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Targeting Proteins into Mitochondria

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INTRODUCTION

From the pioneering insights of the microscopists, spectroscopists, and cell physiologists almost a century ago (16) to the elucidation of intracellular organelle-organelle communication by cell and molecular biologists today, mitochondria continue to be the focal point for biochemical analysis of many intracellular processes. This organelle is now poised with its wealth of biophysical, cell biological, and structural knowledge to complement the tools of the molecular geneticist. This is most evident in the analysis of intracellular protein targeting and the translocation mechanism of proteins across a cellular membrane.

It has been known since the early 1950s that the development of functional mitochondria in yeasts requires Mendelian genes; however, the extent to which nuclear deoxyribonucleic acid (DNA) contributed to mitochondrial development was not appreciated until 20 years later. Indeed, the few mitochondrially encoded proteins in yeasts and higher eucaryotes are subunits of oligomeric enzymes which consist for the most part of proteins encoded by nuclear DNA (13). Based on labeling and genetic studies, the amount of protein contributed by the nuclear and mitochondrial genomes has been evaluated. It has been conservatively estimated that the nucleocytoplasmic system contributes greater than 90% of the protein mass to the organelle.

During the past 8 years considerable effort has gone into analysis of the delivery and localization mechanisms for mitochondrial proteins (53, 67). Our current understanding of the events required to correctly deliver a protein into mitochondria has come largely from studies in *Saccharomyces cerevisiae* and *Neurospora crassa*, which trace the fate of mitochondrial precursors in vivo compared to their behavior in in vitro import assays. The recent explosion of data bearing on the mechanism of import of proteins into mitochondria reflects the fact that they can be conveniently purified as intact, well-characterized organelles. Most importantly, however, they are isolated with the same membrane orientation and compartmentalization that they exhibit in situ. Thus, assays which conveniently measure the import and localization of proteins in vitro do so in the same direction as that which occurs within the cell.

The membrane permeability properties of both inner and outer mitochondrial membranes to various metabolites, cations, and anions are better characterized than those for any cellular membrane. Because of this, the functional integrity of isolated mitochondria can be conveniently and sensitively documented. One can purify intact organelles which are essentially impermeable to protons as determined by a convenient and sensitive assay. These permeability properties and the use of antibiotics which alter membrane ion transport have proven to be valuable assets for the biochemical analysis of protein import into mitochondria. The well-defined biochemistry of the mitochondrial system is currently being combined with genetic manipulations and in vitro import assays to define additional details of the import mechanism. The in vivo analysis of this mechanism has been provided in large part in yeasts as well as in transient expression systems in animal cells. In this review we describe how recent technological and genetic advances in the field of organelle assembly within the past year have been used to further define the mechanism of protein transport into mitochondria. These data will be presented within a brief summary of observations which have been discussed in recent reviews (26, 60, 78).

BIOCHEMICAL ANALYSIS OF PROTEIN IMPORT

Co- Versus Posttranslational Initiation of Import

Early studies by Butow documented the presence of a specific class of 80S cytoplasmic ribosomes bound to the mitochondrial outer membrane (37). Since these bound ribosomes could only be released by the combination of high

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salt and puromycin, the binding was proposed to be mediated by nascent polypeptides which were "frozen" during cotranslational insertion of the growing polypeptide into mitochondria. Subsequent studies which have examined the in vitro completion of F_1-adenosine triphosphatase (ATPase) subunits from this mitochondrially bound ribosome fraction have demonstrated that these organelle-bound ribosomes are enriched over nonbound ribosomes in their content of specific mitochondrial proteins (1). In addition, quantitation of messenger ribonucleic acids (RNAs) from mitochondrially bound and unbound populations of ribosomes for specific mitochondrial and nonmitochondrial proteins provided independent support for this specific enrichment (77). During steady-state growth of cells the mitochondrially bound ribosomes represent only about 10 to 15% of the total cytoplasmic ribosome content. However, >50% of the total messenger RNA for specific mitochondrial polypeptides is present in this bound ribosome fraction. Thus, in vivo, the specific initiation of mitochondrial import can occur, for at least some proteins, prior to their completion. Clearly, soluble mitochondrial precursor proteins can be imported into mitochondria following completion of their synthesis (see below). Thus, the mitochondrial system exhibits flexibility in the timing of synthesis and the initiation of import. It would appear that the signals which initiate mitochondrial import are located within the residues initially synthesized in the nascent peptide and that they remain accessible for membrane binding within the completed protein.

During steady-state growth in *S. cerevisiae*, extra mitochondrial pools of precursors are not detectable. However, precursors have been demonstrated to accumulate under conditions in which the import was prevented by the addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (52, 77). In *N. crassa*, extramitochondrial pools of newly synthesized adenosine 5'-diphosphate/adenosine 5'-triphosphate (ADP/ATP) translocator protein were demonstrated which could be subsequently imported into mitochondria (24). Likewise, in *S. cerevisiae* the cytoplasmic accumulation of the F_1-ATPase β-subunit precursor was apparent, using CCCP to block import. This newly synthesized precursor could be subsequently chased into mitochondria following inactivation of the import block (61).

These analyses clearly demonstrated that the initiation of protein import into mitochondria could occur either prior to or after the completion of protein synthesis. The extent to which a given protein will be imported by either route will depend on the rate at which targeting sequences present on the precursor will recognize the mitochondrial import machinery relative to its rate of completion. One can reduce the rate of precursor synthesis to reduce the cytoplasmic pool of the precursor (61) or, conversely, decrease the mitochondrial binding efficiency to increase the pool of cytoplasmic precursor. In this respect the import of proteins into mitochondria exhibits similarities to the export of mutant proteins from *Escherichia coli* (81). The initiation of export of the wild-type bacterial protein is very efficient and occurs before the completion of the nascent precursor. However, mutations within the protein which reduce its efficiency of export initiation exhibit pools of completed precursor in the cytoplasm which can be subsequently exported at reduced efficiency (66). Thus, for any imported mitochondrial protein or exported proteins from the procaryote, the efficiency of its transport initiation relative to its rate of synthesis will define the extent to which cytoplasmic pools of precursor in each case will be detected.

Precursor Requirement for Import

The specific sorting of mitochondrial proteins in the cytoplasm as either completed or nascent polypeptides for import requires the presence of a specific recognition or targeting signal. Considerable insight into the mechanism of the mitochondrial targeting event was provided with the observation that some mitochondrial proteins, such as the three largest F_1-ATPase subunits, were initially synthesized as higher-molecular-weight precursors (43). Addition of the in vitro synthesized high-molecular-weight precursor containing an estimated 20 to 40 additional amino-terminal residues to isolated mitochondria resulted in their uptake. The isolated mature subunits lacking these transient residues were not sequestered by the isolated organelles. These data clearly demonstrated that mitochondria distinguished the import competency of the high-molecular-weight precursors from the mature proteins. This observation led to the initial suggestion that the additional sequences played an essential role in the uptake mechanism (43).

At the same time, however, it was clearly demonstrated that some mitochondrial proteins such as cytochrome *c* (89) and the ADP/ATP translocator protein (89) were not synthesized as high-molecular-weight precursors yet were efficiently imported into isolated mitochondria. The precursor nature of these mitochondrial proteins was established by the demonstration that they exhibited an extra mitochondrial conformation distinct from the mitochondrially associated mature form of the protein. Antibodies were prepared which specifically recognized the precursor apocytochrome *c* but not the mitochondrially associated holocytochrome *c* (41). The soluble precursor form of the membrane-bound ADP/ATP translocator protein (87), like that of the porin (57), was present as a soluble aggregate which assembled into a different oligomeric state in the membrane. In fact, all of the different mitochondrial precursor proteins described thus far are water soluble and exhibit a conformation or aggregate state which is distinct from the mitochondrially localized protein. With notable exceptions, either covalent modification by endoproteolytic cleavage or attachment of a prosthetic group induces a new conformation of the protein upon binding to mitochondria.

Energy Requirements for Protein Import

The transfer of cytoplasmically synthesized proteins into mitochondria is an energy-dependent process. By using both in vitro and in vivo assays to measure the transport of various precursors into mitochondria, it has been shown that conditions which prevent the generation of an electrochemical gradient of protons across the inner membrane block the uptake of mitochondrial precursors. Independently, two groups were able to show that the addition of respiratory inhibitors, ionophores, or uncouplers of oxidative phosphorylation prevented the uptake of the mitochondrial F_1-ATPase subunit precursors (52) or the ADP/ATP translocator protein (87). The energy dependence of protein import has been documented for a large number of imported proteins and compiled elsewhere (26). The steady-state electrochemical gradient of protons across the active mitochondrial inner membrane has a membrane potential ($\Delta\psi$) and chemical (ΔpH) component (47). Studies to define the relative contribution of each of these components to transmembrane transfer or insertion of cytoplasmic precursors have utilized well-characterized ion-transporting antibiotics which selectively equilibrate either the $\Delta 4$ or the ΔpH component. The

ionophore valinomycin abolishes the membrane potential ($\Delta\psi$) component by equilibration of K^+ ions with those in the suspension medium. This ionophore in conjunction with K^+ ions prevented the in vitro import of the ADP/ATP translocator and ATPase subunit 9 precursors into isolated *N. crassa* mitochondria (72, 79). The ionophore nigericin catalyzes an electrically neutral exchange of either H^+ or K^+ ions across the inner membrane and selectively equilibrates the chemical gradient of protons with K^+ ions without altering the membrane potential. The addition of this cation-exchanging antibiotic did not inhibit the in vitro import of proteins into mitochondria (72), further documenting the primary role of the membrane potential component in mitochondrial import. The definitive experiment demonstrating that the membrane potential alone is the required energy for import was the observation that the import of the ADP/ATP translocator protein could be driven by a valinomycin-mediated K^+ diffusion potential even under conditions in which the membrane was completely permeable (presence of the protonophore CCCP) to protons (58).

