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Apocytochrome *c*: an exceptional mitochondrial precursor protein using an exceptional import pathway

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Summary – The cytochrome *c* import pathway differs markedly from the general route taken by the majority of other imported proteins, which is characterized by the import involvement of namely, surface receptors, the general insertion protein (GIP), contact sites and by the requirement of a membrane potential ($\Delta\psi$). Unique features of both the cytochrome *c* precursor (apocytochrome *c*) and of the mechanism that transports it into mitochondria, have contributed to the evolution of a distinct import pathway that is not shared by any other mitochondrial protein analysed thus far. The cytochrome *c* pathway is particularly unique because i) apocytochrome *c* appears to have spontaneous membrane insertion-activity; ii) cytochrome *c* heme lyase seems to act as a specific binding site *in lieu* of a surface receptor and; iii) covalent heme addition and the associated refolding of the polypeptide appears to provide the free energy for the translocation of the cytochrome *c* polypeptide across the outer mitochondrial membrane.

apocytochrome *c* / mitochondrial precursor protein / import pathway

Introduction

To ensure the specific and efficient delivery of proteins to various cellular subcompartments, the eukaryotic cell has developed a number of devices to control the targeting of proteins to their correct functional destination. These control systems involve both features of the precursor proteins and components of the destination organelles. The accuracy and specificity of these control systems is vital for the maintenance of the cell, as mistargeting of proteins *in vivo* in many instances would be fatal. In the case of mitochondrial protein import, most of the precursor proteins carry amino terminal extension sequences which contain mitochondrial targeting information and receptors exist on the mitochondrial surface to recognize and bind precursor proteins destined for that organelle. Precursor proteins are then imported into mitochondria along complex pathways and are correctly delivered to their functional submitochondrial location. These import pathways can be experimentally divided into a series of independent steps and are common for a large number of proteins. These steps include: a) synthesis of precursor proteins with amino-terminal presequences in the cell cytosol [13–16]); b) maintenance of precursor proteins in a translocation competent form, a process which requires heat shock proteins (hsps), other cytosolic factor(s) and ATP [17–20]; c) binding to proteinaceous receptors on the mitochondrial outer surface [21–23]; d) transfer to the putative general insertion protein (GIP) in the outer mitochondrial membrane, which is believed to contribute to the initial membrane insertion step of precursor proteins destined for all the different mitochondrial subcompartments [24, 25]; e) membrane potential-dependent insertion into or through the inner membrane *via* translocation contact sites [26–29]; f) cleavage of amino-terminal presequences by the matrix processing peptidase, an event assisted by PEP, the processing enhancing protein [30–33]; g) finally, intramitochondrial sorting and assembly into functional complexes assisted by chaperonin-type proteins, in particular hsp60 [34].

Import of cytochrome *c*, however, shares hardly any of the features with the above described general targeting mechanism. Our current working hypothesis on the assembly pathway of cytochrome *c* is illustrated in figure 1 and can be summarized as follows. Cytochrome *c* is synthesized as a precursor protein known as apocytochrome *c* and does not contain an amino-terminal extension sequence, and thus is not proteolytically processed upon import into mitochondria [35–40]. No protease-sensitive components exist on the surface of mitochondria to mediate apocytochrome *c* binding and import [10]. Instead, apocytochrome *c* partially inserts into the outer mitochondrial membrane [2–5, 7] where it is recognized and binds with high affinity to specific binding sites [11, 12, 41, 42] in a complex which includes cytochrome *c* heme lyase (CCHL) [10]. CCHL is the enzyme which is responsible for the covalent attachment of heme to apocytochrome *c* and it displays a dependence on NADH and flavin nucleotides [10]. It is thought that cytochrome *c* heme lyase acts as a specific binding site *in lieu* of a surface receptor [10]. The mitochondria of a mutant

