Journal of Bioenergetics and Biomembranes, Vol. 22, No. 6, 1990

MINI-REVIEW

Biogenesis of Mitochondrial *c*-Type Cytochromes

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Received March 2, 1990

Abstract

Cytochromes *c* and c_1 are essential components of the mitochondrial respiratory chain. In both cytochromes the heme group is covalently linked to the polypeptide chain via thioether bridges. The location of the two cytochromes is in the intermembrane space; cytochrome *c* is loosely attached to the surface of the inner mitochondrial membrane, whereas cytochrome c_1 is firmly anchored to the inner membrane. Both cytochrome *c* and c_1 are encoded by nuclear genes, translated on cytoplasmic ribosomes, and are transported into the mitochondria where they become covalently modified and assembled. Despite the many similarities, the import pathways of cytochrome *c* and c_1 are drastically different. Cytochrome c_1 is made as a precursor with a complex bipartite presequence. In a first step the precursor is directed across outer and inner membranes to the matrix compartment of the mitochondria where cleavage of the first part of the presequence takes place. In a following step the intermediate-size form is redirected across the inner membrane; heme addition then occurs on the surface of the inner membrane followed by the second processing reaction. The import pathway of cytochrome *c* is exceptional in practically all aspects, in comparison with the general import pathway into mitochondria. Cytochrome *c* is synthesized as apocytochrome *c* without any additional sequence. It is translocated selectively across the outer membrane. Addition of the heme group, catalyzed by cytochrome *c* heme lyase, is a requirement for transport. In summary, cytochrome c_1 import appears to follow a "conservative pathway" reflecting features of cytochrome c_1 sorting in prokaryotic cells. In contrast, cytochrome *c* has "invented" a rather unique pathway which is essentially "non-conservative."

Key Words: Cytochrome *c*; Cytochrome c_1; protein transport; heme; protein sorting; biogenesis of mitochondria; cytochrome *c* heme lyase.

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0145-479X/90/1200-0753$06.00/0

Introduction

Mitochondria are the site of respiration and oxidative phosphorylation in eukaryotic cells. These functions are performed by several protein complexes that are bound to the inner membrane, each of them being comprised of a number of different polypeptides. The biogenesis of these complexes is an extremely complicated process that is only partially understood.

Important questions are how these polypeptides are synthesized and modified and how these reactions are regulated; how the different polypeptides are delivered to their functional location and how they are assembled into functional complexes. In addition, the proteins of these complexes are encoded in two different genomes, namely, the nuclear and the mitochondrial. Consequently nuclear-encoded polypeptides, following synthesis in the cytosol, must be translocated across one or two membranes in order to reach their functional destination.

The present review focuses on the biogenesis of two nuclear-encoded components of the mitochondrial respiratory chain, namely cytochromes *c* and c_1. These cytochromes are unique in that they contain a heme group which is covalently bound through its vinyl groups to two cysteines of the corresponding polypeptide (except for the cytochrome *c* of some unicellular organisms, where only one covalent bond is formed). The corresponding precursors, known as the apo forms, become covalently modified during their import pathway. As will be discussed in this review, these modification are intimately related to their import pathway.

Cytochrome *c* is a small molecule, of about 12,000 Daltons, that is not integrated into any of the respiratory chain complexes. It is located in the intermembrane space, reversibly bound to the inner membrane, where it shuttles electrons between cytochrome *c* reductase and cytochrome *c* oxidase.

Cytochrome c_1 is a larger molecule (ca. 28,000 Daltons) and is a component of a larger complex containing at least eight different polypeptides. It is bound to the inner membrane and faces the intermembrane space.

The two *c*-type mitochondrial cytochromes share some characteristics but differ markedly in others. In this review we will describe what is currently known about their import pathways and discuss how different properties may have influenced their respective pathways and their evolution.