These studies provided convincing proof that a membrane potential is an essential step in the import of proteins; however, the function performed by this charge separation across the inner membrane has not been determined. Recent studies indicate that the amino terminus of mitochondrial precursors which exhibit a net positive charge (see below) is necessary and sufficient to direct mitochondrial import. Thus, one might envision the membrane potential across the inner membrane (net negative charge on the matrix face of the membrane) as providing the potential to initially electrophorese the extreme amino terminus or a cluster of positively charged residues through the bilayer to "trigger" import.

Recent in vitro import experiments with *Neurospora* mitochondria have demonstrated that the membrane potential-dependent import of only the amino-terminal residues of the F_1 β-subunit precursor is the committed step in transport of this precursor into mitochondria. When the F_1 β-subunit precursor is bound to energized mitochondria at 4°C, import is initiated but not completed (71). These authors demonstrated that the amino terminus of the protein, apparently trapped in transit at 4°C, was localized within the matrix of the mitochondria while regions of the same protein were accessible to antibody on the cytoplasmic face of the outer membrane. The amino terminus had been processed by a metalloprotease located within the matrix. When these mitochondria were warmed to 24°C, completion of import into the mitochondrial interior was documented. Most importantly, however, addition of ionophore or CCCP to deenergize mitochondria did not inhibit the completion of processed F_1 β-subunit import which had been previously initiated at 4°C. These studies indicate that the membrane potential is required to initiate the import of the mitochondrial precursor but not to complete it.

Determination of alternative roles for the energized membranes in the transport of proteins can be compared to protein export in *E. coli*. The secretion of proteins from *E. coli* requires an energized membrane (59). In addition, the initiation of protein export utilizes a presecretory sequence at the extreme amino terminus which exhibits a net positive charge. However, the polarity of the charge across the membrane with respect to the direction of protein transport from *E. coli* is opposite to that of mitochondria. Therefore, if mitochondria and *E. coli* utilize a similar mechanism to initiate protein transport, a simple electrophoretic model is insufficient to reconcile.

Other possibilities for the role of the energy requirement in mitochondrial import have received only circumstantial support or have been inferred. One proposal is that transient import sites form at "tight" junctions between the inner and outer membrane in an energy-dependent manner. These junctions would be generated by new combinations or conformations of proteins or lipid or both from either membrane. Freeze-fracture plane deflection analysis of mammalian mitochondria indicates that the surface area of apparently fused inner and outer membrane is increased in energized versus deenergized organelles (40). This fused mitochondrial membrane fracture plane was not observed upon simple swelling of the membrane to bring the two membranes close together. Tight associations between mitochondrial membranes were noted in early studies which defined the location of membrane-bound ribosomes harboring nascent peptides at regions of inner-outer membrane contact (37, 38). The recent demonstration that the imported precursors can be apparently "trapped" spanning both membranes (71) is consistent with this possibility.

Intramitochondrial Protein Traffic

All of the proteins constituting the four submitochondrial spaces of the organelle must be programmed for correct localization following their delivery from the cytoplasm. Clearly, proteins delivered to the mitochondrial matrix must be transported through both the inner and outer membranes of the organelle. However, many proteins which reside in either the inner or outer membrane or intermembrane space must contain "programs" which define selective retention or transport through a given membrane. How can a transmembrane protein of the inner membrane synthesized in the cytoplasm complete its transport through the outer mitochondrial membrane? What causes soluble proteins to stop further import at the intermembrane space rather than going to the mitochondrial matrix? These questions have been answered in part by characterization of the precursor nature of proteins located in different submitochondrial compartments. In addition, the identification and localization of mitochondrial components which participate in the maturation of these precursors have aided in the formulation of a model.

Analysis of proteins imported to the intermembrane space has provided and most revealing insights into the role of the membrane potential and processing enzymes in defining submitochondrial localization. Cytochrome *c* is localized to the cytoplasmic surface of the mitochondrial inner membrane by a pathway that is different from that of the other proteins within the intermembrane space. Apocytochrome *c* is synthesized on cytoplasmic ribosomes as a full-length mature apoenzyme (88) which binds to mitochondria at specific sites (27) in an energy-independent manner. Complete transfer of this protein across the outer membrane occurs upon covalent attachment of the heme to cysteine residues near the middle of the apoprotein by enzymes within the intermembrane space (28, 44). The conformational change of the apocytochrome upon covalent addition of heme is proposed to drive the transfer of the protein across the outer membrane.

Other proteins of the intermembrane space such as cytochrome c_1, cytochrome b_2, and cytochrome *c* peroxidase are made as higher-molecular-weight precursors (18, 55, 61) and imported into mitochondria in an energy-dependent fashion. The energy requirement for the localization of these proteins to the intermembrane space indicated that the import was

not just a simple transport of the protein across the outer membrane like cytochrome *c*. Indeed, subsequent studies revealed that the localization of these intermembrane space proteins required the participation of processing enzymes within the mitochondrial matrix and inner membrane. When yeast precursors synthesized in a cell-free protein synthesis reaction were incubated with a processing enzyme preparation from the mitochondrial matrix (5, 45), a processed form of the cytochrome b_2 (10) or cytochrome c_1 (18, 55, 79) precursor was generated which was slightly larger on sizing gels than the isolated mature form. The biological significance of this larger intermediate form was subsequently demonstrated for cytochrome b_2 in two in vivo studies. First, when growing yeast cells were pulse-labeled, a transient intermediate form of the cytochrome b_2 protein, distinct in size from either the precursor or mature form, could be demonstrated. This intermediate form could be generated in vitro by the metalloprotease in the matrix extract and was not observed if the uncoupler CCCP was maintained in the in vivo pulse-labeling reaction (62). The energy dependence of the in vivo processing as well as the demonstration of a matrix enzyme-dependent intermediate indicated that at least a portion of the protein destined for the intermembrane space was transiently localized within the mitochondrial matrix.

Additional biochemical analysis of the intermembrane space localization pathway took advantage of the observation that the covalent attachment of heme to proteins can occur on the cytoplasmic face of the inner membrane (28). Proteins analyzed thus far for localization to the intermembrane space each undergo covalent attachment of heme during their maturation. Using a yeast *hem1* mutant which was unable to synthesize a functional 5-aminolevulinate synthase (the first step in heme biosynthesis), Ohashi et al. (55) demonstrated that the intermediate form of cytochrome c_1 accumulated in isotope dilution experiments. When 5-aminolevulinic acid was supplied to the mutant, thus bypassing the genetic block and allowing the synthesis of heme, the intermediate form of cytochrome c_1 was readily converted to the mature form. The intermediate form of cytochrome c_1 which accumulated cofractionated with the mitochondrial inner membrane, leading to the suggestion that this form of the protein remained firmly attached with the membrane and that the second processing step, which is dependent on the presence of heme, will release the mature protein to the intermembrane space or orient it on the inner membrane.

The localization of proteins to the outer mitochondrial membrane clearly contrasts that of precursor localization to the outer mitochondrial spaces. First, the insertion of proteins into the mitochondrial outer membrane does not require an energized inner membrane (17, 19). A membrane electrochemical potential across the outer mitochondrial membrane is not possible since this membrane is permeable to molecules up to 8,000 daltons (Da) (8). Second, proteins which assemble into the outer mitochondrial membrane are not made as higher-molecular-weight precursors. Porin is the major component of the outer mitochondrial membrane for which a function can be defined (85). It, like many other outer membrane protein precursors, can be made as an apparently mature-sized protein and inserted post-translationally into the outer membrane in the absence of an energized inner membrane (17, 19, 46). In addition, the insertion of the soluble porin precursor into isolated mitochondria or purified outer membrane vesicles from mitochondria rendered the protein resistant to externally added protease. Thus, this protein is apparently oriented in the same manner as the endogenous porin protein following binding (64, 65). Other proteins located in the mitochondrial outer membrane can be inserted by this one-step process such that they adopt their in situ membrane orientation (17). Therefore, in contrast to most other cytoplasmically synthesized components of the organelle, the proteins targeted to the outer membrane may be sequestered in the absence of any apparent covalent modification. However, similar analysis of a 35-kDa outer membrane polypeptide from rat liver mitochondria has revealed that it is made as a larger precursor (74). Additional studies are necessary to further characterize the apparent maturation of this outer membrane protein before evoking a more complex insertion/localization mechanism for outer membrane proteins.

Thus, the delivery of a protein through two membranes to the mitochondrial matrix is programmed in some manner into the precursor. Recent gene manipulation experiments demonstrate that targeting and the initiation of import are programmed into sequences at the extreme amino terminus of the precursor. This "targeting" sequence is often removed within the matrix but may be retained as part of the correctly localized protein. The ability to stop a protein during import for correct localization in either the inner or outer membrane will require additional localization signals (discussed below) which direct the transport and processing machinery within the different organelle compartments.

Machinery of Mitochondrial Import

The characterization of sequences required for the targeting and import of proteins into mitochondria represents an initial step in the analysis of the mechanism of intracellular delivery. The availability of new sequence data for the amino terminus of different mitochondrial precursors and the localization of the targeting signals in several of these proteins reveal some potential structural similarities (see below) and limited primary sequence conservation. It is anticipated that the components present on the mitochondrial surface will be very discriminative. In the best-documented case, the binding of apocytochrome *c* to a specific receptor component has been characterized (27). The receptor component is specific for only the apo form of the enzyme and is not competed for by other precursor proteins. A limited number of cytochrome *c* receptor sites (90 pmol/mg of mitochondrial protein) can be defined on the mitochondrial surface. In different studies the selective inhibition of import or binding of one precursor by another has been demonstrated following very mild treatment of mitochondria with protease (65, 90, 91).