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of *Neurospora crassa*, cyt2-1, are deficient in CCHL activity and are also unable to bind apocytochrome *c* or produce holocytochrome *c* [9, 43]. This result therefore is consistent with the view that CCHL functions as an initial binding site for apocytochrome *c*. Specifically-bound apocytochrome *c* remains accessible to externally added proteases until holocytochrome *c* formation has occurred. Hence, it was concluded that holo-formation is a prerequisite for import of apocytochrome *c* [10, 44–46]. Unlike all other mitochondrial precursors crossing the outer membrane, cytochrome c does not require a membrane potential for translocation [27, 35]. Furthermore, in contrast to all other precursors studied so far, ATP is not required throughout the import process of cytochrome *c* (Nicholson and Neupert, in preparation), thus suggesting that a specific folded, rather than an unfolded, conformation of apocytochrome *c* is required for binding and membrane insertion. Translocation across the outer membrane, directly into the intermembrane space is believed to be driven by the refolding of the polypeptide as a result of the covalent attachment of heme [10, 11, 46].

Targeting of apocytochrome *c* to mitochondria

The presequences of many mitochondrial precursor proteins have been shown to contain the necessary information for mitochondrial targeting. Proteinaceous receptors on the surface of mitochondria, with which precursor proteins specifically interact, play a role in "decoding" the targeting information contained in the precursor protein. Hence correct targeting of proteins to mitochondria involves both the targeting information in the precursor and specific recognition or decoding of this information by receptor sites on the mitochondrial surface. If we consider cytochrome *c* import, however, the situation appears to be very different.

Cytochrome *c* is synthesized as a precursor protein which does not contain an amino-terminal presequence and no protease-sensitive receptors exist on the surface of mitochondria to mediate the initial binding and import of the apocytochrome *c* precursor. How then is cytochrome *c* specifically targeted to mitochondria? It is proposed that, unlike other mitochondrial precursor proteins, no targeting sequence *per se* exists to target apocytochrome *c* to mitochondria. However, a specific overall conformation, *ie* a certain "folded state" of apocytochrome *c*, rather than a targeting sequence, is required for mitochondrial targeting. As will be discussed later, the initial step in cytochrome *c* import is its spontaneous insertion into the outer mitochondrial membrane; once inserted into the lipid bilayer, in the case of mitochondria only, apocytochrome *c* forms a tight complex which includes cytochrome *c* heme lyase. Binding of apocytochrome *c* to mitochondria involves features of both the amino- and carboxy-terminal regions of the apocytochrome *c* polypeptide. Cysteines number 14 and 17 (for *N crassa*) must be exposed to the intermembrane space for interaction with CCHL and subsequent heme linkage. The carboxy terminus of apocytochrome *c* has recently been shown to also be important for targeting of apocytochrome *c* to mitochondria [40]. As a consequence of a defect in cytochrome *c* mRNA splicing, a mutant of *N crassa, cyc1-1*, synthesizes an apocytochrome *c* with an altered carboxy terminus. The polypeptide sequence is changed from amino acid 102 onwards, resulting in an apocytochrome *c* which is 19 amino acids longer than the corresponding wild-type protein, thus the final 27 amino acids are of an unrelated sequence. This alteration in the carboxy terminus renders the apocytochrome *c* incompetent for binding to mitochondria and consequently for import into mitochondria [40]. Results, recently obtained would strongly suggest that alteration in the carboxy terminus of apocytochrome *c*, such as that observed in *cyc1-1* apocytochrome *c*, do not significantly affect the ability of the precursor to spontaneously insert into the mitochondrial membrane (Stuart and Neupert, unpublished results). This indicates that membrane insertion is followed by a more specific step of targeting and as outlined below cytochrome *c* heme lyase appears to be involved in this step. Matsuura *et al*, (1981) postulated that an addressing signal is contained in an apocytochrome *c* fragment which extends from amino acid 66 to the carboxy-terminal end of the protein, which serves to target the apocytochrome *c* to mitochondria [39]. In this respect it is also interesting to note that the carboxy terminus is the most evolutionary conserved part of the cytochrome *c* polypeptide with the exception of the heme binding region. It cannot as yet be concluded from the findings of the study of the *cyc1-1 N crassa* mutant, however, that such a "targeting signal" is located at the carboxy terminus of apocytochrome *c*.

