The precursors associated with mitochondria were degraded by added protease as were the precursors in the supernatant (not shown here) indicating that they were on the surface of the mitochondria in contrast to the processed forms. In the case of cytochrome c_1 processing occurs via an intermediate form (I), which is the major processing product in this *in vitro* transfer. Open arrows: precursors; black arrows: mature proteins.

membrane protein synthesized at the same apparent size as the mature form is the mitochondrial porin, a channel forming protein of the outer mitochondrial membrane (21,22). Apocytochrome c differs greatly in conformation from holocytochrome c, the heme containing polypeptide. This is documented by the non-crossreactivity of antibodies raised against the two forms of cytochrome c (5). There is also evidence that the precursors of the ADP/ATP carrier and of mitochondrial porin differ in conformation from the assembled proteins. Apparently they can exist in a water-soluble form outside the mitochondria.

Transport of Proteins into Mitochondria Occurs Post-translationally

Studies in vivo, in which the appearance of labelled proteins was followed in pulse-chase experiments, have revealed the following features (2,3): a) labelled mitochondrial proteins appear in the mitochondria only after a lag phase when compared to the labelling of cytosolic proteins, b) for different mitochondrial proteins the lag phases are different, c) a sudden block of protein synthesis by poisoning cells with cycloheximide leads to an immediate stop of labelling of total cellular proteins but does not block appearance of labelled proteins in the mitochondria, and d) a precursor product relationship exists between pulse labelled mitochondrial matrix proteins in the cytosolic fraction and in the mitochondrial fraction.

Studies on the distribution of mRNAs for mitochondrial proteins have shown that in most cases they are associated with free, i.e. not membrane bound, polysomes (8,9,22).

All these observations support a post-translational mechanism of transport. Apparently, precursors to mitochondrial proteins are released from cytoplasmic ribosomes as completed polypeptide chains into extramitochondrial pools. The sizes of these pools are different for different proteins. Out of these pools,

precursor polypeptides are then translocated into the mitochondrion.
This mechanism differs largely from the other process of translocation of polypeptides across membranes which has been studied in great detail, the secretion of proteins (24,25). This latter process occurs by a co-translational mechanism, the nascent polypeptide chain being translocated through the membrane. This process of "vectorial translation" is carried out by ribosomes tightly bound to the endoplasmic reticulum membrane. Although basically differing in the mechanism of transmembrane transfer, secretion of polypeptides seems to have certain features in common with the post-translational transfer of mitochondrial proteins (see also below): The newly synthesized protein is identified by a "recognition protein" to be a secretory protein (26-29), the recognized part of the nascent chain being the aminoterminal "signal" sequence (30). Furthermore, recognition and translocation across the membrane appear to be two different steps in both processes.

Recognition of Mitochondrial Precursor Proteins

The post-translational transfer of precursor proteins is a multistep process. Analysis of the individual steps requires *in vitro* systems in which precursors can be transferred into mitochondria in a reaction separate from synthesis. For this purpose precursors are synthesized in cell free systems and the postribosomal supernatant fractions containing the precursors are prepared. On the other hand, mitochondria are isolated from cells by procedures which yield intact organelles. With a number of such *in vitro* systems, using mainly reticulocyte lysates and mitochondria from *Neurospora*, yeast, or rat liver, transport of a number of proteins into their submitochondrial compartments has been achieved (5,7,8,19,23,31-37). In some cases it has been shown that transfer into functional positions involving assembly or partial assembly to oligomeric structures actually occurs. ADP/ATP carrier transferred into

isolated Neurospora mitochondria becomes dimerized; it is able to bind the inhibitor carboxyatractyloside which confers protease resistance and inability to bind to hydroxylapatite to the protein assembled in vitro in the same way as to the mature dimeric carrier (M. Schleyer and W. Neupert, unpublished). For studying the first step, the recognition of precursors by the mitochondria, it must be possible to halt the process after the initial binding. There is not a single unique procedure to achieve that since there is not a unique mechanism of transport for all the various mitochondrial proteins. A few examples will be discussed here:

Precursors whose import depend on a mitochondrial membrane potential.

