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Yta10p is required for the ATP-dependent degradation of polypeptides in the inner membrane of mitochondria

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Abstract Incompletely synthesized polypeptides in the mitochondrial inner membrane are subject to rapid proteolysis. We demonstrate that Yta10p, a mitochondrial homologue of a conserved family of putative ATPases in *Saccharomyces cerevisiae*, is essential for this proteolytic process. Yta10p-dependent degradation requires divalent metal ions and the hydrolysis of ATP. Yta10p is an integral protein of the inner mitochondrial membrane exposing the carboxy terminus to the mitochondrial matrix space. Based on the presence of consensus binding sites for ATP, and for divalent metal ions found in a number of metal dependent endopeptidases, a direct role of Yta10p in the proteolytic breakdown of membrane-associated polypeptides in mitochondria is suggested.

Key words: YTA10; ATPase family; Mitochondrial inner membrane; ATP-dependent proteolysis

1. Introduction

Maintenance of mitochondrial functions depends on the coordinated synthesis and proteolysis of nuclear and mitochondrially encoded proteins. Over recent years basic principles of mitochondrial biogenesis have been recognized [1–3], but the mechanisms regulating the stability of mitochondrial proteins are poorly understood. In different subcompartments of mitochondria, including the matrix, the inner membrane and the intermembrane space, proteolytic systems are present that mediate the turnover of mitochondrial proteins as well as the degradation of misfolded proteins. In the matrix space, a homologue of *Escherichia coli* protease La has been identified [4–6], that degrades misfolded polypeptides in an ATP-dependent manner in cooperation with molecular chaperone proteins [7]. The mitochondrial inner membrane appears to contain a proteolytic system which mediates the degradation of non-assembled subunits of ATP synthase or respiratory chain complexes. This process can be studied most easily by monitoring the stability of mitochondrial translation products. In the absence of a supply of cytoplasmically synthesized proteins, mitochondrial translation products are rapidly degraded [8,9]. Similarily, incompletely synthesized polypeptides are subject to rapid proteolysis. This degradation process is tightly associated with the mitochondrial inner membrane and depends on the hydrolysis of ATP [9]. The identification of components of this proteolytic system, however, is still awaited.

Recently, based on sequence comparisons, a novel ATPase family has been described which is characterized by a highly conserved ATP binding site [10,11]. Members of this family exist in prokaryotic and eukaryotic cells and have been shown to fulfill diverse cellular functions including cell cycle control [12], gene expression [13], membrane fusion [14,15], organellar assembly [16,17], membrane insertion of proteins [18] and protein degradation [19,20]. In mitochondria of *Saccharomyces cerevisiae* three homologues, *BCS1*, *MSP1* (*YTA4*) and *YME1* (*YTA11*), have been identified so far. *BCS1* is required for the assembly of the ubiquinol-cytochrome c reductase complex [21]. The *MSP1* (*YTA4*) gene product is an integral protein of the mitochondrial outer membrane and affects intramitochondrial protein sorting [22]. *YME1* (*YTA11*) has been identifed in a screen for mutants affecting the escape of mitochondrial DNA to the nucleus [23], however, the function of Yme1p (Yta11p) has not been extensively characterized up to now.

In this report, we analysed the function of another mitochondrial homologue of this family of putative ATPases in *S. cerevisiae*, the *YTA10* gene product. *YTA10* is highly homologous to *YME1* (*YTA11*), *YTA12* and *E. coli* FtsH [11,24]. It encodes an integral inner membrane protein that likely contains two transmembrane spanning regions. A large carboxy terminal domain is exposed to the mitochondrial matrix space. Yta10p is required for the ATP and metal ion dependent proteolysis of incompletely synthesized mitochondrial translation products tightly associated with the inner membrane. The carboxy terminal domain of Yta10p carries a consensus binding site for divalent metal ions, that is conserved in Yme1p (Yta11p), Yta12p and *E. coli* FtsH and is found in a number of metallopeptidases, suggesting a direct role of Yta10p in this proteolytic process.

2. Materials and methods

2.1. Yeast strains, isolation of mitochondria and antibody production

The *Δyta10* mutant strain was obtained by a single step disruption procedure as described [24]. *YTA10*-deficient cells and the isogenic wild type strain were grown on YP medium containing 2% galactose and 0.5% lactate to an OD of 1.5. Mitochondria were isolated according to published procedures [25].