General Aspects of Protein Transport into Mitochondria

Mitochondrial proteins that are coded for in the nucleus are initially synthesized on free ribosomes in the cell cytosol (Fig. 1) (Hallermayer *et al.*, 1977; Harmey *et al.*, 1977; Schatz, 1979). Most carry on amino-terminal

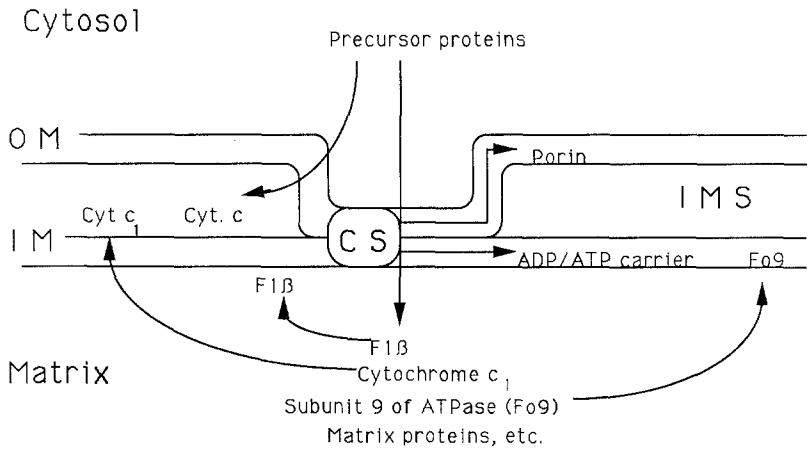

Fig. 1. Import pathways followed by different mitochondrial precursor proteins. Precursor proteins carrying a presequence are translocated through contact sites into the matrix, and then sorted to their final location. Porin, the ADP/ATP carrier, and other precursors share only some steps of the import pathway. Cytochrome *c* does not seem to use any of the components involved in the translocation of other precursor proteins. OM, outer membrane; IM, inner membrane; IMS, intermembrane space; CS, contact site regions.

extension or presequence that acts as a signal sequence for targeting to the mitochondria (for reviews, see Pfanner and Neupert, 1987a; Hartl *et al.*, 1989). An analysis of the primary structures of these presequences shows that no defined consensus sequence is present among them. Nevertheless, all presequences share some common properties, namely, the presence of positively charged residues, the nearly complete absence of negative charges, and the tendency to form an amphipathic α-helix with charged residues on one face and nonpolar groups on the opposite one (Allison and Schatz, 1986; Roise *et al.*, 1986; von Heijne, 1986).

The first step in the import of proteins into mitochondria is the binding to specific receptor sites localized on the mitochondrial surface (Riezman *et al.*, 1983; Zwizinski *et al.*, 1983, 1984; Pfaller *et al.*, 1985; Pfanner and Neupert, 1987b; Pfanner *et al.*, 1987a). The parameters of the binding to receptor sites have been analyzed for some precursors, and it has been shown that this binding is saturable (Hennig *et al.*, 1983; Pfaller and Neupert, 1987) and sensitive to pretreatment of mitochondria with low amounts of proteases (see references in Hartl *et al.*, 1989). More recently, two outer membrane proteins have been identified as receptor sites for a specific set of mitochondrial precursors. Antibodies and Fab fragments directed against these proteins, termed MOM19 and MOM72 (after *mitochondrial outer membrane*, and the respective molecular weight in SDS-PAGE), specifically inhibit the binding of precursors to mitochondria (Söllner *et al.*, 1989, 1990).

It has been demonstrated that MOM19 acts as the receptor site for, e.g., porin, cytochrome c_1, and the subunit 9 of the mitochondrial ATPase, and MOM72 functions as a receptor for the ADP/ATP carrier (Söllner *et al.*, 1989, 1990).

After binding to receptor sites, precursors become initially inserted into the outer membrane, and by doing so become less accessible to exogenously added proteases (Pfaller and Neupert, 1987; Pfanner and Neupert, 1987b). The import of some precursors can be stalled at this stage by performing the import at low temperature and in the absence of membrane potential across the inner membrane. By means of competition experiments with chemical amounts of the porin precursor, it was shown that all precursors tested so far, with the exception of apocytochrome *c*, share a common import site at this stage (Pfaller *et al.*, 1988). This site, presumably represented by a protein that mediates the initial insertion event of precursors in the outer membrane, was then termed GIP ("general insertion protein") (Pfaller *et al.*, 1988).