Membrane insertion properties of apocytochrome *c* are essential for its import

The ability of apocytochrome *c* to spontaneously insert into lipid bilayers has been the focus of research carried out by de Kruijff and co-workers. They have demonstrated in a model-membrane system that apocytochrome *c*, which is relatively positively charged, can interact strongly with negatively-charged phospholipids in an electrostatic manner [3, 5]. Fragments of the polypeptide which have the highest net-positive charge, displayed the highest affinity for the negatively-charged membranes [8]. Furthermore, it could be

demonstrated that it is the amino- and carboxy-terminal fragments of the protein specifically that display the ability to insert into membranes. The amino-terminal fragment, however, is believed to be able to penetrate the bilayer more deeply than the carboxy terminus [8]. The electrostatic interaction of the apocytochrome *c* polypeptide with the phospholipids is thought to induce structural reorganization of the lipids leading to a reduction of the barrier function, thus the anionic phospholipids appear to be sequestered into a micro-environment surrounding the apocytochrome *c* molecule [4, 6, 7]. Circular dichroism (CD) studies revealed that the CD pattern of apocytochrome *c* in an aqueous solution was featureless [5]. During or following the interaction of apocytochrome *c* with the lipid bilayer, α-helical structure was expressed within the amino- and carboxy-terminal regions of the polypeptide [4]. It was postulated that this conformational change is important for the insertion of the protein into the bilayer.

Spontaneous insertion of apocytochrome *c* into the mitochondrial outer membrane thus appears to represent an early and critical step in the cytochrome *c* import process. There may be no specificity of this step for mitochondrial outer membranes or a limited specificity (*eg* exerted by the particular lipid composition). Only in the case of mitochondria, however, is there a specific recognition and tight binding, involving cytochrome *c* heme lyase. This step is followed by the covalent heme attachment and folding-dependent translocation across the outer membrane.

Upon import into mitochondria, all mitochondrial precursor proteins analysed so far, with the exception of cytochrome *c*, must interact specifically with receptor proteins on the outer surface of the mitochondria and then with GIP, to become inserted into the mitochondrial membrane system [21, 22, 25]. This may be considered as a "multiple-check system" which controls the specificity and efficiency of precursor protein entry at multiple stages of import [24, 25]. Hence *in vivo* mistargeting of proteins in the cell would be negligible and consequently the uniqueness of the mitochondrial protein composition would not be threatened. The question thus arises as to how cytochrome *c* is able to take a completely unique import pathway into mitochondria. In order to address this question, we analysed the import pathways of a series of fusion proteins between apocytochrome *c* and mitochondrial presequences. The membrane activity of the apocytochrome *c* domain enabled the fusion proteins to be imported into the mitochondrial matrix in a manner that was no longer dependent on either surface receptors, or GIP-mediated membrane insertion or on NTP-mediated precursor unfolding. Thus, it could be concluded that the presence of the apocytochrome *c* domain enables these fusion proteins to circumvent the receptor/GIP mediated steps of import into the matrix by directly mediating the insertion of the precursor protein into the mitochondrial membrane system. There, the targeting domain of the presequence can respond to the membrane potential ($\Delta\psi$) for complete translocation across the inner membrane [47]. This emphasizes the roles of receptors and GIP in membrane insertion, in addition to the specific recognition of precursor proteins. Combined, these findings help to explain why all other mitochondrial precursor proteins examined thus far require a receptor/GIP system for import. A precursor protein containing a target signal (*ie* a prepiece) but no membrane-insertion activity within the mature part of the polypeptide apparently depends on components in the outer membrane to recognize them and facilitate the insertion of the targeting signal in such a manner that it can respond to $\Delta\psi$. Because apocytochrome *c* is able to spontaneously insert into membranes, it is able to mediate the circumvention of these critical stages of import on behalf of "passenger" sequences.

Import of apocytochrome *c* does not require NTPs

Protein targeting to all membranes, whether they be procaryotic or eucaryotic, share many salient features, a popular one of which at the moment is the notion of correct/proper conformation of precursor proteins so that they may be "translocation competent" (for reviews see [48–50]). A precursor protein is considered to have a translocation-competent conformation when it has an unfolded structure rather than when it has adopted a "foldedness" identical or similar to that of its mature counterpart. A translocation competent conformation is correlated therefore, with a certain openness of structure that is characterized by increased sensitivity to proteolytic digestion [19, 51].

