Dual targeting of yeast catalase A to peroxisomes and mitochondria

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Yeast catalase A (Cta1p) contains two peroxisomal targeting signals (SSNSKF) localized at its C-terminus and within the N-terminal third of the protein, which both can target foreign proteins to peroxisomes. In the present study we demonstrated that Cta1p can also enter mitochondria, although the enzyme lacks a classical mitochondrial import sequence. Cta1p co-targeting was studied in a catalase A null mutant after growth on different carbon sources, and expression of a Cta1p–GFP (green fluorescent protein)-fusion protein or a Cta1p derivative containing either a c-Myc epitope (Cta1p^{myc}) or a SKF-extended tag (Cta1p^{myc-SKF}). Peroxisomal and mitochondrial co-import of catalase A were tested qualitatively by fluorescence microscopy and functional complementation of a $\Delta cta1$ null mutation, and quantitatively by subcellular fractionation followed by Western blot analysis and enzyme

activity assays. Efficient Cta1p import into peroxisomes was observed when cells were cultivated under peroxisome-inducing conditions (i.e. growth on oleate), whereas significant co-import of Cta1p–GFP into mitochondria occurred when cells were grown under respiratory conditions that favour oxygen stress and ROS (reactive oxygen species) accumulation within this organelle. In particular, when cells were grown on the non-fermentable carbon source raffinose, respiration is maximally enhanced, and catalase A was efficiently targeted to the mitochondrial matrix where it presumably functions as scavenger of H₂O₂ and mitochondrial-derived ROS.

Key words: catalase A, mitochondria, peroxisome, protein co-targeting, reactive oxygen species (ROS), *Saccharomyces cerevisiae*.

INTRODUCTION

In all eukaryotic cells, peroxisomes and mitochondria share a great variety of enzymic reactions that are catalysed by isoenzymes present in both organelles. For some of these enzymes it is known that they can be co-targeted to different organelles; an example of such a co-targeting is the yeast peroxisomal citrate synthase Cit2p, an enzyme of the tricarboxylic acid cycle, which contains a cryptic N-terminal signal sequence that functions in both peroxisomal and mitochondrial targeting [1]. Besides enzymes that catalyse related reactions within the tricarboxylic acid and glyoxylate cycles, enzymes involved in the detoxification of oxygen radicals are also present in both organelles, peroxisomes and mitochondria. It is well documented that during oxygen reduction in mitochondria, ROS (reactive oxygen species), such as superoxide radicals (O₂^{•−}) and H₂O₂, accumulate as a result of either autooxidation of ubiquinone of the cytochrome *bc*₁ complex or by spontaneous and/or enzymic dismutation of O₂^{•−} [2,3]. The main scavengers of O₂^{•−} and H₂O₂ in mitochondria are two superoxide dismutases (Mn-SOD and Cu,Zn-SOD), glutathione peroxidase and peroxiredoxins [4–8]. Mitochondrial catalase as a fourth component of antioxidant defence was originally discovered in rat heart mitochondria by Radi et al. [2], and more recently in the yeast *Saccharomyces cerevisiae* [9]. However, until now it was not known which of the two catalase isoenzymes in yeast contributes to this activity, and how it is targeted to this organelle.

The important function of catalase as scavenger of H₂O₂ is convincingly underlined by the fact that catalases are always co-distributed with H₂O₂-producing enzymes in peroxisomes and other organelles [5,6]. In the yeast *S. cerevisiae*, two catalase isoenzymes have been identified: an atypical organelle-associated catalase A, Cta1p, and a typical soluble catalase T, Ctt1p [10,11].

