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Functional complementation analysis of yeast $bc_1$ mutants:
a study of the mitochondrial import of heterologous and hybrid proteins

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Abstract

Previous complementation studies with yeast $bc_1$ mutants, defective in subunits VII or VIII, using heterologous and hybrid subunits, suggested that the requirement for import into mitochondria might significantly restrict the scope of this test for compatible proteins. Prediction algorithms indicate that the N-terminal domain of subunit VII contains all (known) characteristics of a mitochondrial targeting signal, whereas in subunit VIII such a signal is absent from the N-terminal domain, but possibly present in an internal region of the protein. Despite the fact that the characteristics of a mitochondrial import signal are found in the N-terminus of all known subunit VII orthologues, in vitro import experiments show that the protein from human origin is not imported into yeast mitochondria. In vitro import can be restored, however, by replacement of the N-terminal part of the human protein by the N-terminus of the $S.\ cerevisiae$ orthologue, indicative of a requirement for species-specific elements.

Similar experiments were performed with subunit VIII and orthologues thereof, including a hybrid protein in which the N-terminus of the bovine heart orthologue was replaced by that of $S.\ cerevisiae$. The ability of yeast mitochondria to import this hybrid protein, in contrast to the bovine subunit VIII orthologue itself, indicates that also for subunit VIII the N-terminus, in contrast to theoretical predictions, contributes to the targeting signal, most likely via species-specific elements.

Our findings expose the limitations of the currently available criteria for prediction of the presence and location of a mitochondrial targeting sequence and bring out the necessity of performing separate import studies for the interpretation of complementation studies as long as the species-specific characteristics of the import signals have not been identified.

Introduction

For the $bc_1$ complex from $Saccharomyces\ cerevisiae$ 10 subunits have been described until now [25, 43]. Based on mutational studies and by analogy with the 3D-structure of the bovine complex, subunits VII and VIII (14 kDa and 11 kDa, respectively) are likely to be closely associated with cytochrome $b$ ([8], see also [205] and [206]) and a role in the binding of ubiquinol/ubiquinone has been suggested [76, 94, 194]. Both subunits are essential for correct assembly of the complex [129, 182]. Complementation studies, designed to analyze the functionality in yeast of orthologous subunits from other organisms, suggested that at increasing evolutionary distance the import into $S.\ cerevisiae$ mitochondria plays a crucial role and has to be further analyzed [23, 123].

Most mitochondrial proteins are encoded in the nucleus and synthesized as larger precursors in the cytoplasm. These precursor proteins contain a mitochondrial targeting signal that directs
import to the mitochondria. This signal sequence is generally located at the N-terminus of the protein, has a length of about 30 amino acids, is characterized by the presence of predominantly hydrophobic amino acids in combination with one or more positive charges and has the ability to form an amphipathic alpha-helical structure. The sequence is either present as an N-terminal extension of the mature protein, which is cleaved off upon import into the mitochondrial matrix space, or present within the mature protein. Although the location of the latter is generally assumed to be N-terminal, some examples have recently been described of mitochondrial proteins containing a targeting signal in the middle or even at the C-terminus [33, 58, 97]. In no case has a consensus been described, so that no single simple method exists for the recognition of such a signal and the definition of a general method to locate its position.

To overcome this difficulty, several indirect approaches based on known features of mitochondrial targeting signals have been applied to predict whether a targeting sequence is present, and if so, where this signal is located [37, 38, 60, 198]. We have applied several of these prediction algorithms for subunit VII and VIII of the bc\textsubscript{1} complex (complex III) of the respiratory chain from \textit{Saccharomyces cerevisiae}, as well as for several orthologues from other yeasts and higher eukaryotes. Subunit VII from \textit{S. cerevisiae} is a typical example of a protein which contains all features of an N-terminal mitochondrial targeting sequence [198]. In contrast, the N-terminus of \textit{S. cerevisiae} subunit VIII does not show these characteristics and it therefore may be speculated that it contains a mitochondrial targeting signal at another location, the region of amino acid residues 34 to 51 being the preferred candidate.