It has been demonstrated that folded precursor proteins cannot be transported across mitochondrial membranes [18, 28, 52]. On the other hand, hydrolysis of nucleoside triphosphates (NTPs) is also necessary for mitochondrial protein import [17, 19]. It was demonstrated that the sensitivity of precursor proteins in reticulocyte lysate to non-specific proteases is greater in the presence of NTPs [19]. It is thus postulated that the hydrolysis on NTPs is involved in modulating the folding of precursors in the cytosol. The answer to how NTPs are involved in conferring a conformation on a precursor protein that is competent for import, possibly lies in a group of proteins known as heat shock proteins. Heat shock proteins in times of stress are apparently able to bind to exposed areas of denaturated proteins and thereby promote disaggregation and renaturation [53–55]. Recently it has been shown that import into mitochondria requires the involvement of

hsp70 proteins [20, 56] and that these maintain precursor proteins in a translocation competent conformation [57]. It would appear that the heat shock proteins may act in a preventative manner by inhibiting folding, rather than by coming to the rescue of an already-folded protein, by unfolding it.

All mitochondrial precursor proteins studied to-date, require an unfolded conformation in order to be translocation competent and hence display a dependence on NTPs for import into mitochondria. In contrast, however, NTPs are not required throughout the import process of cytochrome *c* (Nicholson and Neupert, in preparation). Hence in the absence of NTPs, apocytochrome *c*, like other mitochondrial precursor proteins studied to-date, folds into a more protease-resistant structure. This "folded" structure was found to be a more import-competent form, in contrast to an unfolded apocytochrome *c* structure. Dissection of the cytochrome *c* pathway revealed that it was the membrane insertion of apocytochrome *c* and its binding to cytochrome *c* heme lyase which was enhanced when the apocytochrome *c* molecule was more "folded". Once bound to mitochondria, however, subsequent holocytochrome *c* formation was relatively unaffected by NTP levels (Nicholson and Neupert, in preparation). Thus it was concluded that a specific folded rather than unfolded conformation of apocytochrome *c* is required for mitochondrial binding and membrane insertion.

The importance of a specific conformation of apocytochrome *c* for its binding to mitochondria is also supported by the observation that apocytochrome *c* that had been treated with urea or cycles of freezing and thawing, was not able to insert into membranes [44].

Cytochrome *c* heme lyase: a protein with a dual function?

Not only does the attachment of the heme group play a vital role for the function of cytochrome *c* as an electron carrier, but it also appears to be crucial for events in the import pathway of cytochrome *c*. Cytochrome *c* heme lyase (CCHL) is the enzyme which catalyses the covalent attachment of heme *via* thioether linkages to the 2 cysteines located near the amino terminus of apocytochrome *c*. When the heme lyase action is inhibited by the presence of the heme analogue deuteroheme, import of apocytochrome *c* is stalled at the level of the high-affinity binding sites. Deuteroheme lacks the 2 vinyl groups present in protoheme and thus cannot form the thioether bridges to cysteines 14 and 17 of apocytochrome *c*. This inhibition is reversible, however, as upon removal of deuteroheme and addition of protoheme, bound apocytochrome *c* can be converted to holocytochrome *c* and is translocated across the outer membrane [11, 12, 44]. It has recently been demonstrated that the import of cytochrome *c* is dependent on the presence of NADH and flavin nucleotides. It appears that the heme must be in the reduced state in order to be covalently attached by CCHL to apocytochrome *c*. NADH and FMN together directly mediate this reduction step [46]. In the absence of NADH, heme attachment does not take place and consequently import of externally bound apocytochrome *c* cannot occur. It was thus concluded that holocytochrome *c* formation is a prerequisite for translocation of cytochrome *c* across the outer mitochondrial membrane. Following holocytochrome *c* formation, the cytochrome *c* polypeptide folds around the newly acquired heme group into its native functional conformation. It is believed that energy derived from the folding process drives the transport of the complete protein across the outer mitochondrial membrane. Thus the transmembrane movement of cytochrome *c* is mechanistically coupled to the heme attachment event.

