Oxa1p mediates the export of the N- and C-termini of pCoxII from the mitochondrial matrix to the intermembrane space

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Abstract Oxa1p is a mitochondrial protein reported to be involved in the assembly of the cytochrome oxidase complex. In the absence of a functional Oxa1p, subunit II of the cytochrome oxidase accumulates as its precursor form (pCoxII). Using mitochondria isolated from a yeast strain bearing a temperature sensitive mutation in the Oxa1p, *pet ts1402*, we have analyzed the function of the Oxa1p protein. We demonstrate that the accumulation of pCoxII in the *pet ts1402* mitochondria does not reflect a compromised Imp1p activity in this mutant. Furthermore, measurement of the membrane potential has shown it to be sufficient to support the export of CoxII from the matrix. Rather, we found that newly synthesized pCoxII accumulates in the matrix of the *pet ts1402* mitochondria, because export across the inner membrane is inhibited in the *pet ts1402* mitochondria. In conclusion, Oxa1p mediates the export of the N- and C-termini of the mitochondrially encoded subunit II of cytochrome oxidase from the matrix to the intermembrane space.

Key words: Mitochondrial protein Oxa1p; Cytochrome oxidase complex assembly

1. Introduction

In the yeast *Saccharomyces cerevisiae* only eight proteins are encoded by the mitochondrial genome. One of these proteins, var1, is a soluble protein, the remaining seven are integral inner membrane proteins. The membrane proteins are subunits of respiratory chain complexes, cytochrome *b* of the cytochrome bc_1 complex, subunits I, II and III of the cytochrome oxidase complex (CoxI, CoxII and CoxIII) and subunits 6, 8 and 9 of the F_1F_o-ATPase complex (ATPase 6, ATPase 8 and ATPase 9) [1–3]. These proteins are made on mitochondrial ribosomes and are inserted into the inner membrane in a manner that requires the complete translocation of hydrophilic charged segments across the lipid bilayer to the intermembrane space [4,5].

We have previously characterized the process of membrane insertion of mitochondrially encoded proteins using CoxII as a model protein [6]. CoxII contains two transmembrane segments and is synthesized as a precursor protein, pCoxII, in the mitochondrial matrix. PCoxII bears an N-terminal presequence which undergoes proteolytic processing by the Imp1p protease [7,8] located on the intermembrane space side of the inner membrane. Synthesis of pCoxII is not obligatorily coupled to its translocation across the inner membrane, an observation which enabled an experimental dissection of the export events [6]. PCoxII undergoes insertion into and across the inner membrane which results in the complete translocation of both the N- and C-termini across the membrane into the intermembrane space (N_{out}-C_{out} topology). The translocation of both N- and C-termini was found to be supported by a membrane potential. In the absence of a membrane potential pCoxII accumulated in the mitochondrial matrix [6].

As this export event involves the translocation of highly charged domains of CoxII, it was speculated that it may be mediated by a specific channel in the inner membrane [5,6]. A possible candidate for such a translocation machinery was recently described, the OXA1/pet 1402 gene product [9,10]. Oxa1p is a polytopic protein of the mitochondrial inner membrane [11–13], and is required for the assembly of cytochrome oxidase and F_1F_o-ATPase complexes [9,10,13–15]. Deletion of the OXA1 gene or mutations in it result in the accumulation of uncleaved pCoxII [9,10]. The accumulation of pCoxII in the absence of a functional Oxa1p could indicate one of three possible defects, (i) the process of export of at least the N-terminal tail (and maybe also the C-terminus) of pCoxII is directly inhibited, (ii) the maturation of exported pCoxII by the Imp1p protease is defective, or finally, (iii) in the absence of a functional Oxa1p, a membrane potential sufficient to support the export of the termini of pCoxII is lacking. The last possibility is not trivial, as deletion of the OXA1 has been reported to have major deleterious consequences for the assembly of both the cytochrome oxidase complex and the F_1F_o-ATPase [13–15].