With the exception of outer membrane proteins and some of the intermembrane space proteins, all other precursor proteins need the presence of a membrane potential for import (Gasser *et al.*, 1982a; Schleyer *et al.*, 1982; Pfanner and Neupert, 1985). The actual way this membrane potential acts is not yet clear; it has, however, been postulated that it functions to promote the translocation of the presequence across the inner membrane. After this step, i.e., when only the initial portion of the precursor is fully translocated into the matrix, the membrane potential can be abolished without any adverse effect on the subsequent import of the rest of the polypeptide (Schleyer and Neupert, 1985; Pfanner and Neupert, 1987b).

When precursors carrying a presequence reach the mitochondrial matrix, the presequence (or at least a part of it in the case of proteins which are destined for the intermembrane space) is cleaved off by a matrix-processing peptidase (MPP) (Conboy *et al.*, 1982; McAda and Douglas, 1982; Böhni *et al.*, 1983; Schmidt *et al.*, 1984; Hawlitschek *et al.*, 1988; Pollock *et al.*, 1988). This enzyme, for its function, depends on a second protein that is structurally related to it, termed PEP ("processing-enhancing protein") (Hawlitschek *et al.*, 1988). The peptidase needs divalent cations for activity and thus is inhibited by chelators such as *o*-phenanthroline and EDTA. It has been shown that the inhibition of the processing peptidase does not abolish the import of precursor proteins (Zwizinski and Neupert, 1983). Consequently proteolytic processing is not a prerequisite for the import of precursor proteins.

By performing the transport of precursors at low temperature, or after prebinding of precursor proteins to antibodies, precursor proteins can be trapped as translocation intermediates, spanning both mitochondrial membranes (Schleyer and Neupert, 1985; Schwaiger *et al.*, 1987). Precursors

trapped in such a manner are still sensitive to externally added proteases, yet have undergone cleavage of their presequence by the matrix-located processing peptidase. An analysis of the length of the spanning region led to the conclusion that the import should occur at sites where the inner and outer membranes are in close contact with each other (Schleyer and Neupert, 1985; Schwaiger *et al.*, 1987). In fact, such contact sites can be morphologically distinguished by electron microscopy (Hackenbrock, 1968; Kellems *et al.*, 1975) and by means of immunolocalization studies, it was observed that precursors spanning both membranes are localized near these contact sites (Schwaiger *et al.*, 1987).

More recently, artificial precursor proteins, such as a fusion protein between the presequence of the subunit IV of cytochrome oxidase and dihydrofolate reductase cross-linked to bovine pancreas trypsin inhibitor (Vestweber and Schatz, 1988) or a fusion protein between cytochrome b_2 and dihydrofolate reductase (Rassow *et al.*, 1989), have been used to quantitate the amount of contact sites in mitochondria. It has been shown that mitochondria contain a finite and constant number of these sites (about 50–70 pmol per mg of protein), and that the saturation of these sites completely inhibits the import of mitochondrial precursor proteins (Vestweber and Schatz, 1988; Rassow *et al.*, 1989).

Once inside the matrix, precursors (or at least some of them) interact with the stress-responsive protein hsp60 (Cheng *et al.*, 1989; Ostermann *et al.*, 1989). This protein is essential for the correct assembly of mitochondrial proteins (Cheng *et al.*, 1989), and when mitochondria are depleted of ATP, newly imported precursors remain bound to hsp60 in an unfolded state (Ostermann *et al.*, 1989). The interaction of newly imported proteins with hsp60 is thought to be important for the assembly and correct sorting of these proteins.

Finally, some proteins that are targeted to the intermembrane space contain a second cleavable presequence that presumably contains the information for the retranslocation across the inner mitochondrial membrane (Hartl *et al.*, 1986, 1987). These second presequences are similar to bacterial leader peptides, and it is presumed that they are recognized by a translocation machinery localized in the inner membrane of mitochondria. No components of this apparatus have, however, been identified up to date.

Among the energy requiring steps along the import pathway of mitochondrial proteins, the requirement for nucleoside triphosphate (NTP) hydrolysis should be mentioned (Pfanner and Neupert, 1986; Chen and Douglas, 1987; Eilers *et al.*, 1987). NTPs seem to be involved in conferring a transport-competent conformation to precursor proteins in the cytosol (Pfanner *et al.*, 1987b; Verner and Schatz, 1987). Members of the hsp70 family may participate in this process (Deshaies *et al.*, 1988). ATP is also

required in the matrix for the release of imported proteins from the above-mentioned hsp60 (Ostermann *et al.*, 1989). Finally, the transfer of the ADP/ATP carrier from its receptor site to GIP has been shown to require the hydrolysis of a high-energy phosphate bond (Pfanner and Neupert, 1987b). Some additional and yet unidentified energy-requiring steps may also be present along the import pathway of mitochondrial precursor proteins.