In order to be able to exclude import deficiency as the reason for lack of complementation, \textit{in vitro} import assays into \textit{S. cerevisiae} mitochondria were performed with subunit VII and subunit VIII orthologues from several species. In this way also the functionality of the targeting sequences of these orthologues was determined, allowing us to speculate about the evolutionary conservation of these mitochondrial targeting signals. In addition, hybrid proteins were constructed in order to study the location of the targeting signal.

\textbf{Materials and Methods}

\textbf{Strains and media}

\textit{Escherichia coli} strain JF 1754 (\textit{lac gal metB leuB hisB hsdR}) was used for DNA manipulations, strain BMH71-18 was used for plasmid transformation after site-directed mutagenesis and strain JM109 was used for the generation of single-stranded DNA. \textit{E. coli} transformants were grown in YT medium (1% (w/v) yeast extract, 1% (w/v) bactotryptone and 0.5% (w/v) NaCl) containing 200 \(\mu\)g/ml ampicillin. The \textit{Saccharomyces cerevisiae} strain DALU273 was made as described in [23]. Disruption of the \textit{QCR8} gene was performed as described before [129], giving DALU80 (\textit{MATa, ura3, lys2, qcr8::LEU2}). \textit{S. cerevisiae} strains DLL70 (\textit{MATa his3 ura3 qcr7::LEU2}) [77], DLL80 (\textit{MATa his3 ura3 qcr8::LEU2}) [129] and DALU80 [23] were used for the transformation of plasmids. Transformation of yeast was performed according to Klebe \textit{et al.} [104]. Transformants were selected on minimal media supplemented with histidine or lysine (20 \(\mu\)g/ml). The respiratory capacity of the transformants was checked on solid
media containing 1% (w/v) yeast extract, 1% (w/v) bactopeptone, 2% (w/v) glycerol and 2% (w/v) ethanol (EG medium). Mitochondria used for the in vitro import experiments were isolated from Saccharomyces cerevisiae strain YPH501 (MATa/α ade2-101/ade2-101 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 ura3-52/ura3-52 trp1-Δ63/trp1-Δ63 lys2-801/lys2-801) [184].

Construction of heterologous and hybrid proteins

An 840 bp HindIII-SalI fragment carrying the QCR8 gene was cloned from plasmid pUC18-HUS [76] into the multiple cloning site of the centromeric E. coli/S. cerevisiae shuttle vector pRS313 [184] to create pRS/Sc8. NcoI restriction sites were created at the respective ATG start codons and ClaI sites directly downstream of the S. cerevisiae QCR8 gene and its orthologue from bovine heart (if not already present). By exchanging the NcoI-ClaI fragment (the ClaI site is located 27 bp after the stop codon), a clone was made in which the bovine heart gene is flanked by S. cerevisiae sequences (named pRS/bh8).

The S. cerevisiae-bovine heart hybrid gene was constructed using the NdeI site in S. cerevisiae QCR8 at nucleotide position 77 (with respect to the ATG) and by introducing an NdeI site in the bovine heart gene at position 47 (with respect to the ATG). The NdeI-ClaI fragment from pRS/Sc8 was subsequently replaced by the NdeI-ClaI fragment from the bovine heart gene giving pRS/Sc-bh8 (residues 1-26 from S. cerevisiae subunit VIII fused to residues 16-81 from bovine heart subunit VIII). By exchanging the NcoI-ClaI fragment from S. cerevisiae with the orthologous Neurospora crassa genes encoding the precursor and mature protein, clones were made in which the N. crassa genes are flanked by S. cerevisiae sequences (named pRS/Nc8-1 and pRS/Nc8-2, respectively).