CCHL is not only essential in the heme addition events. Recent results would strongly suggest that CCHL interacts with apocytochrome *c* at an earlier stage on its import pathway. Mitochondria which were pretreated with low levels of proteases exhibit almost normal levels of cytochrome *c* import [10]. This observation indicates that apocytochrome *c* binding and import is not mediated by protease-sensitive components on the surface mitochondria [10]. When arrested upon its import pathway at the stage of high-affinity binding, apocytochrome *c* forms a salt-resistant complex that includes cytochrome *c* heme lyase [10]. We have demonstrated on the other hand, both enzymatically and immunologically, that CCHL is topologically exposed to the intermembrane space and fractionates with the inner mitochondrial membrane ([10]; Nicholson *et al*, in preparation). A similar finding has also been reported in yeast mitochondria [58]. Apocytochrome *c* that is specifically-bound to mitochondria exists in a membrane spanning configuration. While a large portion of the protein remains accessible to externally added proteases, at least some part(s) of the molecule must penetrate through the outer membrane in order to interact and form the salt-resistant complex with CCHL. Such a topological arrangement of the specifically bound apocytochrome would therefore have important consequences for the submitochondrial localization of CCHL. One could imagine that the bound apocytochrome *c*, which is largely exposed to the outer membrane surface of mitochondria (as judged by acessibility to added proteases) whilst at the same time forming a salt-resistant complex with CCHL could only achieve this conformation if the CCHL was localized in the vincinity of where both the inner and outer

membranes come in very close contact, namely, at translocation contact sites. Indeed, preliminary results from our laboratory would strongly favour this suggestion (Nicholson *et al*, in preparation).

Further evidence that would support the suggestion that CCHL is involved in the initial binding of apocytochrome in addition to its catalytic conversion to holocytochrome, lies in a mutant of *N crassa* known as *cyt2-1*. The mitochondria of this mutant are deficient in CCHL activity, furthermore, high-affinity binding sites of apocytochrome *c* cannot be detected [9, 43]. This suggests a direct interaction between bound apocytochrome *c* and CCHL, however, it cannot rigorously be excluded that other components are involved in mediating apocytochrome *c* binding. This question can only be finally answered when the purified CCHL is reconstituted into liposomes and thereby reconstituting the apocytochrome *c* import system.

Divergent evolution of import pathways for mitochondrial *c*-type cytochromes

Although mitochondrial cytochromes *c* and c_1 share a number of similarities with regard to function, submitochondrial location and prosthetic group, the import pathway of cytochrome *c* has evolved to be strikingly different from that of cytochrome c_1. As outlined above, the import pathway of cytochrome *c* appears to be relatively simple and does not involve the complex multi-step pathways required to import other mitochondrial precursor proteins. The import and assembly pathway of cytochrome c_1, on the other hand, is comparatively complex (fig 1) [59, 60]. Cytochrome c_1 is initially synthesized as a precursor protein with a long amino-terminal prepiece that is processed in 2 steps upon import into mitochondria [26, 61–64]. The precursor binds to a protease-sensitive receptor (the MOM19 protein) and then becomes inserted into the outer membrane, a process which requires the general insertion protein (GIP) [25, 47, 65]. Cytochrome c_1 is imported into the matrix *via* translocation contact sites in a membrane potential ($\Delta\psi$)-dependent fashion [26, 28]. In the matrix the precursor is processed by the matrix processing peptidase to its intermediate-size form (iC_1). The remaining part of the cytochrome c_1 presequence serves to "re-export" cytochrome c_1 across the inner membrane [59, 60]. Intermediate-size cytochrome c_1 (iC_1) thereby becomes exposed to the intermembrane space and the covalent attachment of heme to iC_1 occurs in a reaction catalysed by cytochrome c_1 heme lyase (CC_1HL). Cytochrome c_1 heme lyase is distinct from CCHL, although there is an apparent similarity of functional location and structural similarity of the heme binding regions in the respective apoproteins. Once the heme addition has taken place,