Although these preliminary observations indicate that individual receptors mediate import, it is unlikely that different proteins will be available on the surface of mitochondria to individually bind each of several hundred precursors for import. It is most likely that a limited set of components on the mitochondrial surface will interact with different classes of mitochondrial precursors. In recent studies, the purified 3-ketoacyl-coenzyme A thiolase inhibited the in vitro import of three other precursors to the rat liver mitochondrial matrix (51). This result indicated that a common set of mitochondrial components participates in the matrix delivery of these proteins in rat liver. In addition, a limited number of proteins are present in the outer membrane (64, 74). Most recently, synthetic targeting signal peptides have been chemically synthesized which inhibit the in vitro import of selected precursors into mitochondria (21,

84). These provocative results must be interpreted with some caution since these synthetic peptides may specifically disrupt the mitochondrial inner membrane to discharge the membrane potential required for import. The inhibition observed in these studies may reflect the competition for a threshold membrane potential necessary to drive the import of different proteins rather than competition at a specific binding site.

In addition, soluble factors are proposed to mediate the delivery of proteins for import. This protein factor has been described in rabbit reticulocyte lysate for the mitochondrial import of ornithine transcarbamylase (OTC) precursor in rat (3, 4, 49) and the F_1 β- subunit in yeast cells (56). Recent studies indicate that a protein import factor associates with the OTC precursor before binding of the factor-precursor complex with mitochondria (4). The import factor, however, does not bind to mitochondria in the absence of the OTC precursor. Thus, the association of the precursor with the cytosolic factor may be required for the binding to other components on the outer membrane. The observation that animal cell mitochondrial precursors can be imported into yeast mitochondria (A. Horwich, personal communication) and that precursors from divergent yeasts can be efficiently transported in different species (6) would indicate that the basic features of mitochondrial import have been conserved.

DETERMINANTS FOR MITOCHONDRIAL LOCALIZATION

Gene Fusion Studies

The mitochondrial delivery and compartmentation of a protein are programmed into its primary sequence. Recent studies are beginning to tell us that the context in which sequences within the protein are presented to the mitochondrial transport and processing apparatus will define the location of the protein. Biochemical analysis of receptor components on the mitochondrial surface (see above) has revealed that there are, at most, a limited set of specific molecules available for the binding of mitochondrial precursors. What sequences on mitochondrial precursors are recognized by these receptors to specifically segregate them from the cytoplasm?

Molecular genetics is the major experimental approach which has been used to define and characterize the protein determinants of mitochondrial import. Essentially, genes encoding mitochondrial proteins have been isolated and used to construct gene fusions. These constructs encode various amino-terminal lengths or deletions within the amino terminus of a mitochondrial precursor protein joined to the coding sequence of a nonmitochondrial protein. The ability of these mitochondrial targeting sequences to direct the hybrid protein into mitochondria both in vitro and in vivo defines the sequences sufficient for mitochondrial import. Conversely, deletion of these targeting sequences should prevent the import of the hybrid protein. This gene fusion technology is directly analogous to the approach exploited for the analysis of protein export from *E. coli* (see reference 75).

The isolation of nuclear genes encoding mitochondrial proteins for these gene fusions in yeasts has utilized a combination of genetic and physical methods (12, 60). Complementary DNA clones encoding the human and rat mitochondrial pre-OTC (42, 54) have been characterized. These genes have proven particularly instrumental in defining the import mechanism and mitochondrial targeting signal in animal cells.

In yeast cells, the genes encoding either a mitochondrial matrix protein, the F_1-ATPase β subunit, or an outer membrane protein, the 70-kDa outer membrane protein of mitochondria, were first used to demonstrate the utility of the gene fusion approach for in vivo analysis of mitochondrial delivery (11, 25). In each case, gene fusions were constructed which joined the amino-terminal coding sequences of the yeast mitochondrial protein to the region encoding a large, functional carboxy-terminal fragment of β-galactosidase. The yeast cell biologist can take advantage of the fact that plasmid shuttle vehicles are available which allow the manipulation of yeast-coding DNA in *E. coli* followed by reintroduction of the plasmid DNA into yeast cells as either an autonomously replicating or an integrated genetic element (76). When these gene fusion constructs are expressed in yeast cells, the fate of the hybrid gene product can be conveniently monitored by enzyme analysis in subcellular fractionations. By using this approach, the targeting of the F_1-ATPase β-subunit precursor to mitochondria was shown to reside in the amino-terminal 28 residues (11, 15; A. Vassarotti, W. Chen, C. Smagula, and M. Douglas, J. Biol. Chem., in press). In-frame deletions which remove this amino-terminal region prevent the cofractionation of the hybrid protein with mitochondria. The targeting signal for the 70-kDa outer mitochondrial membrane protein was determined in the same manner and consists for the most part of the first 12 amino-terminal amino acids (25, 31). In an earlier study the location of the mitochondrial targeting signal within the amino-terminal two-thirds of the protein was determined by cofractionation analysis of the truncated 70-kDa outer membrane protein (63).

Gene fusions which link the short coding segments of a mitochondrial protein to *lacZ* are, however, of limited value in the analysis of in vivo targeting function. Analysis of gene fusions to yeast *ATP2* encoding the F_1-ATPase β subunit (15) demonstrated that gene fusions retaining 142 amino-terminal residues or less of the F_1-ATPase β precursor fused to *lacZ* were markedly reduced in their ability to deliver β-galactosidase to mitochondria even though targeting signals were clearly demonstrated to be located within the first 30 residues. These same short *ATP2* coding segments were able to efficiently mislocalize a large carboxy-terminal fragment of the yeast invertase, the product of the *SUC2* gene (15).

The lack of hybrid protein targeting observed with short targeting sequences fused at the amino terminus of β-galactosidase most likely represents interference by the β-galactosidase protein in the posttranslational delivery to mitochondria. The targeting sequence present at the extreme amino terminus of the hybrid molecule was not utilized for the initiation of import until enough of the β-galactosidase fragment had been synthesized to hinder its accessibility to the mitochondrial import apparatus. This interference by *lacZ* fusions for in vivo mitochondrial targeting can be partially overcome by the expression of additional "bridge" residues between the short mitochondrial targeting sequence at the amino terminus and the large β-galactosidase fragment at the carboxy terminus. Recently, Keng et al. (39) were able to show that a fusion of the first nine codons of the yeast *HEM1* product 5-aminolevulinate synthetase to LacI′-LacZ encoded a hybrid protein which was delivered, albeit with low efficiency, to mitochondria in vivo. Thus, the expression of 90 additional bridge residues of the *lac* repressor to separate the mitochondrial targeting residues from the LacZ residues prevented the interference by regions of the protein distal to the targeting sequences.

In Vitro Import

Although in vivo analysis of mitochondrial targeting has proven useful in defining the residues required for mitochondrial import, in vitro mitochondrial import of hybrid proteins is a fast and direct method for biochemical analysis of import which accurately reflects the events defined by in vivo studies. The utility of in vivo hybrid protein targeting, however, will come from mutant analysis which exploits the altered phenotypes of host strains expressing disruptive or potentially disruptive hybrid proteins (see below).

Mitochondria are an important resource for biochemical analysis of protein transport across membranes. They are easily purified as intact organelles with the same membrane orientation and compartmentalization as the organelle in situ. These in addition to their well-defined biophysical properties make them the best biochemically defined membrane system currently available for analysis of protein transport. In general, proteins for import are synthesized as labeled precursors in a cell-free reticulocyte lysate and added to isolated energized mitochondria in an isotonic suspension buffer. Following a brief incubation, import into mitochondria is usually determined by cofractionation analysis and by accessibility of the target protein to exogenous protease.

In the earlier import assays, this analysis required that, following the import incubation, mitochondria be quantitatively immunoprecipitated with specific antibody prior to analysis by gel electrophoresis-autoradiography. Recent advances now make possible the in vitro synthesis of specific mitochondrial precursors as either the wild type or specifically deleted proteins. For this, specific messenger RNA molecules coding selected gene products are synthesized in vitro. ColE1 plasmids are now available which harbor strong bacteriophage promoters for T_7, T_5, or SP6 RNA polymerases adjacent to a convenient polylinker sequence. After the coding DNA of interest is correctly positioned into the polylinker, it can be conveniently propagated and used to generate specific in vitro transcripts, using the appropriate purified bacteriophage RNA polymerase. In this manner specific transcripts can be generated to program cell-free lysates for the synthesis of mitochondrial precursors. Since these transcripts yield in most cases a single labeled translation product, they are ideal for mitochondrial import assays and avoid the need for immunoprecipitation (see references 32–34).

In vitro import of hybrid proteins synthesized in a linked transcription-translation system has been independently utilized to define and analyze the signals necessary for import of the yeast cytochrome oxidase subunit IV (*COX4*) protein and the human mitochondrial OTC. In each case, DNA regions encoding the extreme amino termini of the respective proteins were fused in-frame with the gene encoding the mouse dihydrofolate reductase (30, 32, 33). Additional studies showed that the sequences within the first half of the *COX4* leader peptide (34) were sufficient to direct in vitro import of dehydrofolate reductase into mitochondria. In these studies, mitochondrial subfractionation analysis revealed that the delivery of the hybrid through both the inner and outer membrane had occurred.

In the same manner the 32-residue leader peptide of the human OTC was shown to contain the targeting information necessary for mitochondrial import (30). In subsequent studies, both deletion analysis and analysis of specific mutations within the leader sequence demonstrated that the middle region of the OTC 32-residue leader, most likely residues 8 to 23, contains the information necessary for import. Deletions between residues 8 and 22 as well as a specific glycine for arginine substitution at residue 23 blocked import of the otherwise wild-type OTC precursor into mitochondria (29).