A KpnI-XbaI fragment carrying the S. cerevisiae QCR7 gene was cloned into the multiple cloning site of pSelect (Promega, Madison, USA). Site directed mutagenesis by pSelect Altered Sites (Promega) was performed to create an NcoI site at the first codon and a BamHI site 29 nucleotides downstream of the S. cerevisiae QCR7. A final construct was cloned into the centromeric E. coli/S. cerevisiae shuttle vector pRS316 [184]. From this construct it was possible to substitute the NeoI-BamHI fragment carrying the S. cerevisiae QCR7 by its orthologous genes from potato and man, keeping the S. cerevisiae promoter and terminator sequences intact, resulting in the constructs named pRS/pot7 and pRS/hum7, respectively.

The two hybrid genes were constructed by fusing 111 nucleotides (corresponding to the N-terminal 37 amino acid residues) from the S. cerevisiae QCR7 gene to the genes from man or potato from which the first 78 and 84 nucleotides (corresponding with the N-terminal 26 and 28 amino acid residues) had been removed, which resulted in the constructs named pRS/Sc-hum7 and pRS/Sc-pot7, respectively.

Isolation of mitochondria and gel electrophoresis

Mitochondria were isolated as described in Glick et al. [65]. Protein concentrations were determined according to Bradford [24]. Mitochondrial proteins were separated on denaturing polyacrylamide gels [116] which were stained with Coomassie Brilliant Blue to visualize marker proteins. After staining the polyacrylamide gels were dried and subjected to autoradiography.

Import of proteins into isolated yeast mitochondria

After in vitro transcription with T7 or SP6 RNA polymerase, proteins were in vitro synthesized in rabbit reticulocyte lysates in the presence of 35S-methionine. In vitro import into isolated yeast mitochondria was performed in the presence of 2 mM ATP, 2 mM NADH and bovine serum albumin containing buffer as described by Sollner et al. [189]. Where indicated, the mitochondria were incubated with 200 μg/ml proteinase K for 30 min. at 30°C. The mitochondria were reisolated, washed, and the proteins were separated on denaturing polyacrylamide gels. For dissipation of the membrane potential ΔΨ, 0.5 μM valinomycin, 8 μM antimycin A and 20 μM oligomycin were included during the import reaction [151].

Chemicals

Proteinase K was purchased from Boehringer, Mannheim, Germany. Restriction and other enzymes were purchased from Biolabs or Gibco.
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Results

In vitro import of heterologous and hybrid proteins

To establish whether the lack of functionality of some orthologues of subunits VII and VIII in yeast [23, 123] was possibly due to a deficiency in the import of these orthologues into yeast mitochondria, we investigated whether the import apparatus of *Saccharomyces cerevisiae* mitochondria is able to import subunit VII and subunit VIII orthologues from other organisms, using an *in vitro* import assay with isolated *S. cerevisiae* mitochondria. Control experiments confirmed that the subunits VII and VIII of the *bc*₁ complex from *S. cerevisiae* were imported only in the presence of a membrane potential across the inner membrane ([59, 172, 173, 207], Figure 1a and 1b). Of the two orthologues that were not able to complement yeast strains deficient in subunit VII of the *bc*₁ complex (see Figure 2 for the sequences), the orthologue from potato could be imported into isolated yeast mitochondria, as indicated by the presence of this protein in a location where it is protected against externally added protease (Figure 1a, lane 2). In contrast, the human subunit VII orthologue could not be imported (Figure 1a, lane 2).

In order to determine which domain of subunit VII is essential for import, hybrid proteins were constructed. Since the N-terminus of subunit VII from *S. cerevisiae* shows all features of a typical N-terminal mitochondrial targeting sequence (Table 1), the N-terminal 26 amino acid residues of the human orthologue were replaced by the N-terminal domain (residues 1-37) of the *S. cerevisiae* orthologue. Figure 1a shows that this hybrid protein could be imported into isolated yeast mitochondria in a Δψ-dependent manner and was translocated to a location where it was protected against externally added protease. As a control, the N-terminus (residues 1-28) of the potato subunit VII orthologue was also substituted by the N-terminal domain (residues 1-37) of the yeast orthologue and also this hybrid protein could be imported in an *in vitro* import system. The N-terminus of the human orthologue was not replaced with that of the potato subunit.