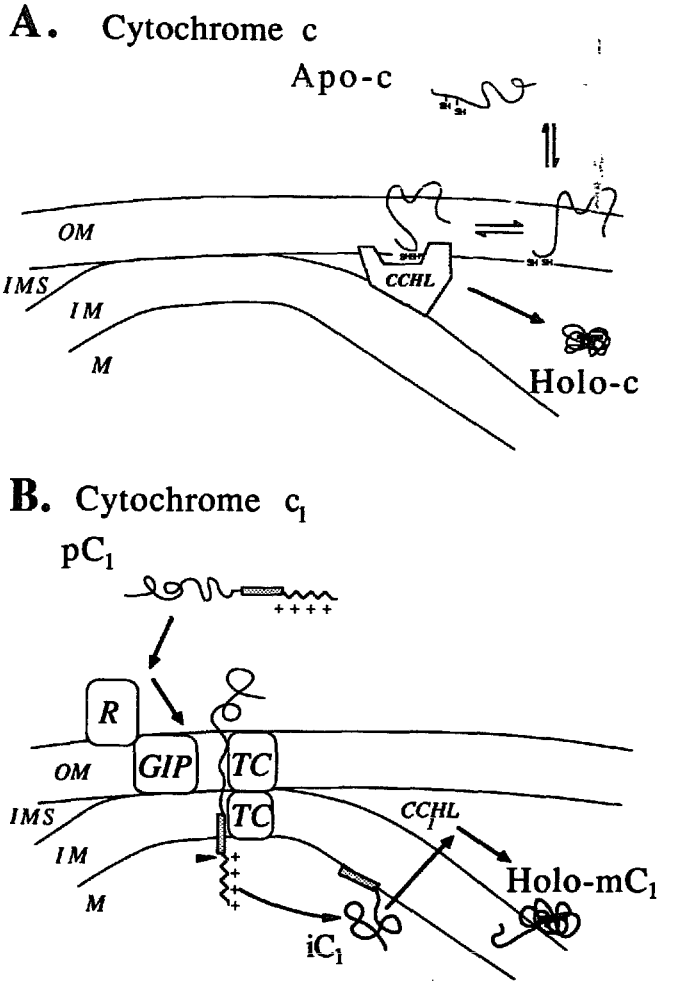

Fig 1. Hypothetical import and assembly pathways of cytochrome *c*, and cytochrome c_1. A. Apocytochrome *c* (Apo-*c*), the precursor of holocytochrome *c* (Holo-c), partially inserts into the mitochondrial outer membrane (OM) where it binds to cytochrome *c* heme lyase (CCHL), which acts as a specific binding site *in lieu* of a surface receptor. Translocation across the outer membrane directly into the intermembrane space (IMS) is believed to be driven by the folding of the holocytochrome *c* polypeptide as a result of the covalent heme addition. B. After synthesis in the cytosol, the precursor of cytochrome c_1 (pC_1) binds specifically to distinct receptor sites (R) on the mitochondrial surface. Following insertion into the outer membrane (OM), mediated by the general insertion protein (GIP), pC_1 is translocated through contact sites (translocational contact sites, Ts) into the matrix (M), in a membrane potential-dependent manner. Processing to an intermediate-size cytochrome c_1 (iC_1) occurs followed by retranslocation across the inner membrane (IM). Conversion of apocytochrome c_1, thus exposed to the intermembrane space (IMS) to its holocounterpart, is catalysed by cytochrome c_1 heme lyase (CC_1HL). The second processing event resulting in the formation of holo mature-size cytochrome c_1 (holo-mC_1) then ensues.

but not before, the second proteolytic processing of cytochrome c_1 occurs resulting in the production of mature-size holocytochrome c_1 [61, 66].