In a similar manner the mitochondrial import of the yeast F_1-ATPase β subunit, *ATP2*, was dependent on sequences located within the first 10 residues of its transient presequence (Vassarotti et al., in press). Thus, deletion and amino acid replacement analysis of three matrix proteins from various sources indicate that the information necessary to direct specific delivery and targeting into the mitochondrial matrix resides within a reasonably small (9 to 12 residues) sequence.

Analysis of Targeting-Import Signals

The mitochondrial targeting sequences are similar in size to the peptide sequence required to sort proteins from the cytoplasm for delivery to the nucleus (23, 50). However, comparison of the mitochondrial targeting sequences defined thus far does not reveal any obvious consensus primary sequence responsible for mitochondrial targeting and import. Amino acid sequences from the extreme amino terminus of other imported proteins (60) reveal that the proposed targeting region exhibits a net positive charge and is with rare exception devoid of acidic residues. Unlike presecretory signal sequences which exhibit charged residues (usually arginine and lysine) flanking a membrane-spanning hydrophobic core sequence (75), the residues which direct the mitochondrial import are punctuated with basic amino acids on the average every four to eight residues (Fig. 1). No hydrophobic core within a mitochondrial targeting sequence has been described thus far. The 70-kDa outer membrane protein contains an "anchor element" adjacent to the targeting sequence (25, 31). All mitochondrial targeting sequences described thus far are located at the amino terminus of the cytoplasmic precursor. Interestingly, they exhibit the potential for the formation of a structure which exhibits sidedness of charge distribution when displayed on a "helical wheel" plot (Fig. 1). This distribution of charge, a structural feature of mitochondrial import signals, suggests the potential for the assembly of stable oligomeric associations within the membrane. There are, however, no experimental data at present to support the role of an amphipathic structure in mitochondrial import or the presence of such structures in the bilayer. It is clear that the import of mitochondrial proteins requires the formation of a specific secondary structure or complex rather than the display of a consensus sequence recognized by the import apparatus.

Further characterization of the structural requirement for targeting proteins to mitochondria will take advantage of specific targeting signal mutants. Several studies have recently described deletions or modifications of the targeting sequence which block import of a given precursor (25, 29; Vassarotti et al., in press). It should not be long before *cis* suppressor mutations are available which restore import of the protein harboring the primary targeting signal defect. A comparison and analysis of independently isolated suppressors in this manner should provide considerable insight into the structure of the mitochondrial targeting signals.

Processing-Dependent Signals

Are the sequences which direct protein import the same sequences necessary for the processing of leader peptide

from the precursor? Answers to this question may vary depending on the protein and organism examined. Horwich et al. (29) showed that deletions within the leader sequence which block the targeting of the OTC precursor to mitochondria also block its processing in vitro by isolated matrix protease. These authors proposed that deletion of residues 9 to 21 or a glycine for arginine substitution at residue 23 of the OTC leader would delete or disrupt the secondary structure required for both targeting and processing by the matrix metalloprotease (9, 48). On the other hand, the processing of the yeast F_1-ATPase β-subunit precursor has been shown to require sequences located within the mature protein, in addition to the targeting signals, within the leader sequence (Vassarotti et al., in press). Small deletions within the *ATP2* gene product centered on residue 36 completely blocked both in vivo and in vitro maturation of the protein at residue 19 but did not block the in vivo targeting function (first 10 residues) or even the assembly of the protein into the functional enzyme within mitochondria. Since the internal deletions were distal to the processing site for the yeast mitochondrial metalloprotease, these authors concluded that a maturation-competent structure at the amino terminus of the F_1-ATPase β-subunit precursor required determinants in addition to those within the leader sequence. Further studies are required to determine if these observations reflect the flexibility of the mitochondrial processing apparatus or the different gene products being examined.

The extent to which the signals and apparatus for mitochondrial import are conserved among different species has been addressed in several studies. The *S. cerevisiae ATP2* gene was able to complement a *Schizosaccharomyces pombe* mutant lacking a detectable F_1-ATPase β subunit (6). This study indicated that the same import and processing signals could function in these divergent yeasts. In addition, the maturation of the yeast F_1-ATPase β-subunit precursor was observed following incubation with a rat liver matrix fraction (5). Most recently, Horwich et al. demonstrated that the human OTC precursor was imported into yeast mitochondria either in vitro or in vivo and was processed by the yeast matrix protease. In this study a specific arginine to glycine substitution at residue 23 of the OTC precursor which blocked import or maturation of the protein in rat liver mitochondria also blocked processing in the yeast system. Thus, the specificity of the targeting and processing signals within mitochondrial precursors, as well as the cellular apparatus which recognizes these signals, appears to have been conserved in these divergent eucaryotes.

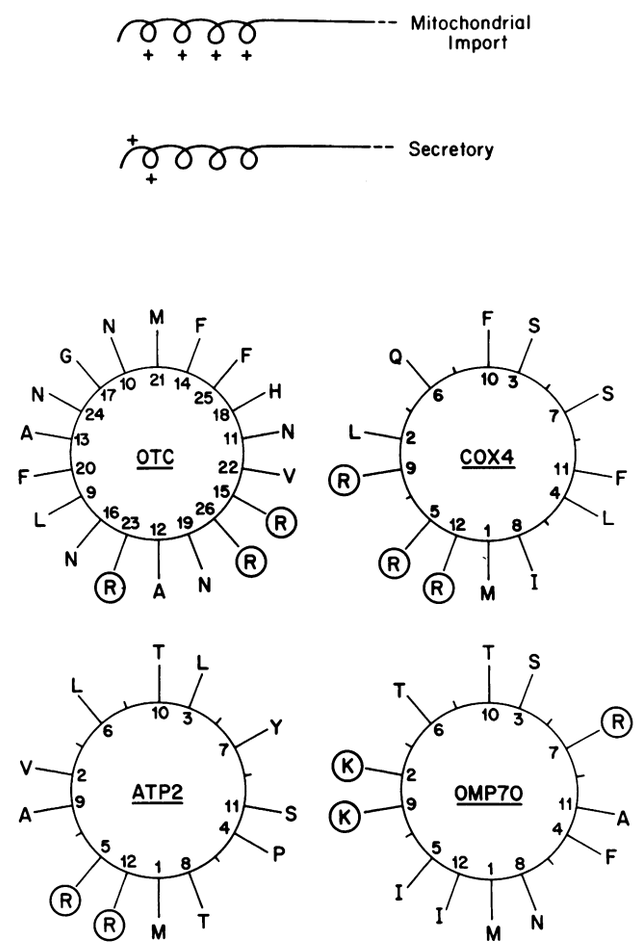

FIG. 1. Mitochondrial targeting sequences exhibiting the capability of forming ampipathetic helices. The sequences for four mitochondrial proteins which have been characterized to target both homologous and nonhomologous proteins into mitochondria are displayed on helical wheel plots (73) (bottom). Only the minimum number of residues are demonstrated to be essential for targeting of OTC (29, 30), *COX4* (cytochrome oxidase subunit 4) (34), *ATP2* (ATPase subunit 2) (Vassarotti et al., in press), and OMP70; the 70-kDa outer mitochondrial membrane (31) protein are shown. Residues that have been demonstrated to be unnecessary for import are not shown for three of the proteins. Charged residues in each case are circled. (Top) The rather uniform distribution of charged residues along the length of the mitochondrial targeting peptide is quite distinct from the signal sequences for secretory proteins, which exhibit clustered charged residues at the extreme amino terminus (35).

Submitochondrial Localization Signals

To date the analysis of import signals has examined the case of cytoplasmic proteins which must be delivered to the mitochondrial matrix. It is anticipated that determinants in the precursor, in addition to import signal, are necessary to program it to other locations within mitochondria. Several earlier observations indicate that the location of a protein sequence within a given cytoplasmic precursor in relation to its targeting signal will determine the destination of the mature protein. A common structural feature exhibited by precursors localized outside the matrix is an uninterrupted stretch of uncharged amino acids sufficient to span a membrane (Fig. 2). Cytochrome *c* is the exception, but as discussed earlier is localized by a different route.