Using a yeast strain harboring a temperature sensitive mutation in the Oxa1p, *pet ts1402* [9], we demonstrate here that Oxa1p plays an important role in mediating the export of the N- and C-termini of pCoxII to the intermembrane space following its synthesis in the mitochondrial matrix.

2. Materials and methods

2.1. Yeast strains and mitochondria isolation

Yeast strains used in this study were wild-type D273-10B (ATCC 24657); the temperature sensitive mutant of the OXA1 gene product, termed *pet ts1402*, and its isogenic wild-type (Sc167) [9] and Δ*sco1* (GR20) [16] and Δ*coxIV* [6]. The wild-type yeast D273-10B was grown on lactate medium at 30°C, Sc167 and *pet ts1402* at 24°C. The Δ*sco1* and Δ*coxIV* yeast strains were grown on YP medium supplemented with 2% galactose and 0.5% lactate. All cells were harvested at an OD_{578} of about 1.5. Mitochondria were isolated as previously described [11] with the exception that zymolyase treatment was performed at 24°C in the case of the *pet ts1402* mutant and its isogenic wild-type.

2.2. Labeling of mitochondrial translation products

In vitro labeling of mitochondrial translation products was performed in isolated mitochondria, as described previously [6,17]. In order to ascertain the localization of the newly synthesized proteins, mitochondria were converted to mitoplasts by hypotonic swelling pri-

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or to initiation of translation, as described previously [6]. Following translation mitochondria (or mitoplasts) were reisolated and washed once and either lysed directly in SDS-sample buffer or treated with proteinase K (100 μg/ml), as indicated. Proteins were separated by SDS-PAGE and visualized by fluorography [6].

2.3. Import of precytochrome b_2 into isolated mitochondria

Precytochrome b_2 was synthesized in the presence of [^{35}S]methionine by coupled transcription/translation in rabbit reticulocyte lysate (Promega Corp.) as described before [18]. Import of radiolabeled precytochrome b_2 into isolated mitochondria was carried out as described before [18]. Following the import incubation, protease treatment and mitoplasting were performed according to published methods [19].

2.4. Assessment of the mitochondrial inner membrane potential

The $\Delta\psi$ of isolated yeast mitochondria was assessed by recording the fluorescence decrease (quenching) of the voltage sensitive dye 3,3'-dipropylthiodicarbocyanine iodide ($DiSC_3(5)$; Molecular Probes) as previously described [20]. The fluorescence value was set at 100 arbitrary fluorescence units, and mitochondria (100 μg protein) were injected into the cuvette to start the measurement. NADH (2 mM) was also present. The membrane potential across the inner membrane of mitochondria was dissipated by the addition of valinomycin (1 μM final concentration). The difference between the fluorescences prior to and after the addition of valinomycin represents a relative assessment of the membrane potential.

2.5. Miscellaneous

Following labeling of mitochondrial translation products, samples were analyzed by SDS-PAGE [21] and fluorography, data were quantified by densitometry (Ultroscan XL, Pharmacia). Protein determination [22], immunoblotting [23] and immunostaining using the ECL chemiluminescence detection system (Amersham) were performed essentially as previously described [11].