The synthetic peptide CEPPEAPAATN corresponding to amino acids 751–761 at the carboxy terminus of Yta10p was coupled to ovalbumin with maleimide activated carrier protein (Imject, Pierce) and used for generation of antibodies in rabbits.

2.2. Subfractionation of mitochondria

To disrupt the mitochondrial outer membrane, mitochondria (67 μg) were resuspended in ice-cold 20 mM HEPES/KOH, pH 7.4, containing 0.5% of fatty acid free BSA (1 ml). The samples were incubated for

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Abbreviations: PCR, polymerase chain reaction; PMSF, phenylmethylsulfonylfluoride; YTA, Yeast Tat binding Analogue.

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30 min at 4°C with gentle mixing every 5 min. After 25 min, potassium chloride was added to a concentration of 80 mM. For control, mitochondria were incubated in isotonic buffer (0.6 M sorbitol, 20 mM HEPES/KOH, pH 7.4, 0.5% of fatty acid free BSA). When indicated, proteinase K was present during swelling at a concentration of 50 μg/ml. Protease action was stopped by adding PMSF to a final concentration of 1 mM followed by 10 min incubation on ice. After swelling, mitoplasts were reisolated by centrifugation for 7 min at 12,000 × *g*. For lysis, mitoplasts were incubated for 20 min at 4°C in ice-cold 20 mM HEPES/KOH, pH 7.4, in the presence of 0.8% digitonin. For carbonate extraction, mitoplasts were resuspended in 0.1 M Na_2CO_3 and 1 mM PMSF, incubated for 30 min on ice, and centrifuged at 226,000 × *g* for 60 min. The soluble and the pellet fractions, after TCA precipitation, were analysed by SDS-PAGE and Western blot analysis using the chemiluminescence based ECL system (Amersham).

2.3. In organello translation

The synthesis of mitochondrially encoded proteins was essentially carried out according to published procedures [26,27]. Mitochondria (60 μg) were incubated in translation buffer (20 mM Tris-HCl, pH 7.2, 0.6 M sorbitol, 150 mM KCl, 15 mM KH_2PO_4, 12.5 mM $MgSO_4$, 4 mM ATP, 0.5 mM GTP, 5 mM α-ketoglutarate, 5 mM phosphoenolpyruvate, 3 mg/ml fatty acid free BSA, 0.012 mg/ml of all amino acids except methionine in the presence of pyruvate kinase (2.4 U/ml) and 5 μCi [^{35}S]methionine (1069 Ci/mmol, ICN) in a volume of 50 μl. The samples were incubated for 20 min at 25°C. Labelling was stopped by adding 20 mM cold methionine and 100 μM puromycin. Mitochondria were reisolated by centrifugation for 12 min at 9,000 × *g* and washed with 0.25 ml of 0.6 M sorbitol, 1 mM EDTA, 5 mM methionine. The samples were centrifuged as above. Mitochondria were lysed by incubation in LiDS-sample buffer (2% lithium dodecylsulfate, 10% glycerol, 2.5% β-mercaptoethanol, 0.02% Bromophenol blue, 60 mM Tris-HCl, pH 6.8) for 45 min at 4°C and analysed by SDS-PAGE and fluorography.

2.4. Proteolysis of incompletely synthesized polypeptides

To generate incompletely synthesized polypeptides, 5 μM puromycin was added to the translation reaction. Under these conditions in wild type mitochondria the efficiency of labelling was reduced to about 40%. After stopping translation with addition of 100 μM puromycin and 20 mM methionine, mitochondria were reisolated by centrifugation for 12 min at 9,000 × *g* and washed three times with 0.25 ml 0.6 M sorbitol, 1 mM EDTA, 5 mM methionine. Mitochondria were resuspended in translation buffer at a concentration of 1.2 mg/ml and further incubated at 37°C to allow proteolysis to occur. At the time points indicated, aliquots were withdrawn and, after addition of trichloroacetic acid (TCA) to a final concentration of 12.5%, further incubated for 30 min at 4°C. The samples were divided into the TCA soluble fraction and the TCA pellet fraction by centrifugation for 10 min at 25,000 × *g*. The radioactivity of both fractions was measured in Ultima Gold (Hewlett-Packard).