Of the orthologues of subunit VIII from *S. cerevisiae*, again the bovine orthologue was not imported into isolated yeast mitochondria [23]. Substitution of the N-terminus (residues 1-16) of the bovine orthologue with that from *S. cerevisiae* subunit VIII (residues 1-26) resulted also in this case in a hybrid protein which could be imported into mitochondria *in vitro* (Figure 1b, lane 2), implying that the N-terminus of subunit VIII from *S. cerevisiae* contains at least part of the targeting signal. The import of other heterologous proteins into yeast mitochondria was also monitored. The subunit VIII orthologue from potato could not be imported into yeast mitochondria (Figure 1b, lane 2). This orthologue was not used for further study and no hybrid protein was constructed.
Table 1. Relations between mitochondrial targeting sequence characteristics, functional complementation analysis and in vitro import experiments with subunit VII and VIII proteins from S. cerevisiae and orthologues. \( \mu H \) = hydrophobic moment for indicated segment; segment = segment of 18 amino acid residues; \( H_{\text{max}} \) = maximal hydrophobicity; surface = surface-seeking capacity; KR/DE = number of lysine+arginine versus number of asparagine+glutamic acid residues; functional = functional complementation analysis of either qcr7 or qcr8 null mutants by subunit VII or subunit VIII heterologous and hybrid proteins, respectively; import = in vitro import into isolated S. cerevisiae mitochondria. Values for \( \mu H \), segment and \( H_{\text{max}} \) were obtained according to prediction algorithms described by [37, 38]. The calculated value for \( H_{\text{max}} \) of S. cerevisiae subunit VII deviates from the value previously calculated by Eisenberg et al. [53], due to the fact that we have taken into account that the first methionine of subunit VII is processed by a methionine aminopeptidase (MAP) present in the cytosol [28, 35], before translocation of the protein into the mitochondria occurs.

Recently, the gene encoding the subunit VIII orthologue from Neurospora crassa was isolated [124]. Surprisingly, in vitro import assays showed that the N-terminal eight amino acid residues of this 11.8 kDa protein are processed upon import into isolated N. crassa mitochondria, resulting in a 10.8 kDa mature protein [124]. The N. crassa protein is the only subunit VIII orthologue known to be synthesized as a larger preprotein and processed upon import into the mitochondria. It was additionally shown that the 10.8 kDa mature protein lacking the N-terminal extension could also be imported into N. crassa mitochondria using an in vitro system, indicating that the extension is not essential for import [124].

In vitro import studies now showed that the 11.8 kDa preprotein from N. crassa could be imported into isolated S. cerevisiae mitochondria as well. The in vitro import of this heterologous protein into S. cerevisiae mitochondria strongly suggests that this protein is also processed into the mature 10.8 kDa protein, even though the S. cerevisiae subunit VIII protein
itself is not processed. The 10.8 kDa *N. crassa* subunit VIII orthologue was also obtained by *in vitro* synthesis and could also be imported into *S. cerevisiae* mitochondria, again confirming that the extension is not essential for import. Due to cloning strategies both *N. crassa* proteins used for import studies have an altered amino acid residue at the second position when compared with the original *N. crassa* subunit VIII orthologue [124] (Figure 2), but this does not seem to have any effect on the import of these *N. crassa* proteins into either *N. crassa* or *S. cerevisiae* mitochondria. The *in vitro* import experiments show that the import of these proteins into *S. cerevisiae* mitochondria is inefficient, but it must be noted that *in vitro* import experiments of these *N. crassa* subunit VIII proteins into *N. crassa* mitochondria are also highly inefficient [124].