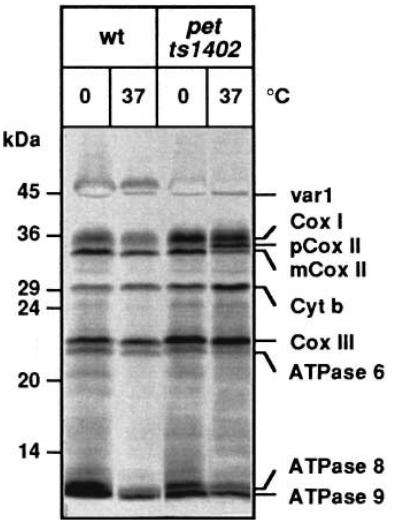

Fig. 1. The phenotype of the *pet ts1402* is inducible in vitro. Mitochondria from wild-type (wt) and *pet ts1402* were preincubated in translation buffer at 0°C or at 37°C for 10 min. In organello translation was then performed in both samples for 20 min at 25°C in the presence of [^{35}S]methionine. The mitochondria were directly analyzed by SDS-PAGE and the resulting fluorograph is presented. Abbreviations: CoxI, CoxII and CoxIII, subunits I, II and III of the cytochrome oxidase complex; Cyt *b*, cytochrome *b*; ATPase 6, ATPase 8 and ATPase 9, subunits 6, 8 and 9 of the F_o-ATPase; p and m, precursor and mature forms of CoxII.

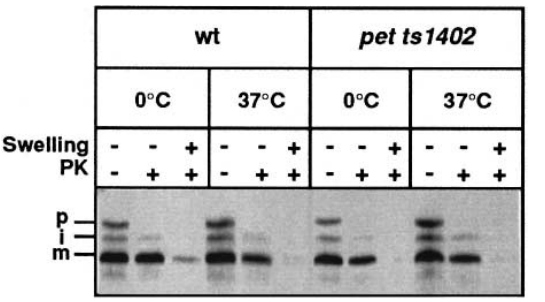

Fig. 2. Precytochrome b_2 is matured by Imp1p and is correctly sorted in the *pet ts1402* mitochondria. Mitochondria from wild-type (wt) and *pet ts1402* were preincubated in import buffer at 0°C or at 37°C for 10 min. Reticulocyte lysate containing radiolabeled precytochrome b_2 was added and import was performed for 30 min at 25°C. After import, mitochondria were re-isolated and either mock-treated or proteinase K (PK) treated (50 μg/ml) under non-swelling or swelling conditions, as indicated. All samples were analyzed by SDS-PAGE, blotted onto nitrocellulose and the resulting radiograph is presented. Immunodecoration of the intermembrane space marker protein, cytochrome *c* peroxidase and the matrix marker protein, Mge1p, was performed to assess the efficiency of the swelling procedure. Swelling was >85% efficient. p, precursor; i, MPP processed intermediate; m, Imp1p processed mature forms of cytochrome b_2.

3. Results

3.1. The temperature sensitive phenotype of pet ts1402 can be induced in vitro with isolated mitochondria

Yeast cells harboring a temperature sensitive mutation of Oxa1p, *pet ts1402*, accumulate the precursor form of the mitochondrially encoded subunit II (pCoxII) of the cytochrome oxidase complex at non-permissive temperatures in vivo [9]. This temperature sensitive phenotype of the *pet ts1402* can be induced in vitro with isolated mitochondria also (Fig. 1). Mitochondria were isolated from the *pet ts1402* yeast strain which had been grown at the permissive temperature of 24°C. Protein synthesis in the presence of [^{35}S]methionine was performed in isolated mitochondria from *pet ts1402* and its isogenic wild-type and maturation of pCoxII was analyzed. When synthesized in mitochondria isolated from wild-type or *pet ts1402* which had been kept at the permissive temperature, pCoxII was efficiently matured by the Imp1p (Fig. 1). In contrast, when the isolated *pet ts1402* mitochondria were exposed to the non-permissive temperature of 37°C prior to translation, newly synthesized CoxII accumulated largely as pCoxII (Fig. 1). Prior exposure to this higher temperature did not have an adverse effect on the maturation of CoxII synthesized in the control wild-type mitochondria.