2.5. Import of precursor proteins into isolated yeast mitochondria

The cDNAs encoding $b_2(167)^{RIC}$-DHFR and Su9 (1–69)-lactalbumin were transcribed using SP6 polymerase and translated in rabbit reticulocyte lysate (Promega) according to published procedures [28]. Import of $b_2(167)^{RIC}$-DHFR and Su9 (1–69)-lactalbumin into isolated yeast mitochondria was carried out as previously described [7]. Reticulocyte lysate (2% of total volume) was added to import reactions (50 μg mitochondria) containing import buffer (50 mM HEPES/KOH, pH 7.2, 0.5 M sorbitol, 80 mM KCl, 10 mM MgOAc, 2 mM K-phosphate, 1 mM $MnCl_2$, 3% fatty acid free BSA) in the presence of 5 mM NADH, 2.5 mM ATP and an ATP-regenerating system (10 mM phosphocreatine, 100 μg/ml creatine kinase) in a final volume of 100 μl. Import of $b_2(167)^{RIC}$-DHFR and Su9 (1–69)-lactalbumin was performed for 20 min at 15°C and halted by adding 0.9 μM valinomycin and chilling on ice. Non-imported precursor proteins were digested by proteinase K (150 μg/ml) for 30 min at 0°C. Digestion was stopped by the addition of 1 mM PMSF. Mitochondria were reisolated by centrifugation for 12 min at 9,000 × *g* and washed once with SEM buffer (10 mM MOPS/KOH, pH 7.2, 250 mM sucrose, 1 mM EDTA) containing 1 mM PMSF. After reisolation mitochondria were resuspended in import buffer in the presence of NADH, ATP and an ATP-regenerating system as above. Samples were then incubated at 30°C to allow degradation of the newly imported proteins. At various time points, mitochondria were reisolated by centrifugation for 10 min at 9,000 × *g* and washed with SEM buffer containing 1 mM PMSF. Samples were analysed by SDS-PAGE and fluorography.

3. Results and discussion

3.1. Yta10p is an integral inner membrane protein

In *S. cerevisiae*, a set of 12 different genes, which comprise the 'YTA-family' (*Y*east *T*at binding *A*nalogs), have been identified using PCR technology [11]. The *YTA10* gene encodes a mitochondrial protein, that is essential for mitochondrial respiratory function [24]. Gene disruption results in mitochondrial dysfunction and the inability to grow on non-fermentable carbon sources (nuclear petite). Yta10p contains two hydrophobic regions (amino acids 116–141 and amino acids 222–245) which have the characteristics of transmembrane segments. To determine the submitochondrial location of Yta10p, mitochondria were subfractionated by osmotic swelling (Fig. 1). Yta10p was not released from mitochondria by opening of the intermembrane space nor degraded by protease added to mitoplasts (Fig. 1; lanes 2 and 3). Detergent lysis of the mitochondrial inner membrane did, however, make Yta10p, as well as the matrix localized Mge1p and the mitochondrial inner membrane marker, the ADP/ATP carrier, accessible to added protease (Fig. 1; lane 4). To test whether Yta10p is an integral protein of the inner membrane, mitoplasts were treated with carbonate at pH 11.5. Yta10p, like the ADP/ATP carrier, was found to be resistant towards extraction with alkaline pH, indicating that Yta10p is an integral part of the inner membrane (Fig. 1; lanes 5 and 6). Interestingly, Western blot analysis, using a peptide-specific antiserum directed towards the carboxy terminal amino acids of Yta10p, revealed that the carboxy terminus of Yta10p was protected from externally added protease in mitoplasts (Fig. 1; lane 3). In contrast, MIM23, an integral protein of the mitochondrial inner membrane that exposes a domain to the