![Image](image_url)

**Figure 1.** *In vitro* import of heterologous and hybrid subunit VII (Figure 1A) and subunit VIII (Figure 1B) proteins into isolated yeast mitochondria. All proteins were *in vitro* synthesized in rabbit reticulocyte lysate in the presence of $^{35}$S-methionine. Isolated yeast mitochondria were incubated with the different rabbit reticulocyte lysates for 30 min. at 30°C, either in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of a membrane potential $\Delta\psi$. Subsequently, the mitochondria of samples in lanes 2 and 4 were treated with 200 $\mu$g/ml proteinase K and reisolated by centrifugation. The imported proteins were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. Sc7 = *S. cerevisiae* subunit VII, hum7 = human subunit VII, pot7 = potato subunit VII, Sc-hum7 = *S. cerevisiae*-human subunit VII hybrid protein, Sc-pot7 = *S. cerevisiae*-potato subunit VII hybrid protein. Sc8 = *S. cerevisiae* subunit VIII, pot8 = potato subunit VIII, Nc8-1 = *N. crassa* subunit VIII, Nc8-2 = *N. crassa* subunit VIII without N-terminal extension, bh8 = bovine heart subunit VIII, Sc-bh8 = *S. cerevisiae*-bovine heart subunit VIII hybrid protein.
Computational prediction of mitochondrial targeting sequences

Extensive analysis of the sequences of mitochondrial proteins has shown that their targeting sequences share several common features, including the presence of positively charged amino acids and the tendency to form amphipathic alpha-helices. Several calculation methods and computational programs have been developed [37, 38, 60] which use these criteria to predict whether the protein will be imported into the mitochondria.

Statistical studies [198] indicate that mitochondrial targeting sequences share a high amphiphilicity, or asymmetry of hydrophobicity as quantified using Eisenberg's hydrophobic moment $\mu H$ [52, 53]. However, the presence of a mitochondrial targeting sequence cannot be predicted on the basis of a high $\mu H$ value alone. In addition to a high $\mu H$ value, the segment of maximal hydrophobic moment must be free of acidic residues and contain at least two K or R residues; putative mitochondrial targeting sequences should have a $\mu H \geq 7.3$ (sometimes $\mu H > 5.0$ is sufficient) [166, 198]. $H_{\text{max}}$ indicates the maximal hydrophobicity calculated over a defined segment of the protein sequence. In prediction programs, the scanning window which is used to define this segment is generally 18 amino acid residues, corresponding to exactly 5 helical turns and approaching the length of the shortest mitochondrial targeting signals identified [37]. Good mitochondrial targeting sequences should have a $H_{\text{max}} \geq 4.5$ [37]. The surface-seeking capacity of a protein is also used to determine the presence of a mitochondrial targeting sequence. A protein, or a segment thereof, can have a surface-seeking capacity which is related to its high amphiphilicity when the peptide is coiled as an $\alpha$-helix. The presence of a large helical hydrophobic moment results in a high surface-seeking capacity of a protein [52].

We have used the calculation methods of Eisenberg et al. [53], von Heijne [198] and Claros et al. [37, 38] to determine the presence and location of the mitochondrial targeting signal in the protein sequence of subunit VII and VIII from S. cerevisiae and of their orthologues from potato, N.crassa, bovine heart and man. These results are compared with those of the in vitro import assays with these proteins.

The results of the calculations presented in Table 1 show high scores for $\mu H$, $H_{\text{max}}$ and the surface-seeking capacity for the N-terminal domain of subunit VII from S. cerevisiae and conserved orthologues, whereas these scores are significantly lower for almost all subunit VIII orthologues, except for the potato and bovine subunit. Comparison of these results with those of the in vitro import experiments shows that the predictions of the computational programs do not correspond with the in vitro import assays in all cases.