3.2. Precytochrome b_2 is matured by the Imp1p protease following import into the pet ts1402 mitochondria

In order to exclude the possibility that the processing by Imp1p protease is defective in the *pet ts1402* mitochondria, the import and maturation of precytochrome b_2 were analyzed. Precytochrome b_2 is synthesized as a precursor protein containing an N-terminal cleavable bipartite sequence which is processed initially by the mitochondrial processing peptidase and matured by the Imp1p protease [7,8]. Radiolabeled precytochrome b_2 was synthesized in a reticulocyte lysate and imported into isolated wild-type and *pet ts1402* mitochondria (Fig. 2). Both processing by Imp1p to its mature size form and sorting to the intermembrane space occurred in the *pet ts1402* mitochondria with similar efficiencies as in the wild-

type mitochondria. Induction of the *pet ts1402* phenotype by prior incubation of the mitochondria to the non-permissive temperature of 37°C had no adverse effect on the Imp1p processing event (Fig. 2). We conclude therefore that the activity of the Imp1p protease was not compromised in the *pet ts1402* mitochondria. We can thus exclude that accumulation of the precursor form of CoxII in these mitochondria is directly due to a non-functional Imp1p protease.

3.3. *Export of pCoxII is inhibited in the pet ts1402 mitochondria*

In order to study the role of Oxa1p in the sorting of pCoxII, we analyzed the topology of newly synthesized CoxII in *pet ts1402* mitochondria by treating mitoplasts derived from the mitochondria with protease. The pCoxII accumulated in the *pet ts1402* mitochondria was localized in the mitochondrial matrix, in contrast to CoxII in wild-type mitochondria where it was correctly sorted, as indicated by both its Imp1p processing and accessibility to protease upon opening of the outer membrane (Fig. 3). Thus export of both the N- and C-terminus of pCoxII are strongly inhibited when the function of the Oxa1p is impaired.

3.4. *Measurement of membrane potential in the pet ts1402 mitochondria*

The export from the matrix of the N- and C-terminal tails of pCoxII requires the presence of a membrane potential [6]. It was therefore necessary to make sure that the inhibition of export of pCoxII observed in the *pet ts1402* mitochondria reflected a direct requirement for Oxa1p rather than a possible indirect effect of maybe a reduced membrane potential. The membrane potential in the *pet ts1402* mitochondria was measured and found to be only slightly reduced in comparison to that of wild-type mitochondria; it was higher than that of other mutant mitochondria bearing a defective COX complex, for example mitochondria isolated from the Δ*coxIV* and

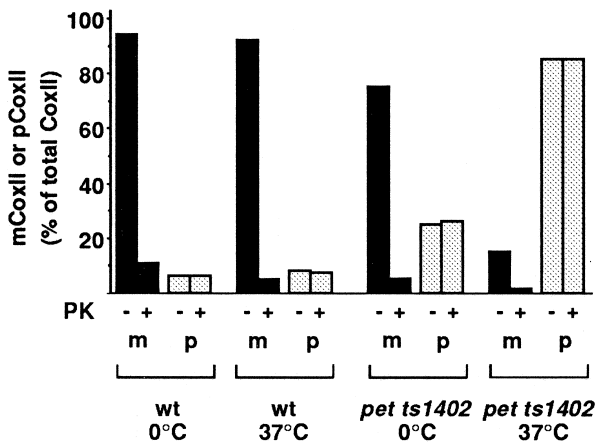

Fig. 3. Export of pCoxII is inhibited in the *pet ts1402* mitochondria. Mitochondria from wild-type (wt) and *pet ts1402* were preincubated in translation buffer at 0°C or at 37°C for 10 min and then were converted to mitoplasts. In organello translation was then performed in both samples for 20 min at 25°C in the presence of [^{35}S]methionine. The mitoplasts were then either treated with proteinase K (PK) or mock-treated. All samples were analyzed by SDS-PAGE and autoradiography. Accessibility of both pCoxII and mCoxII to added PK was quantified by densitometry and is expressed as a percentage of the total CoxII (pCoxII + mCoxII) in the corresponding mock-treated sample.