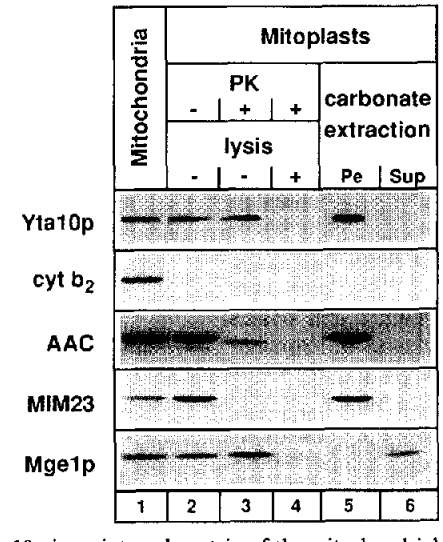

Fig. 1. Yta10p is an integral protein of the mitochondrial inner membrane. Mitochondria were subfractionated as described in section 2. Protein fractions were analysed by SDS-PAGE and Western blot analysis using specific antisera directed against Yta10p, cytochrome b_2 (cyt b_2), ADP/ATP carrier (AAC), mitochondrial inner membrane protein 23 (MIM23) and mitochondrial GrpE (Mge1p). PK, proteinase K; Pe, pellet fraction; Sup, supernatant.