Experiments in vivo and in vitro have revealed that the majority of precursor proteins require a membrane potential across the inner mitochondrial membrane for transport (38-41)(see Fig. 2). Transport was blocked under conditions where the membrane potential was dissipated by the addition of uncouplers (carbonylcyanide m-chlorophenylhydrazone, dinitrophenol) or ionophores (valinomycin/K^+). This was also true when the generation of a potential was inhibited by applying both respiratory chain inhibitors (antimycin A, KCN) together with oligomycin which blocks ATP-driven H^+ ejection catalysed by the F_1F_o ATPase. A high level of ATP in the matrix in the absence of a membrane potential did not support import. This supports the conclusion that not ATP but the membrane potential serves as the primary energy source to drive import of precursor proteins.

In the absence of a membrane potential precursor proteins still become bound to the mitochondria (Fig. 2). This binding has the following characteristics: a) it does not lead to processing of those precursors which have additional sequences, b) the bound precursors are on the mitochondrial surface, i.e. the outer membrane; they can be digested by added proteases at very low concentrations which do not affect mature proteins transferred either in vivo or in vitro, c) they retain proper-

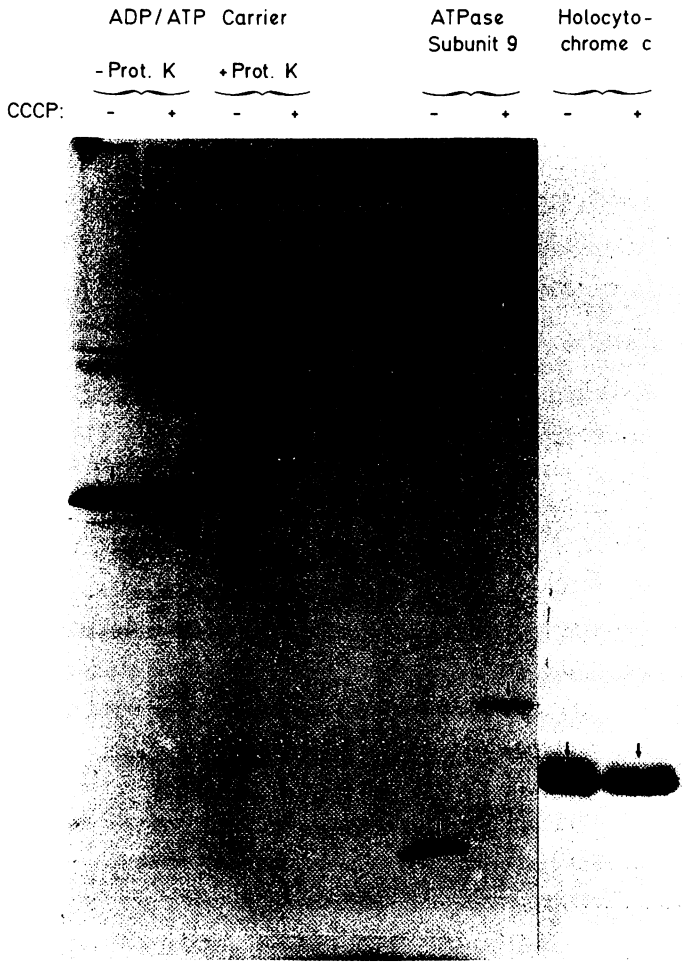

Fig. 2. Transfer in vitro of ADP/ATP carrier and ATPase subunit 9 depends on the mitochondrial membrane potential. Transfer of precursor proteins into isolated Neurospora was performed as described for Fig. 1, with the exception that half of the incubation mixture received 6 µM CCCP (carbonylcyanide m-chlorophenylhydrazone). After 60 min incubation, mitochondria were re-isolated and immunoprecipitation was performed with antibodies against ADP/ATP carrier, ATPase subunit 9 and holocytochrome c. For analysis of the ADP/ATP carrier, aliquots of the mitochondria were also treated with 100 µg/ml proteinase K for 30 min to digest precursor bound to the mitochondria. Immunoprecipitates were analysed by SDS gel electrophoresis and autoradiography.