Import of Cytochrome *c*

The import pathway of cytochrome *c* is quite different from those followed by other precursor proteins. The precursor of cytochrome *c*, apocytochrome *c*, is synthesized in the cell cytosol on free ribosomes (Korb and Neupert, 1978). It contains no cleavable presequence, and no classical mitochondrial targeting sequence can be identified along its primary structure (Stewart *et al.*, 1971; Zitomer and Hall, 1976; Zimmermann *et al.*, 1979). However, it differs from the holo form by the lack of the heme group and by a different conformational arrangement (Fisher *et al.*, 1973; Rietveld *et al.*, 1985). Apocytochrome *c* binds with high affinity to intact mitochondria (Hennig and Neupert, 1981; Zimmermann *et al.*, 1981). The availability of apocytochrome *c* in high amounts (since it can be obtained from the holo form by chemical cleavage of the heme group) has allowed the quantitation of the binding sites ("receptor"). They are present at an amount of ca. 70 pmol/mg of protein and show an association constant of $2.2 \times 10^7\ M^{-1}$ (Hennig *et al.*, 1983). These binding sites differ in a number of properties from those of other precursors. They display a lower affinity, are considerably more abundant, and, in intact mitochondria, are not sensitive to low levels of proteases (Nicholson *et al.*, 1988). Treatment of mitochondria with high amounts of trypsin, which cause damage to the outer membrane barrier, completely abolishes this high-affinity binding (Nicholson *et al.*, 1988). These experiments show that the binding sites for apocytochrome *c* are not exposed on the surface of mitochondria.

The interaction of the precursor with this binding site is then thought to be preceded by an insertion of apocytochrome *c* into the outer mitochondrial membrane. The ability of apocytochrome *c* to insert into lipids has been demonstrated in studies with artifical membranes (Dumont and Richards, 1984; Rietveld *et al.*, 1985, 1986), and has been recently shown to be functional in the first domain of mitochondria (Stuart *et al.*, 1990). In fact, a fusion protein between the first part of the presequence of cytochrome c_1 and the complete apocytochrome *c* molecule is imported to the matrix with high efficiency without the requirement of surface receptors or GIP (Stuart *et al.*, 1990).

Either the insertion through the outer membrane or interaction with the binding site are strongly dependent on the conformational arrangement of apocytochrome *c*. The high-affinity binding of chemically prepared precursor is markedly affected by repeated freezing and thawing. Also the addition of ATP to the import mixture, which is thought to promote processes involved with unfolding of precursors, results in inhibition of the import of cytochrome *c* by interfering with the binding step (Nicholson *et al.*, in preparation). The interaction of apocytochrome *c* with its binding sites is also sensitive to high salt concentrations but, once bound, further steps can occur even in the presence of high salt (Nicholson *et al.*, 1988).

The import pathway of apocytochrome *c* can be interrupted at the binding stage by preventing the heme addition event from occurring. Heme addition, catalyzed by the enzyme cytochrome *c* heme lyase (CCHL) (Taniuchi *et al.*, 1983; Nicholson *et al.*, 1987), can be hindered by the heme analogue deuterohemin (Hennig and Neupert, 1981) or by performing import in the absence of NADH, which is required for the formation of holocytochrome *c* (Nicholson *et al.*, 1988). In fact, these two methods have been used to quantify the binding sites for cytochrome *c* and both result in identical results, namely, 60–90 pmol/mg of protein (Hennig *et al.*, 1983; Nicholson *et al.*, 1988).

The formation of holocytochrome *c* requires that the heme be in the reduced state (Nicholson and Neupert, 1989). In intact mitochondria *in vitro*, this is achieved by the addition of NADH plus a flavin nucleotide (Nicholson and Neupert, 1989). It is not clear if there is a special enzyme in mitochondria for the reduction of heme or if reduced heme is delivered directly from ferrochelatase to the heme lyase in this state.