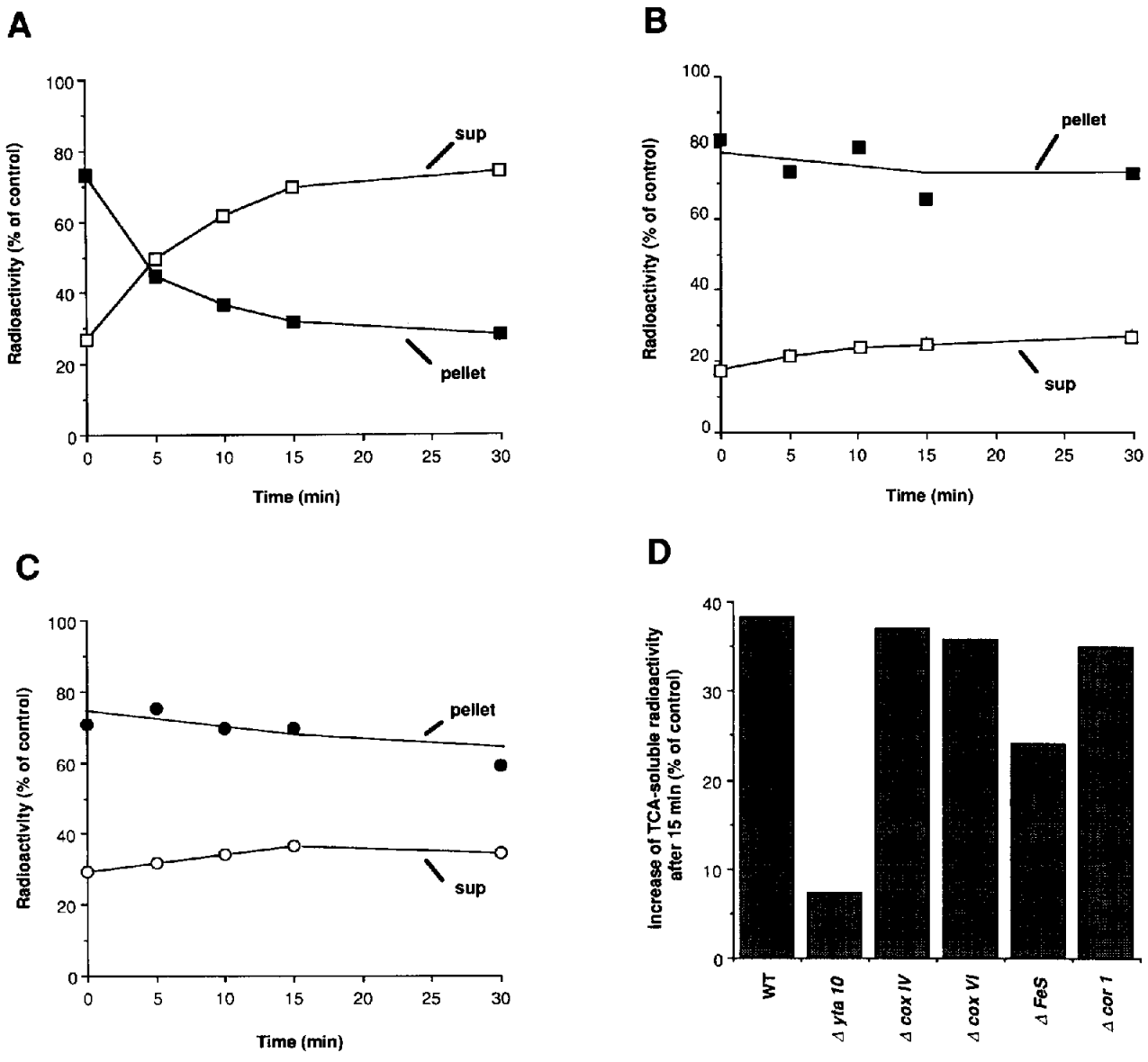

Fig. 2. Degradation of incompletely synthesized mitochondrial translation products. The labelling of mitochondrial translation products was performed as described in section 2. Proteolysis of puromycyl polypeptides was then analysed (A) in wild type mitochondria in the presence of matrix ATP (□)(■), (B) in wild type mitochondria in the absence of matrix ATP (□)(■), (C) in Δyta10 mutant mitochondria in the presence of matrix ATP (○)(●) and (D) in mitochondria isolated from wild type cells (WT) and from cells deficient in Yta10p (*Δyta10*), cytochrome oxidase subunit IV (*ΔcoxIV*), cytochrome oxidase subunit VI (*ΔcoxVI*), Rieske-FeS-protein (*ΔFeS*) or core 1 (*Δcor1*), respectively, in the presence of matrix ATP. Routinously, mitochondria were resuspended in 20 mM Tris-HCl, pH 7.2, 0.6 M sorbitol, 150 mM KCl, 15 mM KH_2PO_4, 12.5 mM $MgSO_4$, 3 mg/ml fatty acid free BSA. To reduce ATP levels after translation, apyrase, at a concentration of 40 U/ml, and oligomycin, at a concentration of 20 μM, were added. Total radioactivity before proteolysis was set to 100%. In *Δyta10* mutant mitochondria, the efficiency of translation was reduced to about 40% compared to wild type. Sup, supernatant fraction.

intermembrane space [29,30], is protease-sensitive under these conditions. This, together with the presence of two putative transmembrane regions in the sequence of *YTA10*, suggests a topology equivalent to that of the *E. coli* FtsH protein [31], with a small amino terminal and a large carboxy terminal domain facing the mitochondrial matrix space.

3.2. *Yta10p is required for the proteolysis of incompletely synthesized mitochondrial translation products in the inner membrane*

Sequence comparisons of Yta10p with Yme1p (Yta11p), Yta12p and *E. coli* FtsH revealed a highly conserved motif between amino acids 558–562 of Yta10p [24]. In this region, two histidine residues are separated by a three amino acid spacer which is reminiscent of the HEXXH motif found in various Zn^{2+} dependent metallo-peptidases [32,33] including bacterial thermolysin [34], rat testes endopeptidase [35,36], mitochondrial intermediate peptidases [37,38], yeast oligopeptidase yscD [39] and bovine endothelin converting enzyme ECE-1 [40]. Therefore, we investigated a potential role of Yta10p in proteolytic processes within mitochondria. As Yta10p is an integral inner membrane protein, it might be part of the proteolytic