Although the import pathways of cytochrome *c* and cytochrome c_1 are markedly different, it appears that they have both evolved from a common starting point. Amino acid sequencing and X-ray crystallographic analysis of cytochromes from a diverse range of both mitochondria and bacteria have supported the notion of the endosymbiotic origin of mitochondria. Mitochondria and purple photosynthetic bacteria, such as members of the *Rhodobacter* species, are probably descendants of a common ancestor. Mitochondrial cytochrome *c* is structurally and functionally very similar to c_2-type bacterial cytochromes *eg* cytochrome c_2 of *Rhodobacter capsulata* [67]. The bacterial equivalent of mitochondrial cytochrome c_1 is also termed cytochrome c_1 (*eg* cytochrome c_1 from *R sphaeroides*) [68]. Like its mitochondrial equivalent, bacterial cytochrome c_1 is an electron transferring subunit of the ubiquinol-cytochrome *c* reductase (bc_1) complex. The bacterial cytochrome c_2 and c_1 are in many aspects similar with regard to their biogenesis. Both cytochromes are synthesized as precursor proteins containing an amino-terminal leader sequence [66, 69]. These leader sequences are characteristic of bacterial leader sequences, namely, they are relatively short (each being only 21 residues), contain either one (cytochrome c_2) or 2 (cytochrome c_1) positive charges at the extreme amino terminus, followed by a hydrophobic core rich in alanines and valine residues. The leader sequences of both cytochromes c_2 and c_1 are cleaved upon export of the proteins across the photosynthetic membrane to their functional location in the periplasm. The bacterial periplasm is the compartment corresponding to the mitochondrial intermembrane space, in that both are topologically opposed to the F_1 part of the ATP synthase. The leader sequences of cytochrome c_2 and c_1 are quite similar to the second domain of the mitochondrial cytochrome c_1 presequence at the amino acid level.

Following the endosymbiotic event, evolutionary gene transfer from the bacterial endosymbiont to the host cell nucleus occurred. Thus, the newly formed eucaryotic cell had to evolve mechanisms to return the gene products to their functional location in the mitochondrion. Rather than completely reconstruct the means by which a cytoplasmically-synthesized mitochondrial protein was imported and assembled, the import pathways are believed to have evolved so as to use evolutionary remnants of the pre-existing "ancestral assembly pathways" of the bacterial endosymbiont ("conservative sorting hypothesis") [59, 60]. During evolution, however, the respective assembly pathways for cytochrome *c* and c_1 have diverged. Whereas cytochrome c_1 import into mitochondria has retained quite a few remnants of its "ancestral" assembly pathway, cytochrome *c* import occurs by a simpler and novel mechanism. How then has cytochrome *c* escaped the conservative sorting that cytochrome c_1 must follow during import?

The membrane insertion properties of apocytochrome *c* may in part explain how the cytochrome *c* import pathway has evolved. Apocytochrome *c* can spontaneously insert into the outer mitochondrial membrane and following covalent heme addition undergoes folding-dependent translocation across the outer membrane. In contrast, because the mature part of cytochrome c_1 does not have membrane insertion activity, the receptor/GIP system is required for specific recognition and membrane insertion. Once initiated along this route, cytochrome c_1 then apparently requires transit along a conservative sorting pathway (*ie via* the matrix) in order to become properly assembled in the intermembrane space. It is postulated here, therefore, that the unique property of apocytochrome *c* to spontaneously insert into membranes confers upon the precursor the ability to circumvent the early classical steps of mitochondrial protein import. The other unique aspect of cytochrome *c* which probably enabled it to escape the conservative sorting pathway that cytochrome c_1 must follow, is the fact that the heme addition event is coupled to the translocation of cytochrome *c* across the membrane. Consequently, the translocation of cytochrome *c*, in contrast to other precursors proteins, does not rely on other exogenous sources of energy such as $\Delta\psi$ or ATP. This is in marked contrast to cytochrome c_1 where the precursor protein is imported and sorted to its final submitochondrial location, facing the intermembrane space, before the heme attachment takes place. Membrane translocation of cytochrome c_1 is not mechanistically coupled to its holo-formation.

In summary, the striking difference in the mechanisms by which cytochrome *c* and c_1 are imported into the intermembrane space probably reflects the divergence of their assembly pathways during the evolution of mitochondria. It is likely that the major factor contributing to this divergence is the difference in their respective membrane insertion properties and the difference in coupling of the membrane translocation of the protein to the heme addition event.

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