For precursors of the intermembrane space proteins, these uncharged stretches are flanked by clusters of basic residues. For example, cytochrome *c* peroxidase and cytochrome b_2, soluble proteins of the intermembrane space, each contain a membrane-spanning region beginning at residues 19 (36) and 23 (22), respectively, flanked by clusters of positively charged residues. A protein targeted to the outer membrane, on the other hand, contains a transmembrane anchor directly adjacent to its targeting sequence (25). The first transmembrane-spanning region of the ADP/ATP translocator, an integral protein of the inner membrane, begins at residue 74 (2). If the import signals for these proteins are located at the extreme amino terminus, then the submitochondrial destination of these different proteins may simply be determined by the membrane at which the transmembrane anchor sequence acts to halt delivery of the

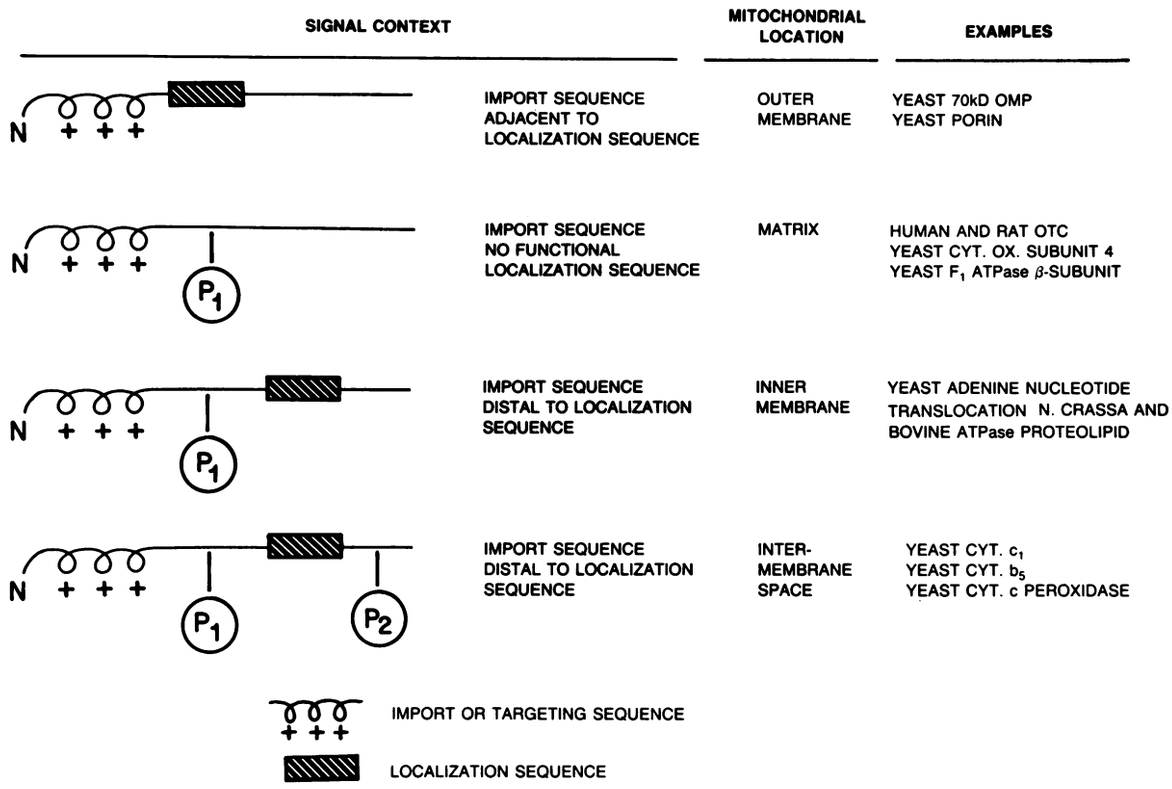

FIG. 2. Determination of destination of the imported protein by relative position of mitochondrial import and localization sequences. The targeting of proteins both to mitochondria and into the mitochondrial matrix utilizes positively charged hydrophilic sequence (import or targeting sequence). An additional element, usually a stretch of uncharged amino acids sufficient to span the membrane bilayer (localization sequence), will detour the further entry of protein into the matrix. The position or localization sequence relative to the import sequence defines the membrane at which the "stop-transfer" signal of the localization sequence is expressed. P_1 and P_2 are sites of cleavage within the precursor. The participation of proteolytic processing catalyzed by a soluble metalloprotease localized in the matrix, P_1, or a membrane-associated heme-dependent protease located in the inner membrane, P_2, occurs for certain proteins but is not necessary in some cases to achieve the correct mitochondrial location.

protein to the matrix. If the import signal and localization signal (anchor sequence) overlap as in the case of the 70-kDa outer membrane protein (25), then the protein is blocked from further entry as soon as it binds to the mitochondrial outer membrane. Deletion of the anchor sequence or its modification so that it was no longer able to form a transmembrane spanning structure allowed further import of the target protein into mitochondria (25, 31).

Gene fusion analysis has shown that the first 115 residues of the yeast adenine nucleotide translocator protein (309 residues), a transmembrane protein of the inner membrane, will deliver β-galactosidase to the mitochondrial inner membrane (2). Since this region of the protein contains well-defined transmembrane anchor sequences, the targeting and anchoring sequences were proposed to be sufficiently distal from each other to initiate the import of a transport-permissive conformation or assembly of the anchor region through the outer membrane. Essentially the same type of reasoning has been used to explain the observation that the hydrophobic proteolipid (ATPase complex, subunit 9 of *N. crassa*) can be maintained as a soluble protein in the cytoplasm and imported through the outer membrane but not the inner membrane (80). This precursor contains a 66-residue hydrophilic leader sequence which targets the protein and is able to prevent illegitimate insertion of the hydrophobic portion of the protein into the wrong intracellular membrane. This "shielding" mechanism may be invoked in the transport of the transient membrane-spanning segments of the cytochrome *c* peroxidase and cytochrome b_2 precursors through the outer membrane. These precursors are then partially transported across the mitochondrial inner membrane, where the second of two proteolytic processing steps releases the mature form of the protein into the intermembrane space (Fig. 3).

The first gene constructions which tested the context function of submitochondrial localization signals demonstrated that the transmembrane anchor of cytochrome c_1 beginning at residue 36 of the precursor will function in conjunction with its import signal to localize a cytosolic protein to the intermembrane space (A. Van Loon, A. Brandli, and G. Schatz, Cell, in press). Mature cytochrome c_1 protrudes from the mitochondrial inner membrane into the intermembrane space. The cleavable presequence, which retained the transmembrane anchor of the yeast cytochrome c_1, localized a fused mouse dehydrofolate reductase protein to the mitochondrial intermembrane space both in vivo and in vitro. However, a hybrid retaining the import signal but lacking the localization sequence (transmembrane anchor) localized dehydrofolate reductase to the matrix (Van Loon et al., in press).

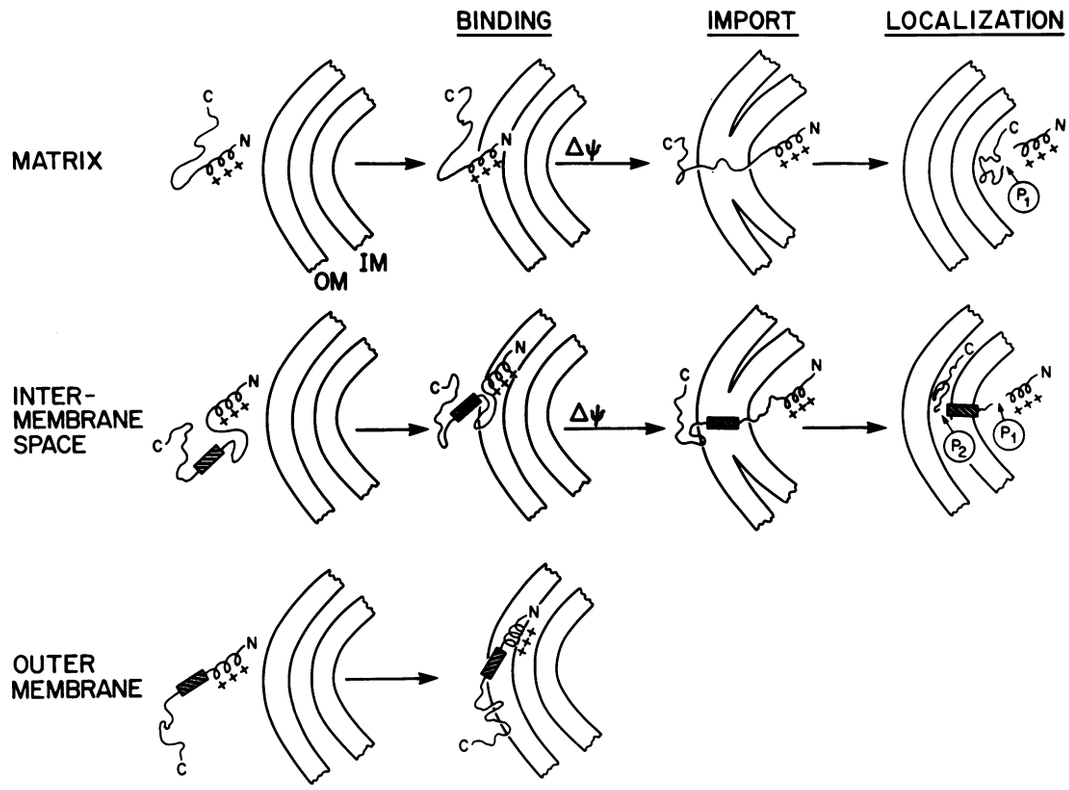

FIG. 3. Participation of separate determinants of mitochondrial import and localization. In the absence of additional localization determinants the mitochondrial targeting or import sequence will direct the matrix delivery of any protein through both the outer membrane (OM) and inner membrane (IM). The binding of proteins to mitochondria which are destined for any submitochondrial location does not require an energized membrane. The import of proteins beyond the outer mitochondrial surface is dependent upon a membrane potential ($\Delta\psi$), except for cytochrome *c*. The expression of a transmembrane localization sequence at the inner but not the outer membrane locates the protein to the mitochondrial inner membrane. A metalloprotease in the matrix, P_1, and the inner membrane protein, P_2, will release a soluble or inner membrane-associated protein within the intermembrane space.

GENETIC ANALYSIS OF MITOCHONDRIAL IMPORT

Considerable emphasis has been placed in the past 2 years on utility of genetics to unravel the molecular details of the mitochondrial import process. This approach has been important in expanding the analysis of protein export in the procaryote and has been successfully exploited to broaden our understanding of protein secretion in the eucaryote (69). The ease with which genes encoding modified targeting and localization signals can be reintroduced into cells has already made possible a detailed analysis of the sequence requirements for mitochondrial protein targeting and localization within the cell. The area of protein import which will serve as the major focus of genetic analysis is the identification and characterization of mutants which exhibit targeting, localization, and processing defects for mitochondrial precursors. For example, Matner and Sherman (44) have described yeast cytochrome *c* mutants which are able to make apocytochrome *c* but are unable to express a functional protein. Analysis of these mutants has revealed that they lack any apparent mitochondrial enzymatic activity for heme attachment and contain extremely low levels of the mitochondrial apocytochrome *c*. Further analysis of nuclear mutants blocked in mitochondrial assembly will help to define specialized import and assembly pathways which may be unique to a given protein. We have previously discussed the special case of cytochrome *c*, which utilizes a pathway distinct from that of other proteins localized to the intermembrane space.