When the addition of heme is inhibited, apocytochrome *c* remains accessible to externally added proteases (Nicholson *et al.*, 1988). This would mean that, although it is inserted into the outer membrane and interacts with a nonsurface exposed binding site, apocytochrome *c* is not fully translocated. When heme addition takes place, then holocytochrome *c* is found in the intermembrane space (Hennig and Neupert, 1981; Nicholson *et al.*, 1988). Altogether, these results suggest that translocation is coupled to heme addition. Furthermore it was proposed that the formation of holocytochrome *c*, which promotes a drastic change in the conformation of the polypeptide chain, can act as the driving force for the complete translocation of the cytochrome across the outer mitochondrial membrane (Hennig and Neupert, 1981; Nicholson *et al.*, 1988).

The addition of heme to apocytochrome *c* can also occur in detergent extracts of mitochondria (Nicholson *et al.*, 1988). Notably, the reaction in this soluble system is also sensitive to salt, but when the apo form is prebound at low salt, then the conversion to the holo form can occur in the presence

of high salt (Nicholson *et al.*, 1988). This would suggest that the salt-sensitive binding step observed in intact mitochondria could be the binding to CCHL itself. This in turn would imply that the heme lyase acts as, or comprises a part of, the cytochrome *c* "receptor". A further possibility is that binding of apocytochrome *c* to another component, prior to the interaction with the heme lyase, is also required in the soluble system. To further support the first hypothesis, it has been observed that a *Neurospora crassa* mutant which lacks CCHL activity is not only deficient in cytochrome *c* import, but also in the specific binding of apocytochrome *c* (Nargang *et al.*, 1988).

The availability of this mutant, and of a similar one in yeast, has allowed the cloning and the determination of the primary structure of CCHL from both organisms (Dumont *et al.*, 1987; Drygas *et al.*, 1989, Nargang *et al.*, 1988). The proteins show 32% homology (49% if conservative substitutions of amino acid residues are included), with the most conserved regions clustered in the C-terminal half of the polypeptide. Notably, there is a seven amino acid motif at the amino terminus that is identical in both proteins, followed by a region of unconserved residues. There is no cleavable presequence and no typical mitochondrial targeting sequence in their structure (Dumont *et al.*, 1987; Drygas *et al.*, 1989).

CCHL is a membrane-bound enzyme. It is not released from mitochondria upon opening the intermembrane space with digitonin (Nicholson *et al.*, 1988) and not even by sonication in the presence of salt (Hergersberg and Neupert, unpublished). However, if a protease treatment is performed following disruption of the outer membrane barrier, the CCHL protein becomes digested and loss of activity is also monitored, thus showing that CCHL is exposed to the intermembrane space (Nicholson *et al.*, 1988; Hergersberg and Neupert, unpublished).

If CCHL is able to bind apocytochrome *c* that is inserted into the outer membrane, then CCHL should be bound to the outer mitochondrial membrane. However, when outer and inner membrane vesicles prepared by swelling and sonication are separated on a sucrose gradient, CCHL does not migrate with outer membrane markers (Hergersberg *et al.*, unpublished). The location of CCHL thus seems to be unclear, although the most logical explanation is that it is located at sites where both membranes come in close contact with each other. If this turns out to be the case, this protein could then be used as a marker to isolate and characterize contact sites.

Our current view on the cytochrome *c* import pathway is depicted in Fig. 2. The import of cytochrome *c* to the intermembrane space does not show any energy-requiring step. If the driving force for translocation is, as postulated, the folding of the polypeptide caused by heme addition, then the generation of an unstable intermediate (i.e. unfolded holocytochrome *c*) would need some input of energy. Since direct energy requirements have not