According to the prediction algorithms, subunit VIII from S. cerevisiae does not contain an N-terminal targeting signal, but instead the programs predict an internal targeting signal near the center of the protein sequence of subunit VIII (amino acid residues 34 to 51). The in vitro import studies indicate that additional features are important for the import of proteins into mitochondria, which are not taken into account by the prediction algorithms.
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Figure 2. Multiple sequence alignment of the subunit VII (Figure 2a) and subunit VIII (Figure 2b) orthologues. sc7 = S. cerevisiae subunit VII [42]; hum7 = human subunit VII [190]; pot7 = potato subunit VII (S. tuberosum) [26]; sc8 = S. cerevisiae subunit VIII [129]; pot8 = potato subunit VIII (S. tuberosum) [27]; bov8 = bovine heart subunit VIII (B. taurus) [21]; nc8-1 = N. crassa subunit VIII [124]; nc8-2 = N. crassa subunit VIII without N-terminal extension [124].

Sequences were aligned using the GCG PileUp program (gap weight 3.00; gap length weight 0.10) [56]. Conserved amino acids are indicated by a black box, similar amino acids are indicated by a grey box. Gaps are indicated (.)

**Functional complementation analysis**

The *in vitro* import experiments of the heterologous and hybrid subunit VII and VIII proteins were compared with the ability of these proteins to function *in vivo*, by analyzing the extent of functional complementation of the corresponding *S. cerevisiae* null mutants. Disruption of the *QCR7* or *QCR8* gene results in strains that lack a functional *bc1* complex, thereby preventing growth on non-fermentable carbon sources such as ethanol/glycerol (EG) [129, 182].

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Heterologous and hybrid subunit VII and VIII genes were placed under the control of the S. cerevisiae QCR7 or QCR8 promoter respectively, and cloned in the centromeric shuttle vector pRS316 [184] (see Materials and Methods). When a qcr7 null mutant is transformed with a single copy plasmid carrying the wild type QCR7 gene from S. cerevisiae, the growth deficiency on EG medium is restored, whereas plasmids containing either the human, the potato or the hybrid genes do not restore this growth phenotype ([182]; see Table 1). Since the subunit VII orthologue from potato and the S. cerevisiae-human and the S. cerevisiae-potato hybrid proteins can be imported into yeast mitochondria in an in vitro system, lack of functional complementation is probably due to incorrect assembly or functionality of the bc1 complex, rather than inefficient import of the heterologous or hybrid subunit VII proteins.

Transforming a qcr8 null mutant with a single copy plasmid carrying the wild type QCR8 gene from S. cerevisiae results in a restored growth phenotype on EG medium ([129]; see Table 1). Plasmids containing either the bovine or the potato orthologue are unable to restore the growth deficiency and the plasmid containing the yeast-bovine hybrid orthologue only very slightly restored the growth deficiency, as has been shown previously [23]. Lack of functional complementation by the orthologues from bovine heart and potato is most likely caused by a lack of import into mitochondria, in agreement with the results of the in vitro import assays. The plasmid encoding the mature N. crassa orthologue is not able to restore the growth deficiency of the qcr8 null mutant, while partial complementation can be obtained by the plasmid encoding the precursor form of this orthologue [124]. Both the precursor protein and the mature protein lacking the N-terminal eight amino acids can be imported in vitro, although with a very low efficiency.

Discussion

Recently, the complete genome of the yeast Saccharomyces cerevisiae has been sequenced [51] and the information concerning the DNA and protein sequences of all genes is available in databases. Of many proteins it is not known what their function is and which organelle they reside in. To determine the cellular localization of such proteins, programs as PSORT [141] have been developed, which provides a prediction of probable localization sites based on an analysis of various sequence features related to protein sorting signals. Better definition of the characteristics of mitochondrial targeting signals or definition of a consensus sequence will help on the formulation of predictive approaches to localize such a targeting signal in a protein sequence and this will benefit the specificity of programs (e.g. PSORT) that predict cellular localization of proteins.