Since a limited set of mitochondrial proteins is probably used for the import and processing of a large number of cytoplasmic precursors, mutations which block the function of these components will probably be lethal to the cell. With this in mind, conditional mutants blocked for mitochondrial assembly at the restrictive temperature have been isolated (83). In this study a collection of yeast mutants temperature sensitive for growth at 37°C were subsequently screened for the synthesis of precursor forms of the F_1-ATPase β subunit and cytochrome c_1. Additional biochemical and genetic characterization revealed that mitochondrial precursor accumulation in these mutants was not due to a breakdown of the membrane potential across the inner membrane at the nonpermissive temperature and that at least two independent temperature-sensitive mutations had been defined by this selection. One of the mutants, *mas1* (mitochondrial assembly), has more recently been shown to contain only a fraction of the normal matrix metallo-endoprotease activity at nonpermissive temperature (82). Since the in vitro import of proteins into *mas1* mitochondria appears to be resistant to high temperature, the authors conclude that the mutation affects the thermolability of the matrix protease itself or components which regulate its activity. If this is indeed a mutation in the metalloprotease, it represents the first genet-

ically defined component of the mitochondrial import-processing pathway.

Another genetic approach which may prove useful in the identification of the mitochondrial import machinery is the isolation and characterization of mutants able to restore the import of yeast cytoplasmic precursors with defective import signals. This strategy has been successfully exploited in *E. coli* to define components of the protein export apparatus for the *E. coli lamB* protein (14). Now that the import signals have been defined for several proteins and mutant targeting signals have been constructed in some cases (29; Vassarotti et al., in press), the selection of host-linked suppressor strains is not far behind.

Gene fusions may provide yet another independent approach to identify components of the mitochondrial import apparatus. In vivo import of certain fused gene products disrupts the function of normal mitochondria (11, 15, 20; Vassarotti et al., in press). Mitochondrial targeting of a gene fusion product encoded by *ATP2* at its amino terminus and either *E. coli lacZ* (11) or *SUC2* (15) at its carboxy terminus to the mitochondrial inner membrane blocks the normal function of the organelle. If the yeast host strain is cured of the plasmid encoding the hybrid protein, functional mitochondria are restored. The hybrid protein-dependent phenotype (lack of growth on a nonfermentable substrate) is best correlated with the delivery of a threshold level of the *ATP2-lacZ*-encoded hybrid to the mitochondrial inner membrane, where it becomes jammed in the bilayer. The presence of this protein in the inner membrane renders it sufficiently leaky to protons so that the respiratory and energy-transducing components of the membrane cannot maintain an adequate electrochemical gradient to drive mitochondrial-dependent ATP formation. Thus the host harboring the *ATP2-lacZ* fusion is unable to grow on a nonfermentable carbon source such as glycerol. The levels of mitochondrial respiratory and ATPase activities in the strain harboring the gene fusion were reduced slightly; however, the ATP-$^{32}P_i$ exchange activity was reduced to <10% of that in the absence of the hybrid (20). This assay is the most sensitive measure of the integrity of the inner mitochondrial membrane since an ATPase-dependent generation of protein gradient is required to catalyze the exchange reaction at equilibrium. Thus, this phenotype due to the targeting of hybrid protein has been exploited to define mutants which still express high levels of the hybrid protein yet reduce the amount of hybrid protein targeted to the mitochondrial inner membrane. To initiate a similar selection for *cis*- and *trans*-acting components mediating mitochondrial import, *HEM1-lacZ* fusions under the control of a regulated GAL upstream activation sequence have been used to generate a high-level expression of the hybrid protein (39). These studies also demonstrated an altered growth phenotype dependent on the carbon source. Mutants selected by using a hybrid protein-dependent phenotype may define components of the mitochondrial import machinery which interact with the cytoplasmic precursor prior to its insertion into the inner membrane.

CONCLUSIONS AND FUTURE PROSPECTS

Information on the molecular details of the mitochondrial import mechanism is presently accumulating at a very rapid rate. A number of independent and converging observations appear to mutually support the proposed structure of targeting signals. On the horizon, genetic handles will be instrumental in defining the cellular components mediating the mitochondrial import pathway. Recent observations have enlightened us on some details of the import mechanism and point out where investigators and reviewers to follow will concentrate their efforts.

Considerable attention has most recently focused on the characterization of targeting or import signals which initiate the primary cytoplasmic sorting and membrane transport event. This import or targeting signal is necessary and sufficient to direct any protein covalently coupled to it directly through two membranes to the matrix (15, 30, 32, 39). Detours from the common import course to the matrix require additional signals (submitochondrial localization signals or localization signals) within the cytoplasmic precursor in the proper position or context to restrict further entry of the protein at either of the two boundary membranes (Fig. 2). The selection of inner versus outer membrane may reflect the location of the localization signal on the precursor relative to the targeting sequence in much the same manner as an internal stop transfer sequence prevents the further transport of a precursor protein from *E. coli* (7).

The recent elucidation of mitochondrial import signals from different sources which can function in a heterologous system indicates that a common protein structure is potentially formed or can be induced in the cytoplasmic or membrane-bound precursor. The location of the import signal of cytoplasmic precursors at the exteme amino terminus rather than within the protein indicates that cytoplasmic precursors may bind in vivo as nascent polypeptides which have yet to complete their synthesis, although they can clearly be imported following completion of their translation (60). A common structural feature of the import signal which distinguishes it from a presecretory signal is the potential for forming a structure which segregates charged residues (Fig. 1). This suggests that within the hydrophobic environment of the bilayer oligomeric forms of the ampipathic protein and import apparatus may be formed (M. Briggs and L. Gierasch, Adv. Protein Chem., in press).

What is not understood at present is how such a structure mediates the specific segregation of the precursor to mitochondria. Soluble factors are proposed to localize the cytoplasmic precursor to the outer membrane (3, 4, 49, 56), whereas binding to some receptor component within the membrane is proposed to complete the precursor segregation. Once localized at the surface of the mitochondria, the transport of the precursor into the mitochondrial matrix most likely occurs at regions of contact between the inner and outer membrane (71). Whether the protein traverses the bilayers as a linear, globular, or oligomeric structure has not been resolved at present. The ability of precursor sequences distal to the targeting signal to define submitochondrial localization suggests that at least domains near the amino terminus of the precursor will be transported through the import apparatus at different times (Van Loon et al., in press).

The membrane potential across the inner membrane is proposed to generate the transport-competent junctions between the inner and outer membrane (58, 71). Whether this energy-dependent step acts to generate tight junctions between membrane protein components or to destabilize the lipid bilayers of the inner and outer membrane or both has not been established. This step is required after the energy-independent binding of the precursor protein to the outer membrane (19, 65). Thus, it is envisioned that the targeting sequence at the extreme amino terminus will exhibit two basic functions. First, it provides for a specific targeting function, binding to specific sites at the outer mitochondrial

membrane or to soluble components which mediate protein delivery to the membrane. Second, the targeting sequence may act to catalyze the formation of a membrane potential-dependent junction between the inner and outer membrane or to discharge a preexisting junction (Fig. 3). This is envisioned at present as an effect of the presequence to discharge the contact joint between the membranes. Synthetic peptides of mitochondrial target sequences will disrupt a bilayer (21). This discharge of the membranes due to the translocation of the presequence is apparently blocked at low temperature (71). It is noteworthy that the mechanism of transmembrane precursor protein delivery must allow for the transport of large soluble proteins while maintaining an active coupling membrane with a low permeability to protons and cations. The molecular details of this protein transport event will certainly serve as the focus of future studies, using a variety of tools in the repertoire of the mitochondriologist.