Both enzymes are encoded by different genes (*CTA1* and *CTT1*), are highly expressed under conditions that induce peroxisome formation and are localized to different compartments: Ctt1p is a cytosolic enzyme, whereas Cta1p is primarily localized in peroxisomes [12–15]. Previous studies in yeast already suggested that mitochondria possess an independent mechanism for decomposing exogenous H₂O₂ [16,17], and that this organelle is also the site for endogenous detoxification of H₂O₂ by cytochrome *c* peroxidase [18]. Although to date, yeast catalase A has been primarily considered as a peroxisomal protein, we recently demonstrated catalase activity in yeast mitochondria [9], confirming previous studies in mammals in which catalase activity in rat heart mitochondria was identified as a key antioxidant defence mechanism for myocardial tissue [2]. We therefore speculated that catalase activity in yeast mitochondria might have a similar important function *in vivo* by co-operating with mitochondrial SODs in the detoxification of H₂O₂ in this organelle. These findings and the fact that mitochondria and peroxisomes share a variety of enzymic reactions, motivated us to study yeast mitochondrial catalase activity in more detail. In the present study, it is shown that yeast catalase A is not only targeted to peroxisomes, but is also efficiently co-imported into mitochondria, in particular, when cells are cultivated under respiratory growth conditions when Cta1p functions as scavenger of mitochondrial derived H₂O₂.

EXPERIMENTAL

Strains and culturing conditions

Yeast strains used in this study were the *S. cerevisiae* catalase T null mutant MSCTA1 [MAT α *leu2 arg1 ura3 trp1* $\Delta ctt1::P_{CTA-lacZ}$ (*URA3*)] and the catalase A and catalase T double mutant

Abbreviations used: Cox3, cytochrome c oxidase subunit III; CPY, carboxypeptidase Y; Cta1p, catalase A protein; GFP, green fluorescent protein; Pgk, phosphoglycerate kinase; ROS, reactive oxygen species; SC, synthetic complete minimal; SOD, superoxide dismutase; YEPD, yeast extract peptone dextrose.

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GC1-8B (MATa *leu2-3,112 ura3 trp1 ctal-2 ctt1-1*) [19], both kindly provided by Dr Marek Sconenzy (Polish Academy of Science, Warsaw, Poland). The catalase A null mutant strain Y03615 (MATa *his3Δ1 leu2Δ0 ura3Δ0 Δctal::kan MX4*) and its isogenic Cta1⁺ wild-type strain BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) were obtained from the Euroscarf yeast strain collection (Frankfurt, Germany). Yeast strains were grown at 30 °C either on complex YEPD (yeast extract peptone dextrose) medium or on SC (synthetic complete minimal) medium containing 0.67 % yeast nitrogen base (without amino acids, supplemented with ammonium sulphate) with either 2 % glucose or 1 % raffinose as carbon source. Yeast transformation was carried out by the lithium acetate method [20], and transformants were selected on synthetic YNB (yeast nitrogen base) medium supplemented with the appropriate amino acid/base composition of the strain. Liquid oleic acid medium used for the induction of peroxisomes contained 0.5 % oleic acid and 0.05 % Tween 80 as carbon source. Cells were grown on a rotary shaker at 30 °C to late exponential growth phase (10⁸ cells/ml). *Escherichia coli* DH5α cells grown in Luria–Bertani medium were used for plasmid propagation [21].