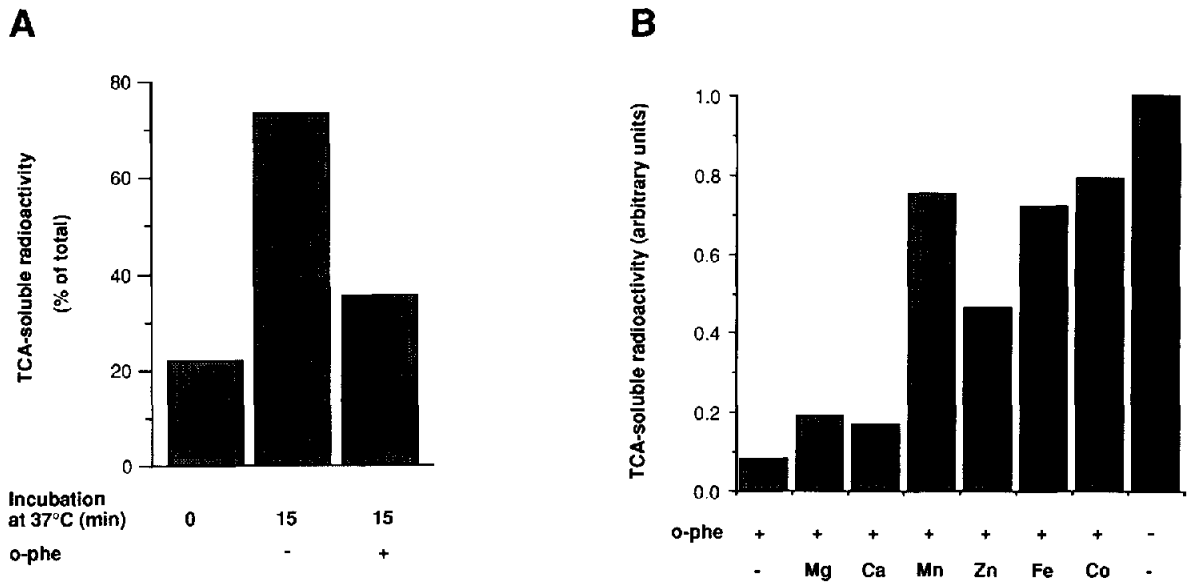

Fig. 3. Dependence of the proteolytic breakdown on the presence of divalent metal ions. (A) Inhibiton of proteolysis by *o*-phenanthroline. The synthesis of mitochondrially encoded proteins was carried out as described in section 2 except that the concentration of Mg^{2+}-ions was reduced to 5 mM. After translation, mitochondria were resuspended in buffer as above in the presence (lane 3) or absence (lane 2) of 1 mM *o*-phenanthroline and 5 mM EDTA. Then, proteolysis was allowed to occur for 15 min at 37°C. Total radioactivity before proteolysis was set to 100%. (B) Reactivation of proteolysis by addition of divalent metal ions. Proteolysis was allowed to occur for 15 min at 37°C in the presence of *o*-phenanthroline as in (A). The samples were supplemented with 10 mM $MgCl_2$ (Mg), $CaCl_2$ (Ca), $MnCl_2$ (Mn), $ZnCl_2$ (Zn), $FeCl_2$ (Fe) or $CoCl_2$ (Co), respectively. In a control reaction, no metal ion was added (lane 1). The increase of radioactivity in the TCA soluble fraction after 15 min in the absence of *o*-phenantroline (lane 8) was set to 1.0. o-phe, *o*-phenanthroline.

system that mediates the degradation of non-assembled mitochondrial translation products in the mitochondrial inner membrane.

In *S. cerevisiae*, eight proteins are mitochondrially encoded. With the exception of the ribosomal Var1 protein, all of these are subunits of the ATP synthase or respiratory chain complexes in the mitochondrial inner membrane [41]. If mitochondrial translation is carried out in the presence of [^{35}S]methionine and limited concentrations of puromycin, only incomplete polypeptides, that do not assemble into functional active complexes and therefore are subject to rapid degradation, are synthesized. This observation was exploited to analyse a potential role of Yta10p in proteolysis. Mitochondria were isolated from wild type and *Δyta10* mutant cells and the synthesis of mitochondrially encoded proteins was carried out in organello in the presence of ^{35}S-labelled methionine and 5 μM puromycin. Both, in the presence and absence of Yta10p, the translation products were found to be resistant to extraction at alkaline pH or with 2 M KCl, indicating a tight membrane association (data not shown). To monitor the proteolytic breakdown of incompletely synthesized polypeptides, the amount of radioactivity released into the TCA-soluble fraction was determined during the subsequent incubation. As shown in Fig. 2A, in wild type mitochondria, incomplete polypeptide chains were degraded with a half time of about 6 min. In agreement with earlier observations [9,42], this process was found to depend on the hydrolysis of ATP in the mitochondrial matrix (Fig. 2B; data not shown). In mitochondria isolated from *YTA10* deficient cells, the rate of the proteolytic breakdown was severely reduced (Fig. 2C), demonstrating the requirement of Yta10p for the degradation of incompletely synthesized polypeptides in the mitochondrial inner membrane. To exclude indirect effects as a result of the growth phenotype of the *YTA10* deletion, we tested the proteolysis of puromycyl polypeptides in mitochondria isolated from a variety of nuclear petite mutants (Fig. 2D). The degradation of incompletely synthesized polypeptides was either not affected or only slightly reduced by the nuclear pet mutations tested, demonstrating the specificity of the *YTA10* deletion.