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LITERATURE CITED

1. **Ades, I., and R. Butow.** 1980. The transport of proteins into yeast mitochondria. Kinetics and pools. J. Biol. Chem. **255:** 9925–9935.
2. **Adrian, G., M. McCammon, D. Montgomery, and M. Douglas.** 1986. Sequences required for the delivery and localization of the ADP/ATP translocator to the mitochondrial inner membrane. Mol. Cell. Biol. **6:**626–634.
3. **Argan, C., C. Lusty, and G. Shore.** 1983. Membrane and cytosolic components affecting transport of the precursor for ornithine carbamyltransferase into mitochondria. J. Biol. Chem. **258:**6667–6670.
4. **Argan, C., and G. Shore.** 1985. The precursor to ornithine carbamyl transferase is transported to mitochondria as a 5S complex containing import factor. Biochem. Biophys. Res. Commun. **131:**289–98.
5. **Bohni, P., G. Daum, and G. Schatz.** 1983. Import of proteins into mitochondria. Partial purification of a matrix-located protease involved in cleavage of mitochondrial precursor polypeptides. J. Biol. Chem. **258:**4937–4943.
6. **Boutry, M., and M. Douglas.** 1983. Complementation of Schizosaccharomyces phombe mutant lacking the β subunit of the mitochondrial ATPase by the ATP2 gene of S. cerevisiae. J. Biol. Chem. **258:**15214–15219.
7. **Coleman, J., M. Inukai, and M. Inouye.** 1985. Dual function of the signal peptide in protein transfer across the membrane. Cell **43:**351–360.
8. **Colombini, M.** 1979. A candidate for the permeability pathway of the outer mitochondrial membrane. Nature (London) **279:** 643–645.
9. **Conboy, J., W. Fenton, and L. Rosenberg.** 1982. Processing of pre-ornithine transcarbamylase requires a zinc-dependent protease localized to the mitochondrial matrix. Biochem. Biophys. Res. Commun. **105:**1–7.
10. **Daum, G., S. Gasser, and G. Schatz.** 1982. Import of proteins into mitochondria. Energy-dependent, two step processing of the intermembrane-space enzyme, cytochrome b_2 by isolated yeast mitochondria. J. Biol. Chem. **257:**13075–13080.
11. **Douglas, M., B. Geller, and S. Emr.** 1984. Intracellular targeting and import of an F_1-ATPase β-subunit β-galactosidase hybrid protein into yeast mitochondria. Proc. Natl. Acad. Sci. USA **81:**3983–3987.
12. **Douglas, M., and M. Takeda.** 1985. Nuclear genes encoding mitochondrial proteins. Trends Biochem. Sci. **10:**192–194.
13. **Dujon, B.** 1982. Mitochondrial genetics and functions, p. 505–635. *In* J. Strathern, E. Jones, and J. Broach (ed.), Molecular biology of the yeast Saccharomyces: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Emr, S., S. Hanley-Way, and T. Silhavy.** 1981. Suppressor mutations that restore export of a protein with a defective signal sequence. Cell **23:**79–88.
15. **Emr, S., A. Vassarotti, J. Garret, B. Geller, M. Takeda, and M. Douglas.** 1986. The aminoterminus of the yeast F_1-ATPase β-subunit precursor functions as a mitochondrial import signal. J. Cell Biol. **102:**523–533.
16. **Ernster, L., and G. Schatz.** 1981. Mitochondria: a historical review. J. Cell Biol. **91:**227–255.
17. **Frietag, H., M. Janes, and W. Neupert.** 1982. Biosynthesis of mitochondrial porin and insertion into the outer mitochondrial membrane of *Neurospora crassa*. Eur. J. Biochem. **126:**197–202.
18. **Gasser, S., A. Ohashi, G. Daum, P. Bohni, J. Gibson, G. Reid, T. Yonetani, and G. Schatz.** 1982. Imported mitochondrial proteins cytochrome b_2 and cytochrome c_1 are processed in two steps. Proc. Natl. Acad. Sci. USA **79:**267–271.
19. **Gasser, S., and G. Schatz.** 1983. Import of proteins into mitochondria. *In vitro* studies on the biogenesis of the outer membrane. J. Biol. Chem. **258:**3427–3430.
20. **Geller, B., M. Britten, C. Biggs, M. Douglas, and S. Emr.** 1983. Import of ATP2-lacZ gene fusion proteins into mitochondria, p. 607–619. *In* R. Schweyen, K. Wolf, and F. Kaudewitz (ed.), Mitochondria 1983: nucleo-mitochondrial interactions. Walter de Gruyter, New York.
21. **Gillespie, L., C. Argan, A. Taneja, R. Hodges, K. Freeman, and G. Shore.** 1985. A synthetic signal peptide blocks import of precursor proteins destined for the mitochondrial inner membrane or matrix. J. Biol. Chem. **260:**16045–16048.
22. **Guiard, B.** 1985. Structure, expression and regulation of a nuclear gene encoding a mitochondrial protein: the yeast L(+)-lactate cytochrome c oxido-reductase (cytochrome b_2). EMBO J. **4:**3265–3272.
23. **Hall, M., L. Hereford, and I. Herskowitz.** 1984. Targeting of *E. coli* β-galactosidase to the nucleus in yeast. Cell **36:**1057–1065.
24. **Hallermayer, G., R. Zimmermann, and W. Neupert.** 1977. Kinetic studies on the transport of cytoplasmically synthesized proteins into the mitochondria in intact cells of *Neurospora crassa*. Eur. J. Biochem. **81:**523–532.
25. **Hase, T., U. Muller, H. Riezman, and G. Schetz.** 1984. A 70 kd protein of the yeast mitochondrial outer membrane is targeted and anchored via its extreme amino terminus. EMBO J. **3:** 3157–3164.
26. **Hay, R., P. Bohni, and S. Gasser.** 1983. How mitochondria import proteins. Biochim. Biophys. Acta **779:**65–87.
27. **Hennig, B., H. Koehler, and W. Neupert.** 1983. Receptor sites involved in post-translational transport of apocytochrome c into mitochondria: specificity, affinity and number of sites. Proc. Natl. Acad. Sci. USA **80:**4963–4967.
28. **Hennig, B., and W. Neupert.** 1981. Assembly of cytochrome c. Apocytochrome c is bound to specific sites on mitochondria before its conversion to holocytochrome c. Eur. J. Biochem. **121:**203–212.
29. **Horwich, A., F. Kalonsek, W. Fenton, R. Pollock, and L. Rosenberg.** 1986. Targeting of pre-ornithine transcarbamylase to mitochondria: definition of critical regions and residues in the leader peptide. Cell **44:**451–459.
30. **Horwich, A., F. Kalousek, I. Mellman, and I. Rosenberg.** 1985. A leader peptide is sufficient to direct mitochondrial import of a chimeric protein. EMBO J. **4:**1129–1135.
31. **Hurt, E., U. Muller, and G. Schatz.** 1985. The first twelve amino acids of a yeast mitochondrial outer membrane protein can direct a nuclear encoded cytochrome oxidase subunit to the mitochondrial inner membrane. EMBO J. **4:**3509–3518.
32. **Hurt, E., B. Pesold-Hurt, and G. Schatz.** 1984. The amino-terminal region of an imported mitochondrial precursor polypeptide can direct cytoplasmic dehydrofolate reductase into the mitochondrial matrix. EMBO J. **3:**3149–3156.
33. **Hurt, E., B. Pesold-Hurt, and G. Schatz.** 1984. The cleavable