ties of the precursorsin the cytosolic space, e.g. both cytosolic and mitochondria bound ADP/ATP carrier bind to hydroxyapatite, whereas the carrier in the inner membrane, transferred either *in vivo* or *in vitro*, does not bind, d) binding occurs at low temperature when transfer is very slow, e) binding is tight; once bound, the precursors cannot be removed by washing the mitochondria, f) binding apparently requires Mg^{++}ions.
The question whether binding in the absence of a membrane potential occurs to specific sites which are involved in the transport process is somewhat difficult to tackle. It has not been possible so far to prepare a precursor protein in pure form and in sufficient quantities to study ligand-receptor interaction in detail. However, the following experiments clearly support the view that binding occurs to sites which mediate the specific uptake of precursor proteins (C. Zwizinski, M. Schleyer, W. Neupert, unpublished). Mitochondria were treated with antimycin A and oligomycin so that the membrane potential was destroyed. Then precursors were bound from a reticulocyte lysate supernatant. Mitochondria were washed and resuspended in a supernatant not containing precursors. Then a membrane potential was built up by the addition of ascorbate plus N,N,N',N'-tetramethylphenylene-diamine, which feed electrons into the respiratory chain beyond site II which was blocked by antimycin A. Transfer of ADP/ATP carrier into a protease protected position and processing of ATPase subunit 9 did occur under these conditions. Excess unlabelled precursors added to the transfer mixture competed for uptake with the labelled precursor when added before binding to mitochondria, but not when added to mitochondria which had the radioactive precursors prebound.
All these data show that binding to specific sites on the mitochondria is a first step in the transport process which presumably serves in recognition of mitochondrial precursors by mitochondria. The data further suggest that import occurs via these receptor sites.
Precursors whose import does not require a membrane potential:

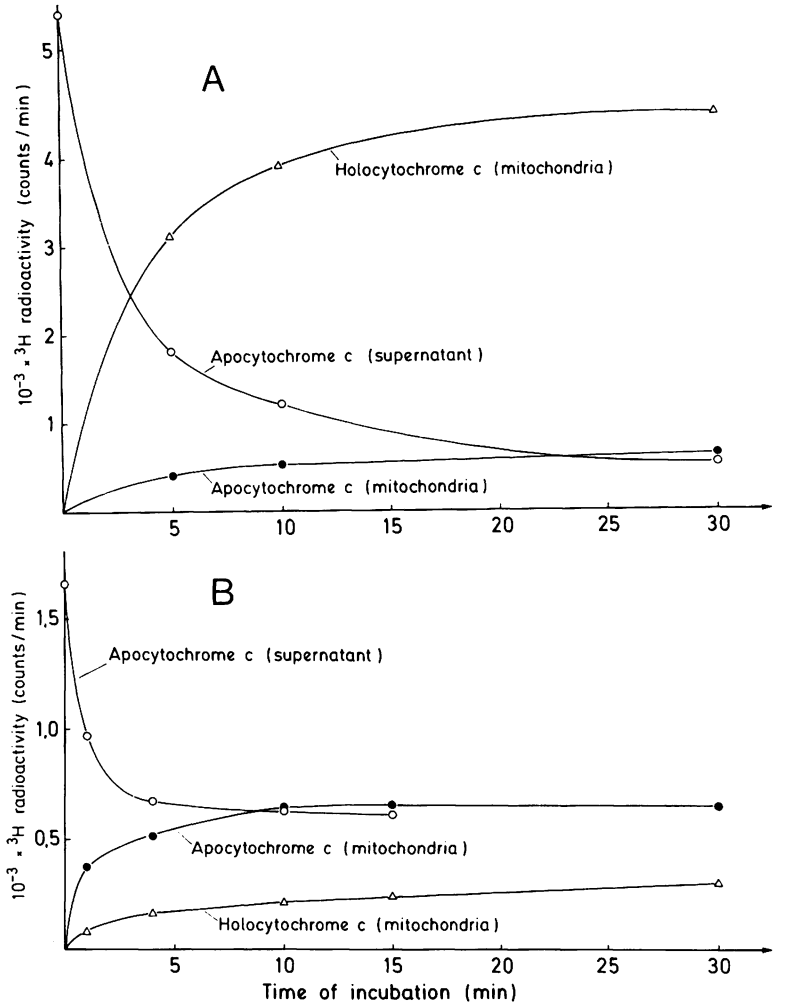

Fig. 3. Conversion of apocytochrome c to holocytochrome c and transport into mitochondria. Apocytochrome c was synthesized in a Neurospora cell free homogenate in the presence of [^{3}H]-leucine and the postribosomal supernatant was prepared. This was incubated together with isolated Neurospora mitochondria (A) in the absence and (B) in the presence of 10 nmol/ml deuterohemin. After the times indicated, mitochondria and supernatant were separated again by centrifugation. Apocytochrome c and holocytochrome c were immunoprecipitated with specific antibodies from supernatant and from mitochondria. The immunoprecipitates were analysed by SDS gel electrophoresis and the radioactivity in the cytochrome c peaks was measured. No significant amounts of in vitro synthesized holocytochrome c were found in the supernatant.

cytochrome c and the mitochondrial porin.

Cytochrome c is a peripheral membrane protein attached to the cytoplasmic face of the inner membrane. As mentioned before, it is synthesized as apocytochrome c (5,19). Apocytochrome c is transferred into isolated mitochondria, whereby the heme group is covalently attached and the polypeptide chain is refolded. This process is not dependent on a membrane potential (see Fig. 2). Blocking of the transfer process can, however, be achieved by interference with the attachment of the heme group (18). Deuteroheme, which lacks the two vinyl groups to which cysteines 14 and 17 of apocytochrome c become linked, inhibits the formation of holocytochrome c (see Fig. 3). Under this condition, apocytochrome c becomes bound to the mitochondria. The following line of evidence speaks for a binding to specific sites on the outer mitochondrial membrane: a) binding occurs to a position in which apocytochrome c is sensitive to added protease, b) labelled Neurospora apocytochrome c bound to Neurospora mitochondria can be displaced by excess chemically prepared unlabelled Neurospora apocytochrome c (Fig. 4), c) displacement efficiency decreases when apocytochrome c from other species is applied, with apocytochrome c from a procaryotic organism, Paracoccus denitrificans, not effecting any displacement; also, holocytochrome c cannot displace apocytochrome c at all (see Table 1), d) binding is tight; apocytochrome c cannot be removed by washing; titration of binding of ^{14}C labelled apocytochrome c prepared by reductive methylation yielded an apparent K of $7 \cdot 10^{-7}$ M (H. Köhler, B. Hennig, W. Neupert, unpublished), e) binding is directly related to transfer of cytochrome c across the outer membrane. When mitochondria, after having bound apocytochrome c in the presence of deuterohemin, are resuspended in a medium containing excess protohemin, the bound apocytochrome c is transferred into mitochondria and converted to holocytochrome c.

These observations make it clear that apocytochrome c is bound to a specific recognition site on the mitochondrial surface. The binding site on apocytochrome c is probably located in the