Vector construction and genetic techniques

CTA1 and its C-terminal fusion to GFP (green fluorescent protein) (*CTA1-GFP*) were amplified by PCR-mediated overlap extension essentially as described for the construction of hybrid genes [22], using chromosomal DNA isolated from the catalase wild-type strain *S. cerevisiae* BY4741 as template and the following primer pairs: CTA1-F (5'-CCC TCGAG ATG TCG AAA TTG GGA CAA GAA AAA AAT GAA G-3') and CTA1-R (5'-CAT GCT TCT GAG CTT TCG AGT AAC TCC AAA TTT GTG AGC AAG GGC GAG GAG CTG TTC ACC GGG-3') for *CTA1*; primers GFP-F (5'-CCC GGT GAA CAG CTC CTC GCC CTT GCT CAC AAA TTT GGA GTT ACT CGA AAG CTC AGA AGC ATG-3') and GFP-R (5'-AGA TCT TTA CTT GTA CAG CTA GTC CAT GCC GAG AGT G-3') were used for the amplification of GFP from plasmid pET-24a(+)GFP [23]. Both PCR fragments were gel purified and subsequently used as template with primers CTA1-F and GFP-R to amplify a *CTA1-GFP* gene fusion, in which GFP was fused in frame to the C-terminus of Cta1p. DNA sequence analysis of all constructs, performed by fluorescence cycle sequencing on an automated DNA sequencer (LI-COR 4200), showed the predicted wild-type sequences within the Cta1p protein fragment and at the fusion junction (results not shown). The *CTA1-GFP* fragment was cloned into the *XhoI*–*Bgl*III site of the 2μ plasmid pDT-PGK [24]. The resulting vector (pCTA1-GFP) contains the *CTA1-GFP* gene fusion under transcriptional control of the constitutive phosphoglycerate kinase promoter *PGK1* [23]. The two C-terminally Myc-tagged derivatives of Cta1p (Cta1p^{myc} and Cta1p^{myc-SKF}) were PCR amplified in the same way by using the 5' primer CTA1-F (see above) and either of the two reverse 3' primers CTA1-cmyc (5'-GGG ATCCT CAC AAA TCT TCT TCA GAA ATC AAT TTT TGT TCA AAT TTG GAG TTA CTC GAA AGC TCA GAA GCA TGT TTT G-3') and CTA1-cmycSKF (5'-AGA TCT TCA AAA TTT GGA CAA ATC TTC TCA GAA AAT CAA TTT TTG TTC AAA TTT GGA G-3') respectively. In addition to the 2μ-based *CTA1* multi-copy expression vectors, centromeric single-copy YCp derivatives were also used which gave an identical Cta1p targeting after expression *in vivo* (results not shown). The only difference between *CTA1* single-copy expression versus *CTA1* multi-copy expression was seen in a more intensive Cta1p protein signal on Western blots after 2μ-driven Cta1p expression; we therefore used 2μ vectors

for catalase A expression throughout our studies. As positive control for peroxisomal protein targeting, the coding sequences for DsRed and GFP were amplified from plasmids pDsRed-N1 (ClonTech) and pET-24a(+)GFP [23] respectively, by using PCR primers that added a classical PTS (peroxisomal targeting sequence; Ser-Lys-Leu) to the C-terminus of both proteins. The fluorescent proteins DsRed^{SKL} and GFP^{SKL} were constitutively expressed from the yeast *PGK1* promoter as described above for the Cta1p–GFP-fusion protein.

Subcellular fractionation and Nycodenz gradients

For cell fractionation, yeast cells were grown overnight to mid-log phase (2 × 10⁷ cells/ml), essentially as described previously [25]. Briefly, spheroplasts were generated by a 90 min incubation at 30 °C in 10 mM Tris (pH 7.5), 0.8 M sorbitol, 2 mM DTT and 10 mM CaCl₂ containing 20 mg of Zymolyase 20-T at 1 mg/g of wet mass of yeast. Spheroplasts were harvested by centrifugation (10 min at 2000 g), washed twice in a solution of 0.8 M sorbitol, 20 mM Hepes/KOH (pH 7.0), 50 mM potassium acetate and 2 mM EDTA, and carefully resuspended in 1 ml of chilled lysis buffer (0.2 M sorbitol, 20 mM Hepes/KOH, pH 7.0, 50 mM potassium acetate, 2 mM EDTA) plus protease inhibitors (protease inhibitor cocktail; Roche Mannheim). Cell lysis was performed with the aid of a 5 ml Dounce glass homogenizer for 15 strokes, and unlysed cells, nuclei and cell debris were removed by centrifugation at 3000 g and 4 °C for 10 min. The supernatant containing the crude yeast cell organelles was again centrifuged at 15000 g and 4 °C for 20 min, and the crude organelle fraction was resuspended in a total volume of 5 mM Mes/KOH buffer (pH 6.0) containing 0.24 M sucrose (to provide osmotic protection for the peroxisomes) and 1 mM EDTA. Purity of such an organelle fraction was routinely determined by Western blot analysis (see also Figure 1C) and activity assays of marker enzymes, such as peroxidase, glucose-6-phosphate dehydrogenase, hexokinase, succinate dehydrogenase, Mn-SOD, D-amino acid oxidase and isocitrate lyase, indicating that the organelle fraction was highly enriched for yeast mitochondria (results not shown). For the separation of cell organelles, in particular for separating peroxisomes from mitochondria, the crude organelle fraction was layered on top of a Nycodenz (Sigma) step gradient consisting of 17 %, 25 % and 35 % Nycodenz in the same buffer, essentially as described by Lee et al. [1]. The Nycodenz gradient was centrifuged at 133000 g and 4 °C for 90 min in an SW50Ti rotor (Beckman) and the gradient was subsequently fractionated from the bottom. Each fraction (1 ml) was diluted 5-fold with Mes/KOH buffer (5 mM, pH 6.0, containing 0.24 M sucrose and 1 mM EDTA), and the Nycodenz was removed by a final centrifugation step at 15000 g and 4 °C for 20 min. For Western blot analysis and enzyme assays, gradient purified organelles were ruptured by the addition of Triton X-100 to a final concentration of 1 %. Yeast mitoplasts were isolated from Nycodenz-purified mitochondria by a hypotonic swelling–shrinking treatment as described previously [26].