prepiece of an imported mitochondrial protein is sufficient to direct cytosolic dehydrofolate reductase into the mitochondrial matrix. FEBS Lett. **178**:306–310.
34. **Hurt, E., B, Pesold-Hurt, K. Suda, W. Oppliger, and G. Schatz.** 1985. The first twelve amino acids (less than half of the presequence of an imported mitochondrial protein) can direct mouse cytosolic dehydrofolate reductase into the yeast mitochondrial matrix. EMBO J. **4**:2061–2065.
35. **Inouye, S., S. Wang, J. Sekizawa, S. Halegana, and M. Inouye.** 1977. Amino acid sequence for the peptide extension on the prolipoprotein of *E. coli* outer membrane. Proc. Natl. Acad. Sci. USA **74**:1004–1008.
36. **Kaput, J., S. Goltz, and G. Blobel.** 1982. Nucleotide sequence of the yeast nuclear gene for cytochrome c peroxidase precursor. Functional implications of the pre-sequence for protein transport into mitochondria. J. Biol. Chem. **257**:15054–15058.
37. **Kellems, R., V. Allison, and R. Butow.** 1974. Cytoplasmic type 80S ribosomes associated with yeast mitochondria. II. Evidence for the association of cytoplasmic ribosomes with the outer mitochondrial membrane *in situ*. J. Biol. Chem. **249**:3297–3303.
38. **Kellems, R., V. Allison, and R. Butow.** 1975. Cytoplasmic type 80S ribosomes associated with yeast mitochondria. IV. Attachment of ribosomes to the outer membrane of isolated mitochondria. J. Cell Biol. **65**:1–14.
39. **Keng, T., E. Alani, and L. Guarente.** 1986. The amino-terminal nine residues of the δ-aminolevulinate synthetase directs β-galactosidase into the mitochondrial matrix. Mol. Cell. Biol. **6**:355–364.
40. **Knoll, G., and D. Brdiczka.** 1983. Changes in freeze-fractured mitochondrial membranes correlated to their energetic state. Biochim. Biophys. Acta **733**:102–110.
41. **Korb, H., and W. Neupert.** 1978. Biogenesis of cytochrome c in *Neurospora crassa*. Synthesis of apocytochrome c, transfer to mitochondria and conversion to holocytochrome c. Eur. J. Biochem. **91**:609–620.
42. **Kraus, J., P. Hodges, C. Williamson, A. Horwich, F. Kalousek, K. Williams, and L. Rosenberg.** 1985. A cDNA clone for the precursor of rat mitochondrial ornithine transcarbamylase: comparison of rat and human leader sequences and conservation of catalytic sites. Nucleic Acids Res. **13**:943–952.
43. **Maccecchini, M.-L., Y. Rudin, G. Blobel, and G. Schatz.** 1979. Import of proteins into mitochondria: precursor forms of the extramitochondrially made F_1-ATPase subunits in yeast. Proc. Natl. Acad. Sci. USA **76**:343–347.
44. **Matner, R., and F. Sherman.** 1982. Differential accumulation of two Apo-iso-cytochromes c in processing mutants of yeast. J. Biol. Chem. **257**:9811–9821.
45. **McAda, P., and M. Douglas.** 1982. A neutral metallo endoprotease involved in the processing of an F_1-ATPase subunit precursor in mitochondria. J. Biol. Chem. **257**:3177–3182.
46. **Mihara, K., G. Blobel, and R. Sato.** 1982. *In vitro* synthesis and integration into mitochondria of porin, a major protein of the outer mitochondrial membrane of *S. cerevisiae*. Proc. Natl. Acad. Sci. USA **79**:7102–7106.
47. **Mitchell, P.** 1985. Molecular mechanics of proton translocating F_oF_1 ATPases. FEBS Lett. **182**:1–7.
48. **Miura, S., M. Mori, Y. Amaya, and M. Tatibana.** 1982. A mitochondrial protease that cleaves the precursor of ornithine carbamoyltransferase. Purification and properties. Eur. J. Biochem. **122**:641–647.
49. **Miura, S., M. Mori, and M. Tatibana.** 1983. Transport of ornithine carbamoyltransferase precursor into mitochondria. Stimulation by potassium ion, magnesium ion and a reticulocyte cytosolic protein(s). J. Biol. Chem. **258**:6671–6674.
50. **Moreland, R., H. Nam, L. Hereford, and H. Fried.** 1985. Identification of a nuclear localization signal of a yeast ribosomal protein. Proc. Natl. Acad. Sci. USA **82**:6561–6565.
51. **Mori, M., H. Matsui, S. Miura, H. Tatibana, and T. Hashimoto.** 1985. Transport of proteins into mitochondrial matrix: evidence suggesting a common pathway for 3-ketoacyl-CoA thiolase and enzymes having presequences. Eur. J. Biochem. **149**:181–186.
52. **Nelson, N., and G. Schatz.** 1979. Energy-dependent processing of cytoplasmically made precursors to mitochondrial proteins. Proc. Natl. Acad. Sci. USA **76**:4365–4369.
53. **Neupert, W., and G. Schatz.** 1981. How proteins are transported into mitochondria. Trends Biochem. Sci. **6**:1–4.
54. **Nguyen, M., C. Argan, C. Lusty, and G. Shore.** 1986. Import and processing of hybrid proteins by mammalian mitochondria *in vitro*. J. Biol. Chem. **261**:800–805.
55. **Ohashi, A., J. Gibson, I. Gregor, and G. Schatz.** 1982. Import of proteins into mitochondria. The precursor of cytochrome c_1 is processed in two steps, one of them heme-dependent. J. Biol. Chem. **257**:13042–13047.
56. **Ohta, S., and G. Schatz.** 1984. A purified precursor polypeptide requires a cytosolic protein fraction for import into mitochondria. EMBO J. **3**:651–657.
57. **Pfaller, R., H. Freitag, M. Harney, R. Benz, and W. Neupert.** 1985. A water-soluble form of porin from the mitochondrial outer membrane of *Neurospora crassa*: properties and relationship to the biosynthetic precursor form. J. Biol. Chem. **260**: 8188–93.
58. **Pfaller, N., and W. Neupert.** 1985. Transport of proteins into mitochondria: a potassium diffusion potential is able to drive the import of ADP/ATP carrier. EMBO J. **4**:2819–2825.
59. **Randall, L.** 1983. Translocation of domains of nascent periplasmic proteins across the cytoplasmic membrane is independent of elongation. Cell **33**:231–240.
60. **Ried, G.** 1985. Transport of proteins into mitochondria, p. 295–336. *In* P. Kanuf and J. Cook (ed.), Current topics in membranes and transport, vol. 24. Membrane protein biosynthesis and turnover. Academic Press, Inc., New York.
61. **Ried, G. A., and G. Schatz.** 1982. Import of proteins into mitochondria. Extramitochondrial pools and post-translational import of mitochondrial protein precursors *in vivo*. J. Biol. Chem. **257**:13062–13067.
62. **Ried, G., T. Yonetani, and G. Schatz.** 1982. Import of proteins into mitochondria. Import and maturation of the mitochondrial intermembrane space enzymes cytochrome b_2 and cytochrome c peroxidase in intact yeast cells. J. Biol. Chem. **257**: 13068–13074.
63. **Riezman, H., T. Hase, A.P.G.M. van Loon, L. Grivell, K. Suda, and G. Schatz.** 1983. Import of proteins into mitochondria: A 70 kilodalton outer membrane protein with a large carboxy-terminal deletion is still transported to the outer membrane. EMBO J **2**:2161–2168.
64. **Riezman, H., R. Hay, S. Gasser, G. Daum, G. Schneider, C. Witte, and G. Schatz.** 1983. The outer membrane of yeast mitochondria: isolation of outside-out sealed vesicles. EMBO J. **2**:1105–1111.
65. **Riezman, H., R. Hay, C. Witte, N. Nelson, and G. Schatz.** 1983. Yeast mitochondrial outer membrane specifically binds cytoplasmically-synthesized precursors of mitochondrial proteins. EMBO J. **2**:1113–1118.
66. **Ryan, J., and P. Bassford.** 1985. Post-translational export of maltose-binding protein in *E. coli* strains harboring mal E signal sequence mutations and either $pR1^+$ or pR1 suppressor alleles. J. Biol. Chem. **260**:14821–14837.
67. **Schatz, G., and R. Butow.** 1983. How are proteins imported into mitochondria. Cell **32**:316–318.
68. **Schatz, G., and T. Mason.** 1974. The biosynthesis of mitochondrial proteins. Annu. Rev. Biochem. **43**:51–87.
69. **Schekman, R., and P. Novick.** 1982. the secretory process and yeast cell-surface assembly, p. 361–379, *In* J. Strathern, E. Jones, and J. Broach (ed.), Molecular biology of the yeast Saccharomyces: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
70. **Schleyer, M., and W. Neupert.** 1984. Transport of ADP/ATP carrier into mitochondria. The precursor imported *in vitro* acquires functional properties of the mature protein. J. Biol. Chem. **259**:3487–3491.
71. **Schleyer, M., and W. Neupert.** 1985. Transport of proteins into mitochondria. Translocational intermediates spanning contact sites between outer and inner membranes. Cell **43**:339–350.
72. **Schleyer, M., B. Schmidt, and W. Neupert.** 1982. Requirement of a membrane potential for the posttranslational transfer of

proteins into mitochondria. Eur. J. Biochem. **125**:109–116.
73. **Schulz, G., and R. Schirmer.** 1979. Principles of protein structure. Springer-Verlag, New York.
74. **Shore, G., F. Power, M. Bendayan, and P. Carignan.** 1981. Biogenesis of a 35-kilodalton protein associated with outer mitochondrial membrane in rat liver. J. Biol. Chem. **256**: 8761–8766.
75. **Silhavy, T., S. Benson, and S. Emr.** 1983. Mechanisms of protein localization. Microbiol. Rev. **47**:313–344.
76. **Struhl, K.** 1984. The new yeast genetics. Nature (London) **305**:391–397.
77. **Suissa, M., and G. Schatz.** 1982. Import of proteins into mitochondria. Translatable mRNAs for imported mitochondrial proteins are present in free as well as mitochondria-bound cytoplasmic polysomes. J. Biol. Chem. **257**:13048–13055.
78. **Teintze, M., and W. Neupert.** 1983. Biosynthesis and assembly of mitochondrial proteins, p. 89–115. *In* E. Elson, W. Frazier, and L. Galser (ed.), Cell membranes: methods and reviews. Plenum Press, New York.
79. **Teintze, M., M. Slaughter, H. Weiss, and W. Neupert.** 1982. Biogenesis of mitochondrial ubiquinol: cytochrome c reductase (cytochrome bc_1 complex). Precursor proteins and their transfer into mitochondria. J. Biol. Chem. **257**:10364–10371.
80. **Viebrock, A., A. Perz, and W. Sebald.** 1982. The imported preprotein of the preteolipid subunit of the mitochondrial ATP synthase from *Neurospora crassa*. Molecular cloning and sequencing of the mRNA. EMBO J. **1**:565–571.
81. **Wickner, W., and H. Lodish.** 1985. Multiple mechanisms of protein insertion into and across membranes. Science **230**: 400–432.
82. **Yaffee, M., S. Ohta, and G. Schatz.** 1985. A yeast mutant temperature-sensitive for mitochondrial assembly is deficient in a mitochondrial protease activity that cleaves imported precursor polypeptides. EMBO J. **4**:2069–2074.
83. **Yaffee, M., and G. Schatz.** 1984. Two nuclear mutations that block mitochondrial protein import in yeast. Proc. Natl. Acad. Sci. USA **81**:4819–4829.
84. **Yoshida, Y., T. Hashimoto, H. Kimura, S. Sakakibara, and K. Tagawa.** 1985. Interaction with mitochondrial membranes of a synthetic peptide with a sequence common to extra peptides of mitochondrial precursor proteins. Biochem. Biophys. Res. Commun. **128**:775–780.
85. **Zalman, L., H. Nikaido, and Y. Kagawa.** 1980. Mitochondrial outer membrane contains a protein producing nonspecific diffusion channels. J. Biol. Chem. **255**:1771–1774.
86. **Zimmermann, R., B. Hennig, and W. Neupert.** 1981. Different transport pathways of individual precursor proteins in mitochondria. Eur. J. Biochem. **116**:455–460.
87. **Zimmerman, R., and W. Neupert.** 1980. Transport of proteins into mitochondria. Posttranslational transfer of ADP/ATP carrier into mitochondria *in vitro*. Eur. J. Biochem. **109**:217–229.
88. **Zimmerman, R., U. Paluch, and W. Neupert.** 1979. Cell-free synthesis of cytochrome c. FEBS Lett. **108**:141–146.
89. **Zimmermann, R., U. Paluch, M. Sprinzl, and W. Neupert.** 1979. Cell-free synthesis of the mitochondrial ADP/ATP carrier protein of *Neurospora crassa*. Eur. J. Biochem. **99**:247–252.
90. **Zwizinski, C., M. Schleyer, and W. Neupert.** 1983. Transfer of proteins into mitochondria. Precursor to the ADP/ATP carrier binds to receptor sites on isolated mitochondria. J. Biol. Chem. **258**:4071–4084.
91. **Zwizinski, C., M. Schleyer, and W. Neupert.** 1984. Proteinaceous receptors for the import of mitochondrial precursors. J. Biol. Chem. **259**:7850–7856.