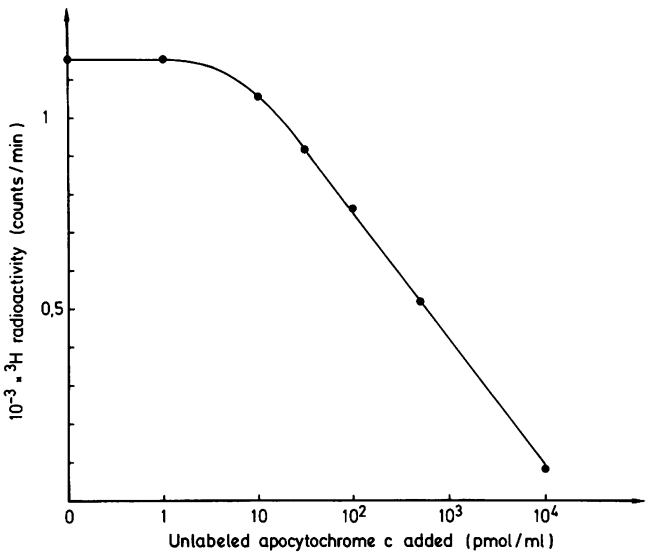

Fig. 4. Displacement of labelled apocytochrome c bound to mitochondria by excess unlabelled apocytochrome c. Neurospora mitochondria were incubated in the presence of 10 nmol/ml deuterohemin with a supernatant of a Neurospora cell free homogenate containing ^{3}H-labelled apocytochrome c. After 15 min incubation, mitochondria were reisolated. They were resuspended in the supernatant fraction of a Neurospora homogenate and aliquots were incubated for 15 min with the indicated concentration of unlabelled apocytochrome c. Mitochondria were reisolated and apocytochrome c was immunoprecipitated. The immunoprecipitates were subjected to SDS gel electrophoresis and the radioactivity in the cytochrome c peaks was determined.

C-terminal half of the molecule (19)(B. Hennig and W. Neupert, unpublished). Mitochondrial outer membrane porin is also a protein which does not need a potential across the inner membrane for its insertion (23). In this case binding to the surface can be distinguished from membrane integration by incubating mitochondria with precursor at low temperature (0-4°C). Under these conditions, the precursor is attached to the surface so that it is still sensitive to added protease, in contrast to the protein transferred into the membrane. Precursor can be bound at low temperature and the mitochondria then re-

Table 1. Displacement of apocytochrome c from mitochondrial receptor sites. Species specificity for apocytochrome c. ^{3}H-labelled Neurospora apocytochrome c was bound to isolated Neurospora mitochondria as described in Fig. 4. Displacement of the labelled apocytochrome c by unlabelled apocytochrome c from different species was determined by titrating with apocytochrome c from various species (see Fig. 4).

Displacement by	% Labelled apocytochrome c displaced
Apocytochrome c	
Neurospora	99
Yeast	75
Horse heart	72
Parsnip	55
Paracoccus	0
Holocytochrome c	
Neurospora	0

isolated, washed, resuspended in a non-synthesis lysate and warmed up to 25°C. The bound precursor is thereby inserted into a protease resistant position (H. Freitag and W. Neupert, unpublished). As with the examples described above, these observations imply the existence of receptor sites on the mitochondrial surface.

How many different receptors are there on the mitochondria? It seems highly unlikely that every protein has its own receptor. On the other hand, experiments described below clearly suggest that there is more than one type of receptor. The major drawback in studying this question is that precursors are usually not available in amounts which allow the performance of competition experiments. There is, however, one exception: apocytochrome c can be prepared from mature holocytochrome c in practically unlimited amounts. Competition experiments were performed with apocytochrome c in cell free systems involving reticulocyte lysate supernatants and isolated Neurospora mitochondria. They showed that the recognition of all other mito-

chondrial precursor proteins tested so far (e.g. ADP/ATP carrier, ATPase subunit 9, cytochrome c_1, porin) apparently does not occur by the cytochrome c receptor (40) It is not known whether all the other precursors use one receptor or whether there are several classes.

Universality of Recognition and Uptake

The *in vitro* systems for protein transport allow the study of species specificity in the components of the transport machinery. It has turned out that a number of *Neurospora* proteins such as the Fe/S subunit of complex III, subunit 9 of F_1F_o ATPase, ADP/ATP carrier, and porin can be transferred into mitochondria from yeast, rat liver or guinea pig heart (35) (B.Schmidt, M. Schleyer and W. Neupert, unpublished). The same energy requirements were observed as with *Neurospora* mitochondria. These observations imply that the transport mechanism is highly conserved in evolution and that mitochondria recognize the precursors of widely different species. In the case of cytochrome c, affinities of apocytochrome c from different species for the *Neurospora* receptor can be deduced from the concentrations required to displace bound *Neurospora* apocytochrome c. As mentioned above, there are clear differences. Nevertheless, cytochrome c import into heterologous mitochondria can be demonstrated (42).