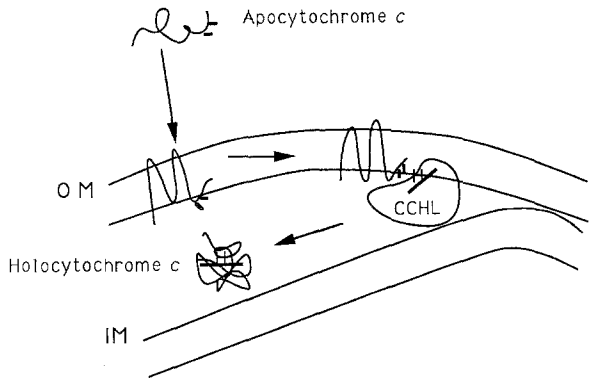

Fig. 2. Hypothetical import pathway of cytochrome c. Apocytochrome c initially inserts into the outer mitochondrial membrane, and then forms a tight complex with a binding protein ("receptor") that is not exposed on the mitochondrial surface, presumably cytochrome c heme lyase (CCHL); addition of heme results in the translocation of the cytochrome c across the outer mitochondrial membrane. The location of cytochrome c heme lyase bound to the outer mitochondrial membrane is hypothetical (see discussion in the text). H, heme; OM, outer membrane; IM, inner membrane.

been identified, it can be speculated that the necessity for reduced heme would represent such requirements. Then, the heme reduction event, which consumes NADH, would be an energetic cost for cytochrome c translocation.

Import of Cytochrome c_1

Cytochrome c_1 follows a more general import pathway (Fig. 3). Its precursor, apocytochrome c_1, contains a cleavable presequence in which two different domains can be identified (Gasser *et al.*, 1982b); Ohashi *et al.*, 1982; Teintze *et al.*, 1982; Sadler *et al.*, 1984; Römisch *et al.*, 1987). The amino-terminal half is a typical mitochondrial matrix targeting sequence, which is positively charged and contains nonpolar residues distributed along it. The second half of the presequence contains a stretch of hydrophobic residues which resembles the leader peptides of exported bacterial proteins (Sadler *et al.*, 1984; van Loon *et al.*, 1986; Hartl *et al.*, 1987; Römisch *et al.*, 1987; Hartl and Neupert, 1990).

After synthesis in the cell cytosol, apocytochrome c_1 binds to a receptor exposed on the mitochondrial surface, in this case MOM19 (Söllner *et al.*, 1989). Then, transfer to GIP (Pfaller *et al.*, 1988; Stuart *et al.* 1990) and then import into the matrix occurs (Hartl *et al.*, 1987; Nicholson *et al.*, 1988). Once in the matrix, the first half of the presequence is removed by the action of the

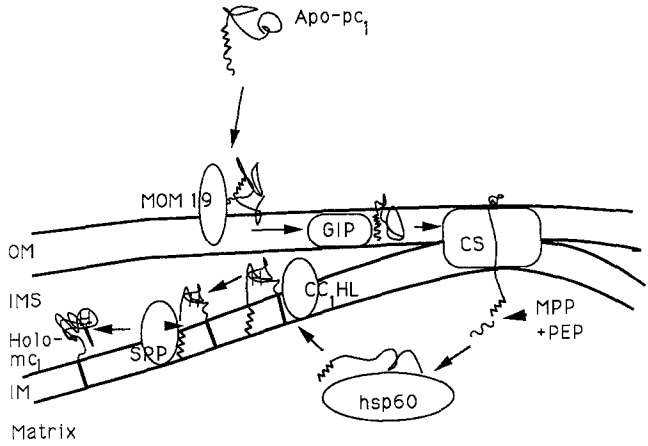

Fig. 3. Hypothetical import pathway of cytochrome c_1. Details are given in the text. Apo-pc_1, apo form of cytochrome c_1 precursor; CC_1HL, cytochrome c_1 heme lyase; CS, contact sites; GIP, general insertion protein; H, heme; holo-mc_1 mature size holocytochrome c_1; hsp60, heat shock protein of 60 kD. IM, inner membrane; IMS, intermembrane space; MPP, mitochondrial processing peptidase; OM, outer membrane; PEP, processing-enhancing protein; SPP, second processing peptidase;

matrix-processing peptidase, thus forming an intermediate form of apocytochrome c_1 (Gasser *et al.*, 1982b; Teintze *et al.*, 1982; van Loon *et al.*, 1986; Hartl *et al.*, 1987).