Computational programs designed to localize a mitochondrial targeting signal in a protein sequence are necessarily based on experimentally defined characteristics of such a signal and
our data show that more elements are involved in the definition of a targeting signal than those so far known. Not only are additional species-specific elements important for import, as is also shown by the studies of Tanudji et al. [191], but also fully unknown elements, as is illustrated by the finding that replacement of the N-terminus of bovine subunit VIII with that of the yeast orthologue results in a hybrid protein that can be imported into yeast mitochondria, although the N-terminus does not contain any as yet identifiable targeting sequence. 

The computational programs predict that the N-terminus of subunit VII from *S. cerevisiae* as well as its orthologues from potato and man contains all (known) features of such a signal. In *vitro* import assays show that the orthologue from potato can be imported into yeast mitochondria, indicating that its mitochondrial targeting signal is functional in yeast, implying an evolutionary conservation of the targeting signal of subunit VII between yeast and potato. However, the human orthologue cannot be imported by the import apparatus from yeast, unless the N-terminal 26 amino acid residues are replaced by the first 37 amino acid residues from the *S. cerevisiae* orthologue, thereby confirming that the N-terminus of this protein contains the mitochondrial targeting signal but at the same time demonstrating species-specificity in one or more steps of the import process. Alignment of the amino acid sequences of the N-terminal domains shows a higher identity between *S. cerevisiae* and potato than between *S. cerevisiae* and man. The differences found in the *in vitro* import experiments with the potato and human orthologues imply a correlation between the evolutionary divergence of the import apparatus and the ability of the yeast mitochondrial import machinery to recognize the targeting signals to import orthologous proteins from other species. 

Recently, Tanudji et al. [191] reported some species specificity of import, although less than that found for processing. These authors found that deletion of the N-terminal 17 amino acids from the cleavable presequence of the soybean alternative oxidase resulted in almost full disappearance of import into soybean mitochondria, whereas import into rat liver mitochondria was inhibited by only 50%. The residual α-helical amphiphilic element is apparently sufficient for import into rat liver mitochondria, but not for import into soybean mitochondria. Subunit VII of the yeast *bc*₁ complex does not have a cleavable presequence, but also for this protein Malaney et al. [130] have reported that the N-terminal 20 amino acid residues are not essential for import into the mitochondria *in vivo*. At first sight this result may appear to conflict with the data presented here, the N-terminal domain of *S. cerevisiae* subunit VII providing the mitochondrial targeting signal to non-imported heterologous subunits. Assuming that the heterologous or hybrid proteins that are not imported into mitochondria in an *in vitro* system (our studies), are also not imported in an *in vivo* system (Malaney et al.), the most probable explanation is that Malaney *et al.* deleted maximally 20 amino acids, while in our studies an N-terminus of 37 amino acids is used, the residual α-helical amphiphilic element after deletion of 20 amino acids residues being enough to support import. Although according to prediction
algorithms the mitochondrial targeting signal of subunit VII is located in the N-terminal segment comprising amino acid residues 1-18, increasing the scanning window from 18 to 40 amino acid residues shows high H_max and µH scores for the whole segment from residues 1-40 (data not shown). This implies that the complete domain consisting of residues 1-40 may contribute to a functional mitochondrial targeting signal and Malaney et al. indeed found that increasing deletions resulted in decreased levels of the mutant protein in the mitochondria, but apparently more than 20 amino acids have to be deleted for full inhibition of import, which is in agreement with our observation that the N-terminal 37 amino acids of subunit VII contains a mitochondrial targeting signal when studied in in vitro import experiments.