3.3. *The degradation of incompletely synthesized polypeptides is dependent on divalent metal ions*

In further experiments, we tested the requirement for divalent metal ions for the proteolysis of incompletely synthesized polypeptides. After completion of translation, the membrane permeable chelating agent *o*-phenanthroline was added, then proteolysis was allowed to occur for 15 min. In the presence of *o*-phenanthroline, the proteolytic breakdown was strongly inhibited (Fig. 3A; lane 3 vs. 2). If Fe^{2+}-, Co^{2+}- or Mn^{2+}-ions were added subsequently and the samples incubated for an additional 15 min, the proteolytic activity was restored almost completely (Fig. 3B). Zn^{2+}-ions showed only a partial effect under the conditions tested, whereas the addition of Mg^{2+}- or Ca^{2+}-ions did not result in efficient reactivation of proteolysis (Fig. 3B). Interestingly, the complexing agent EDTA, which cannot penetrate the mitochondrial inner membrane, did not inhibit the proteolytic breakdown of puromycyl polypeptides (data not shown), indicating that metal ions are required in the mitochondrial matrix space. These results are in large parts consistent with recent observations by Yasuhara et al. [43], who analysed the degradation of completely synthesized mitochondrial translation products, and suggest the participation of a metalloprotease in the degradation of polypeptides associated with the mitochondrial inner membrane.

The presence of a segment in the matrix localized domain of

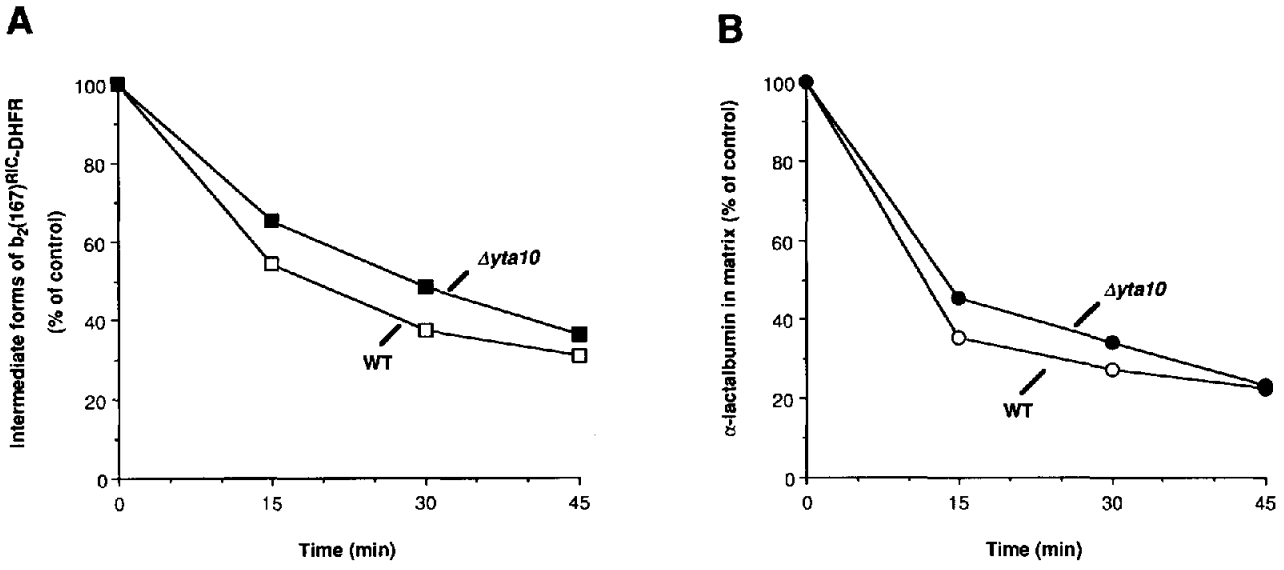

Fig. 4. Proteolysis of misfolded proteins in the mitochondrial matrix does not depend on Yta10p. Degradation of newly imported (A) $b_2(167)^{RIC}$-DHFR and (B) Su9 (1–69)-lactalbumin in the matrix of mitochondria isolated from wild type (open symbols) or *Δyta10* mutant cells (filled symbols). Import of the precursor proteins and subsequent analysis of proteolysis in the matrix was as described in section 2. α-lactalbumin (B) or intermediate forms of $b_2(167)^{RIC}$-DHFR (A) before proteolysis were set to 100%.