The function of the second part of the presequence is then to direct the retranslocation of the intermediate form across the inner mitochondrial membrane. It was initially suggested that this hydrophobic signal acts to stop the transfer of the precursor during import, so that the mature part would remain exposed to the intermembrane space (van Loon and Schatz, 1987). The fact that the intermediate form was found to be fully translocated into the matrix showed that export actually takes place (Hartl *et al.*, 1987; Stuart *et al.*, in preparation). This mechanism is also followed by other precursors that contain a bipartite-signal peptide and has been termed "conservative sorting," because after transfer into the matrix, these precursors are further transported by a mechanism that resembles the export pathways from bacteria, the postulated evolutionary ancestors of mitochondria (Hartl *et al.*, 1986, 1987).

During its transit into the matrix, apocytochrome c_1 probably interacts with hsp60, which has been recently shown to bind newly imported precursors (Ostermann *et al.*, 1989). Although not yet demonstrated, it is most likely that a direct transfer from this protein to the retranslocation machinery occurs, thereby preventing misfolding which would hinder the transport to the intermembrane space.

Following retranslocation, the intermediate form of apocytochrome c_1 becomes exposed to the intermembrane space, and formation of holocytochrome c_1 takes place. This step is catalyzed by the enzyme cytochrome c_1 heme lyase (CC_1HL), which is different from CCHL (Nicholson *et al.*, 1989). The c_1 heme lyase is also exposed to the intermembrane space and most likely bound to the inner membrane. As demonstrated for the CCHL, CC_1HL also requires heme in the reduced state, and the reduction can be accomplished in intact mitochondria by the addition of NADH and a flavin nucleotide (Nicholson *et al.*, 1989). Both the unprocessed and the intermediate forms of cytochrome c_1 can be used as substrates by CC_1HL (Nicholson *et al.*, 1989).

It was recently shown that a fusion protein between the cytochrome c_1 presequence and apocytochrome c can be imported into mitochondria along the cytochrome c_1 pathway under certain conditions. (Stuart *et al.*, in preparation). However, when imported along the cytochrome c_1 pathway, this fusion protein was not converted to the holo form. It was demonstrated that CC_1HL cannot use apocytochrome c as a substrate, thus showing a distinct specificity from CCHL (Stuart *et al.*, in preparation).

After holocytochrome c_1 formation, processing to the mature-sized protein occurs (Teintze *et al.*, 1982; Nicholson *et al.*, 1989). Prevention of heme addition, either by the action of deuterohemin or by omitting NADH from the import reaction, completely inhibits the last proteolytic step (Teintze *et al.*, 1982; Nicholson *et al.*, 1989). It is most likely that a conformational change in the cytochrome c_1 molecule, as a result of the heme addition, is necessary to expose the cleavage site to the action of the second processing peptidase.

Although the addition of heme to apocytochrome c can be measured in a detergent extract of mitochondria, the same has not been possible with cytochrome c_1. It is most likely that apocytochrome c_1 must be delivered to the heme lyase directly by another component of the translocation machinery (Nicholson *et al.*, 1989), thus allowing the right conformation for binding to the enzyme to be preserved. Hence pre-import of apocytochrome c_1 into the intermembrane space in the absence of NADH appears to be a prerequisite for measuring CC_1HL activity in a detergent-solubilized system. As mentioned before, the nature of the components required for retranslocation across the inner membrane, with the probable exception of hsp60, is not currently known.

Evolutionary Aspects

The import pathways of cytochromes c and c_1 have evolved in different ways. In purple photosynthetic bacteria, which according to the

endosymbiotic theory would have a common origin with mitochondria, the two related cytochromes, namely c_2 and c_1, are most likely transported by similar mechanisms. This is suggested by the fact that they contain very similar cleavable presequences (Gabellini *et al.*, 1985; Daldal *et al.*, 1986; Gabellini and Sebald, 1986).

It is clear then that in mitochondria cytochrome c_1, like some other intermembrane space proteins, retained this transport pathway, which now is coupled to the import into the matrix from the cytosol promoted by an addition of a "matrix-targeting presequence" (Figs. 1 and 3).

The import pathway of cytochrome *c*, on the other hand, has been the subject of a marked evolution. Two events can be seen as the main steps of this evolution. One of them is the acquisition of insertion properties by the apocytochrome *c* molecule (Rietveld *et al.*, 1985, 1986; Stuart *et al.*, 1990). The other one is the change in some properties of CCHL, which allowed the binding of the apo form coming from the outside of mitochondria to occur (Nicholson *et al.*, 1988).