Functional complementation analysis shows that the subunit VII orthologues from potato, man and the corresponding hybrid proteins cannot restore the respiratory-deficient phenotype of an S. cerevisiae qcr7 null mutant in vivo, implying either that these proteins are not functional in yeast, or that the bc1 complex is not assembled correctly. Since it has been shown that the orthologue from potato is imported into yeast mitochondria in an in vitro system, this lack of complementation is most likely due to instability or incorrect assembly or functionality of the hybrid bc1 complex and not a result of inefficient import. Lack of complementation of the potato subunit VII is in agreement with the finding that several single point mutations in the QCR7 gene from S. cerevisiae already lead to a mutant with a respiratory negative phenotype, indicating that only a minor change in the subunit VII protein already gives rise to a bc1 complex that is no longer functional ([123] and H.B., unpublished results). In contrast, a large number of subunit VIII mutants is fully or partially respiratory active [31, 78, 125] and orthologues of subunit VIII from various organisms show at least partial complementation of a qcr8 null mutant [23, 124].

Subunit VII orthologues from organisms much closer related to S. cerevisiae, such as Kluyeromyces lactis [140] and Candida utilis [22], are able to complement a qcr7 null mutant, indicating a correlation between evolutionary distance from S. cerevisiae and the ability of functional complementation. Since this distance is much larger between S. cerevisiae and man, the evolutionary divergence of the import apparatus of these organisms may account for the lack of complementation of the qcr7 null mutant and for the lack of in vitro import into S. cerevisiae mitochondria of the human orthologue of subunit VII.

The N-terminal domains of subunit VIII from S. cerevisiae and N. crassa are not predicted to contain an N-terminal mitochondrial targeting sequence, unlike those of the potato and bovine heart orthologues. However, in vitro import experiments show that the N. crassa orthologue can be imported into yeast mitochondria, whereas the orthologues from potato and bovine cannot. The in vitro import experiments with the potato orthologues show a large difference between subunit VII and subunit VIII. Furthermore, the N-terminal domains of both subunit VII and VIII orthologues from potato contain a mitochondrial targeting signal, but only the subunit VII
orthologue can be imported into yeast mitochondria, implying either a difference in the stringency of recognition of the targeting signal by the yeast import apparatus, or an evolutionary divergence of the import apparatus in such a way that the targeting signal of subunit VII can, but that of subunit VIII cannot be recognized by the yeast import apparatus. This may be explained by the observation that the mitochondrial targeting signal of potato subunit VII displays a higher amount of lysine and arginine residues (KR/DE=3/1) than that from *S. cerevisiae* (KR/DE=2/1), thereby compensating for a decreased import efficiency due to evolutionary divergence of the potato targeting signal. Potato subunit VIII, however, displays a lower amount of these positively charged residues (KR/DE=2/0) compared with *S. cerevisiae* (KR/DE=3/0) and this may contribute to the lack of import into yeast mitochondria.

Functional complementation analysis shows that the heterologous subunit VIII proteins from bovine heart, potato and *N. crassa* are not able to complement [23, 123, 124] the respiratory-deficient phenotype of a qcr8 null mutant. For the bovine and potato orthologues this lack of complementation can be directly linked to the inefficient import of these proteins in yeast. The finding that the mature protein from *N. crassa* does not complement, while the preprotein does, indicates that the presequence is indeed required for correct sorting, as is proposed previously [124]. The sorting signal, apparently, is specifically required for the *N. crassa* subunit, whether it has to function in yeast or in *N. crassa* itself. An *S. cerevisiae*-bovine hybrid protein, in which the N-terminus of the bovine heart subunit VIII orthologue is replaced by that of *S. cerevisiae*, can be imported into isolated yeast mitochondria. The ability of this hybrid protein to partially complement a qcr8 null strain indicates that it can also be imported into yeast mitochondria *in vivo*. This implies, although not identified by prediction theorems, that the N-terminus of subunit VIII from *S. cerevisiae* contains at least part of a mitochondrial targeting signal, which enables this protein to be imported into the mitochondria. Although the computational programs predict a targeting signal at the center of the protein sequence, the currently used parameters did not identify an N-terminal targeting signal.

Our results expose the limitations of currently used criteria to predict the presence and location of a mitochondrial targeting signal and that in some cases targeting signals are not recognized as such and in other cases additional species-specific features are involved in the import of proteins into mitochondria.

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