Yta10p, encoding a consensus metal binding site, strongly suggests that complexing of metal ions by *o*-phenanthroline inhibits Yta10p function. Divalent metal ions such as Zn^{2+} are bound to a third amino acid in addition to the two histidine residues in the HEXXH motif. In many cases, this is a glutamic acid residue which is separated from the histidine ligands by a spacer of about 20 amino acids [32]. In Yta10p, no glutamic acid is present at this position. However, as considerable variation in the position of the third binding site has been observed depending on the metalloprotease family [32], structural data are required to determine the location of this binding site in Yta10p. Possible candidates are glutamic acid residues at position 603 and 604 which are conserved in Yta10p, Yme1p (Yta11p), Yta12p and *E. coli* FtsH. It should be mentioned, that in *YTA10* a cysteine residue (at position 565) is located close to the HEXXH binding motif [11]. Similarily, the rat testes endopeptidase contains a cysteine residue in proximity to the active site that was suggested to cause the unique sensitivity of this metallopeptidase towards thiol blocking agents [35]. Interestingly, proteolysis of incompletely synthesized polypeptide chains can be inhibited by the cysteine-modifying agent *N*-ethylmaleimide (data not shown).

3.4. *Yta10p does not affect proteolysis of misfolded proteins in the mitochondrial matrix*

The carboxy terminal domain of Yta10p containing the putative ATP- and metal-binding sites is exposed to the mitochondrial matrix. Therefore, we examined the requirement of Yta10p for the degradation of newly imported, misfolded proteins in the matrix space. As model proteins, we used a hybrid protein containing the first 167 amino acids of cytochrome b_2 fused to cytosolic DHFR, which is missorted to the matrix because of specific mutations in the sorting signal [$b_2(167)^{RIC}$-DHFR] [44], and bovine α-lactalbumin which was fused to a mitochondrial presequence allowing its import into isolated yeast mitochondria [7]. Both proteins have been found recently to be degraded by the matrix localized PIM1 protease, a homologue of *E. coli* protease La, in an ATP-dependent manner [7]. To test a role of Yta10p in this proteolytic process, both fusion proteins were imported into *Δyta10* mutant mitochondria at low temperature to slow down proteolysis. Further incubation of the mitochondria after completion of import results in the degradation of the misfolded proteins in the matrix. Disruption of *YTA10* neither affects the efficiency of the import reaction (data not shown) nor the subsequent proteolytic breakdown of the misfolded proteins (Fig. 4). In the absence of Yta10p, intermediate forms of $b_2(167)^{RIC}$-DHFR (Fig. 4A) and α-lactalbumin (Fig. 4B) were degraded with similar rates as in wild type mitochondria. Therefore, independent proteolytic systems appear to exist in the mitochondrial inner membrane and matrix space.

The results presented in this report demonstrate the requirement of Yta10p for the degradation of incompletely synthesized polypeptides tightly associated with the mitochondrial inner membrane. In the absence of Yta10p, the rate of proteolysis of puromycyl fragments of mitochondrial translation products is severely reduced. Several lines of evidence suggest that Yta10p itself might exhibit proteolytic activity. First, in agreement with the dependence of the proteolytic breakdown on ATP hydrolysis and the presence of divalent metal ions, consensus binding sites for ATP and divalent metal ions have been identified in the *YTA10* gene. Yta10p contains a HEXXH motif which is conserved in Yme1p (Yta11p), Yta12p and *E. coli* FtsH, and is characteristic for a variety of metal dependent endopeptidases [32,33]. Second, consistent with the predicted topology of Yta10p, ATP and divalent metal ions are required in the matrix for proteolysis. Carboxyatractyloside, an inhibitor of the ADP/ATP carrier in the inner mitochondrial membrane, blocks proteolysis of mitochondrial translation products, indicating a specific requirement for ATP in the matrix space [43]. Similarly, only binding of divalent metal ions by a membrane-permeable chelating agent results in inhibition of the proteolytic process.

Direct proof of a proteolytic activity of Yta10p, however, will require reconstitution experiments in vitro using purified Yta10p.

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