On considering the evolution of the cytochrome *c* pathway, one important question is why this evolution took place. The new pathway is simpler and perhaps more favorable from an energetic point of view. However, these features would be only marginally important, especially because the conservative sorting pathway is maintained for a whole set of different proteins. Apocytochrome *c* can be routed to the intermembrane space along the conservative pathway, when fused to a cytochrome c_1 presequence (Stuart *et al.*, in preparation).

One can then speculate that the changes that led to the new pathway were rather simple. One important feature about cytochrome *c* is that is does not need to be assembled with other proteins into a specific multi-subunit complex. Cytochrome c_1, on the other hand, may use a mitochondrial import/export machinery to be correctly assembled with the other polypeptides of the ubiquinol–cytochrome *c* reductase complex.

Concerning the insertion properties of apocytochrome *c*, an interesting question is whether or not they are already present in the bacterial cytochromes. A comparative study of mitochondrial and bacterial cytochromes could help to establish which regions of the polypeptide are important for this insertion activity.

Considering CCHL, evolution saw alterations in the topology and, perhaps, the development of different, though related, enzymes acting in heme addition to cytochromes *c* and c_1. It is not known if one or two heme lyases are present in bacteria. Change in topology would appear to have directed the CCHL to the outer membrane or, more probably, to contact site regions. This migration would have implicated the acquisition of some localization signals for the binding to specific regions in the membrane, or to

a specific polypeptide(s), perhaps a pre-existing one. These facts could explain the postulated association of CCHL with contact sites, although apparently no functional association exists between this enzyme and the other putative components of these specialized structures.

Conclusion

The two mitochondrial cytochromes with covalently attached heme follow extremely different pathways in their transport to the mitochondrial intermembrane space. These differences would arise not only from the molecular properties of the corresponding precursors (i.e., the apo forms), but also from the different final location of the two cytochromes and the nature of the heme addition steps.

Further research should be devoted to the characterization of both pathways at a molecular level. In the case of cytochrome c_1, the question concerns the pathway followed by the majority of imported precursors. A still widely open field is that of the retranslocation from the matrix and across the inner membrane. Almost nothing is known about the components that participate in this retranslocation process. It may be postulated that hsp60 would play a role, as probably do other components that were recently suggested to be involved in the release of proteins from hsp60 (Ostermann *et al.*, 1989). Are these components located in the matrix or in the inner membrane? What role do they play? How is apocytochrome c_1 delivered to CC_1HL and then finally to the second processing peptidase? What are the characteristics of this enzyme? One would expect that it is related to bacterial leader peptidase, as it is the thylakoidal peptidase involved in removing the second part of the presequence of precursors directed to the chloroplast lumen (Halpin *et al.*, 1989). Finally, how does the assembly of the membrane-located complexes occur? Is the insertion of the different polypeptides directed in an ordered fashion from the matrix by the same assembly machinery presumed to be involved in the oligomerization of soluble proteins, or are there unidentified membrane-bound components necesary for the assembly process?

On the other hand, what is the basis for the membrane insertion properties of apocytochrome c? Current data indicate that a specific conformation of the apoprotein is required. Are there conserved residues or regions of the apopolypeptide that are important for import and not for its function as an electron carrier? Can apocytochrome c carry "passenger" proteins across the outer membrane? It is known that two N-terminal additions (the complete cytochrome c_1 presequence or a part of it) do not alter the insertion properties of apocytochrome c (Stuart *et al.*, 1990 and

manuscript in preparation). Do bacterial cytochromes have these properties? Where exactly is CCHL located? And more specifically how is it located in relation to the outer membrane? Are there different domains of CCHL involved in activity, apocytochrome *c* binding, and sorting to a specific sub-localization? The answer to some of these questions will promote a deeper understanding of the transport pathway of cytochrome *c*, and some of the results may turn out to be of interest for the understanding of some aspects of the translocation machinery of other precursor proteins.

Acknowledgments

The authors are grateful to the Alexander von Humboldt-Stiftung for granting a fellowship to D. H. Gonzales, and to R. A. Stuart and N. Pfanner for valuable help in preparing this manuscript. This work was supported by the Sonderforschungsbereich 184, the Genzentrum der Universität München, and the Fonds der Chemischen Industrie.

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