A question of special interest in this context is whether certain mitochondria can import proteins from other species which do not occur in the cells from which the mitochondria are isolated. The answer to this question is apparently yes; e.g. it was found that yeast mitochondria import and process correctly the precursor to ATPase subunit 9 (extension 6500 daltons) (B. Schmidt, B. Hennig and W. Neupert, unpublished). In yeast this protein is coded by a mitochondrial gene, synthesized by mitochondrial ribosomes without any additional sequence, and inserted from the matrix side into the inner membrane. The yeast and *Neurospora* proteins are homologous, 40 out of the 76 and

81 amino acids, respectively, being identical. They apparently have the same function in the oligomycin sensitive ATPase(43).It seems that for import into yeast mitochondria, the Neurospora precursor utilizes a pathway which is designed for other proteins and still reaches the right submitochondrial compartment. This may indicate that recognition and transmembrane transfer are absolutely specific for mitochondrial proteins, but that on the other hand the transfer machinery may handle many different proteins having common devices for recognition and assembly.

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DISCUSSION

Voordouw: Does the isoelectric point of precursors to mitochondrial matrix or inner membrane proteins differ from that of the mature proteins in a systematic way that can be related to the direction of transport?

Neupert: There are reports that a number of mitochrondrial precursor proteins are more basic than the respective mature forms. On the other hand, this does apparently not hold for all imported mitochondrial proteins. One could envisage a mechanism in which a ΔpI does play a role and which implies the requirement for a membrane potential positive outside. However, I do not see how simply a difference in pI between precursor and mature forms could determine the direction and specificity of polypeptide transport across membranes.

Kreil: Sabatini and Margoliash have reported, that cytochrome *c*-uptake is blocked by a fragment derived from the COOH-terminal of cytochrome *c*, which implies that the receptor recognizes a region in this part of the molecule. Do you have data in this context?

Neupert: Neurospora apocytochrome *c* can be cleaved at a tryptophan residue roughly in the middle of the molecule with BNPS-skatol (experiments by Dr. B. Hennig). The C-terminal fragment has some activity in inhibiting uptake

and conversion of labelled apocytochrome *c*, whereas the N-terminal fragment is completely inactive. However, the concentrations required are very high as compared to the uncleaved apocytochrome *c*. The same is true for the experiments with bromocyanogen fragments of cytochrome *c* by Morimoto, Sabatine and co-workers. Therefore in our view it remains to be shown whether these fragments really interact specifically with a component of the cytochrome *c* assembly pathway, or whether we see an unspecific effect of a sticky polypeptide fragment.

Kreil: Do apocytochrome *c* and other precursors of mitochondrial proteins compete for the same receptor?

Neupert: We have tested whether apocytochrome *c* competes for uptake of the precursors to the following proteins: ADP/ATP carrier, ATPase subunit 9, cytochrome c_1 and porin. In all cases the answer was no.

Wirtz: You go by the hypothesis that the outer mitochondrial membrane has the receptors for the proteins to be inserted into the inner mitochondrial membrane. What kind of recognition mechanism does the inner membrane have?

Neupert: The model which I am proposing implies that the transfer of proteins into the inner membrane occurs by a process in which translocation across outer and inner membrane is a single step. This, of course, would require some sort of interaction between the two membranes. As I pointed out, in de-energized mitochondria the precursors by all available criteria stay at the surface of the mitochondria, i.e. do not cross the outer membrane.

Jovin: Is there a stoichiometric relationship between the number of receptors in the outer membrane for apocytochrome *c* and the number of inactive cytochrome *c* molecules finally integrated into the mitochondrion? That is, does the receptor act 'catalytically' in the sense of promoting the transport of many cytochrome *c* molecules?