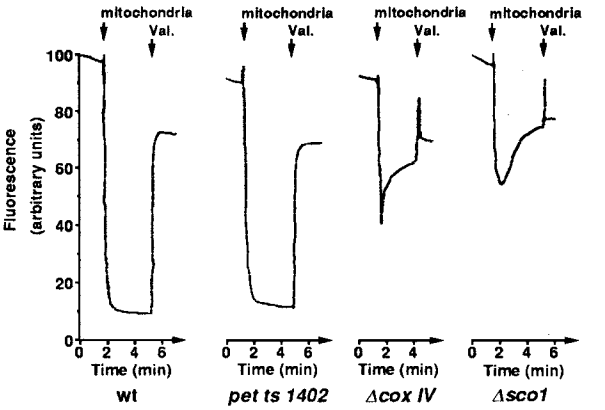

Fig. 4. Membrane potential measurements of mitochondria. The membrane potential of mitochondria isolated from the *pet ts1402* yeast mutant and its corresponding wild-type, together with those isolated from the Δ*sco1* and Δ*coxIV* yeast strains were measured, as described in Section 2. Additions of mitochondria (100 μg of protein) and valinomycin (Val., 1 μM) are indicated.

Δ*sco1 petite* yeast strains (Fig. 4). These latter mutant mitochondria, however, displayed wild-type levels of sorting of both nuclear and mitochondrially encoded proteins [6]. Thus, the membrane potential in the *pet ts1402* mitochondria is sufficient to support the export of proteins from the matrix to the intermembrane space.

4. Discussion

Oxa1p is a polytopic protein located in the inner membrane of mitochondria in the yeast *S. cerevisiae* [11–13]. Disruption of the OXA1 gene gives rise to respiratory deficiency, resulting in a *petite* growth phenotype [9,10]. Previous studies have indicated that the assembly of both the cytochrome oxidase and the F_1F_o-ATPase complexes were affected in the absence of a functional Oxa1p [9,10,13–15]. The exact function of the Oxa1p protein, however, was unclear until now. Recently it was reported that yeast cells bearing a disrupted OXA1 gene, and hence lacking the Oxa1p protein, accumulate CoxII proteins and mitochondrially expressed CoxII derivative proteins in their matrix [24].

We show here, using a yeast strain harboring a temperature sensitive mutant of Oxa1p, *pet ts1402*, that export of both the N- and C-termini of CoxII directly requires the functional presence of Oxa1p. We demonstrate that the phenotype of the *pet ts1402* mutant could selectively be induced in vitro by incubating the isolated mitochondria briefly at the non-permissive temperature of 37°C. Upon doing so, newly synthesized CoxII accumulates as its precursor form and is localized entirely in the mitochondrial matrix. Export of pCoxII could still occur when synthesized in the *pet ts1402* mitochondria kept at the permissive temperature, albeit with a lower efficiency than in the corresponding wild-type mitochondria. We chose to work with a temperature sensitive mutant of the Oxa1p protein rather than with mitochondria isolated from a yeast strain bearing a disrupted OXA1 gene. In the latter case it is often difficult to determine whether inhibitory effects observed are directly or indirectly due to the loss of the protein of interest, especially when they have such a pleiotropic phenotype as the Δ*oxa1* deletion [13–15]. We can conclude from

our studies here, however, that the CoxII export inhibition observed in the *pet ts1402* mitochondria is directly due to the lack of function of Oxa1p.

Is Oxa1p a specific assembly factor of subunit II of cytochrome oxidase or does it represent a general mitochondrial export machinery? Other membrane proteins encoded by the mitochondrial genome must also undergo membrane insertion, coupled with the export of hydrophilic segments to the intermembrane space. These proteins display a variety of topologies with respect to the orientation of the N-termini across the inner membrane. We are currently testing whether the insertion of these proteins is affected in the *pet ts1402* mitochondria also. In addition, a number of nuclear encoded proteins of the inner membrane undergo membrane potential dependent export, following their prior import into the mitochondrial matrix [5,6,11,25]. The export of the N-terminal tails of these proteins from the matrix to the intermembrane space is particularly well characterized [5,6,11,25]. It will be intriguing to establish whether Oxa1p plays a pivotal role in the export of these nuclear encoded proteins too.

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