Neupert: The amount of holocytochrome *c* in Neurospora is about 1000 $pmol.mg^{-1}$ protein, the rate of synthesis (log phase) about 3 $pmol.mg^{-1}.min^{-1}$, the number of high affinity binding sites for apocytochrome *c* is roughly 100 $pmol.mg^{-1}$. So the number of binding sites appears to be rather high. We have reasons to assume that there are much fewer binding sites for other precursor proteins. It is quite likely that recognition sites act 'catalytically', otherwise the cell would have to synthesize and degrade enormous amounts of receptor molecules and there is no indication for that from pulse-chase labelling experiments.

Veeger: Have you measured the actual value of the membrane potential and if so is there a minimum potential needed for the uptake? Is the ΔpH also involved?

Neupert: Until now we have not been able to determine the actual membrane potential, since transport *in vitro* is carried out in a rather complex system (reticulocyte lysate supernatant). From a number of observations we

have got the impression that probably a relatively low potential, as compared to that required for ATP synthesis, is necessary for protein import. Nigericin does not inhibit the import, therefore a ΔpH does not seem to be involved.

Veeger: The difficulty I have is a system in which every subunit is transported via its own recognition site. That would require a large number of these sites plus the additional problem that subunits recognize each other. Which is your view?

Neupert: A determination of the number of different recognition sites requires that the different precursors are available in sufficient quantities to carry out competition experiments. At present, only one precursor can be prepared in such quantities, namely apocytochrome *c*. Competition experiments with apocytochrome *c* tell us that there must be at least two different recognition sites. We have observed that precursor proteins from Neurospora can be transferred *in vitro* into mitochondria of organisms phyologenetically as distant as rat(liver), guinea pig (heart) and yeast. This would suggest a degree of universality of the system and may mean that the diversity of the recognition sites is rather limited.

Brass: Is there any sensitive test available which would allow testing of the biological function of the precursor protein imported into isolated mitochondria (mutants)?

Neupert: The amounts transported in *in vitro* experiments are very small (usually less than a picomol). Therefore it is not possible to measure enzymatic activity etc. There are, however, observations which indeed suggest that after transfer *in vitro*, precursors acquire properties of the functional proteins. E.g. the ADP/ATP carrier acquires the property of not binding to hydroxyapatite which is a characteristic feature of the mature assembled protein. Also, the ADP/ATP carrier transferred in the presence of a membrane potential appears to interact with the specific inhibitor carboxyatractyloside, in contrast to the free precursor or to precursor bound to mitochondria in the absence of the membrane potential. Furthermore, we have found that part of the ATPase subunit 9, transferred and processed *in vitro*, can be immuno-precipitated from Triton-solubilized mitochondria with antibodies against the F_1-ATPase, suggesting some sort of (partial) assembly with other components of the ATPase complex.

Schweizer: Is it possible to abolish uptake or binding of imported proteins by treating the mitochondria with proteinases (damage of the postulated receptors) before adding the pre-synthesized proteins which have to be imported? Are all imported proteins subjected to this inhibition?

Neupert: Yes. Treatment of mitochrondria with trypsin abolishes binding and uptake of precursors. It appears that insertion of the porin into the outer membrane is much less sensitive to protease treatment than *e.g.* transport of the ADP/ATP carrier and ATPase subunit 9 into the inner membrane.

Bade: Is the synthesis of mitochondrial proteins/peptides coded by nuclear and mitochrondrial DNA coordinated (regulated)?

Neupert: There must be mechanisms which coordinate the synthesis of nuclear and mitochondrial gene products. Enzyme complexes of the inner mitochondrial membrane such as cytochrome oxidase, ubiquinol-cytochrome *c* reductase and oligomycin-sensitive ATPase contain subunits from both genetic systems in stoichiometric relationships. A number of mechanisms have been proposed operating at the various levels of gene expression. However, it seems to me that the interplay between the two systems will not be understood before we know more about the molecular mechanisms which govern the assembly of mitochondria.