Protein determination, enzyme assays and Western blotting

Protein content in the purified fractions of peroxisomes and mitochondria was determined with the bicinchoninic acid protein assay kit (Sigma-Aldrich) using BSA as a standard. The levels of D-amino acid oxidase, glutamate dehydrogenase and adenylate kinase were assayed spectrophotometrically, essentially as previously described [9]. Catalase activity was determined by UV spectroscopy at 240 nm by the decomposition of H₂O₂

[27]. The reaction was performed in 50 mM K_2HPO_4 , pH 7.0, containing 20 mM H_2O_2 and 10 μ g of total cellular protein in a final volume of 2 ml. Specific catalase activity was calculated from the equation

$$\text{Catalase activity (units/mg)} = (\Delta A_{240[15\text{ s}]} \times 4) / \text{mg of protein}$$

where $\Delta A_{240[15\text{ s}]}$ is the change in A_{240} over 15 s. SDS/PAGE and immunoblotting were performed as described previously [28]. The following primary antibodies against the indicated marker proteins of *S. cerevisiae* were obtained from Molecular Probes (Leiden, Netherlands): mouse monoclonal anti-GFP, mouse monoclonal anti-Cox3p (cytochrome c oxidase subunit III), mouse monoclonal Pep12p (endosomal membrane protein), mouse monoclonal anti-Pgk1p (phosphoglycerate kinase), mouse monoclonal anti-yeast porin (mitochondrial outer membrane marker), mouse monoclonal anti-c-Myc and mouse monoclonal anti-CPY (carboxypeptidase Y) antibodies. Alkaline-phosphatase-conjugated anti-mouse IgG was used as secondary antibody (Sigma-Aldrich), and colorimetric signal detection of the immunoprecipitate was performed using a NBT/BCIP stock solution (Roche, Mannheim) diluted 1:500 in staining buffer. Polyclonal goat antibodies against wild-type yeast Cta1p, kindly provided by Dr Andreas Hartig (Department of Biochemistry and Molecular Cell Biology, Campus Vienna Biocenter, Vienna, Austria) (see also [33]), turned out to be unspecific and cross-reactive when being used as a probe in immunoblots from Nycodenz gradient fractions (results not shown). Since antibodies against yeast catalase A are not commercially available, Cta1p targeting and subcellular localization was analysed by SDS/PAGE and Western analysis in a yeast $\Delta cta1$ null mutant either expressing Cta1p^{myc} or Cta1p^{myc-SKF} (see above) and subsequently probed with monoclonal anti-c-myc antibody.

Fluorescence microscopy

Exponentially growing yeast cells either expressing Cta1p-GFP, GFP^{SKL} or DsRed^{SKL} were washed twice with PBS buffer (pH 7.4) and subsequently examined using an BX51 fluorescence microscope (Olympus) under standard GFP/DsRed settings [29]. Fluorescent living yeast cells were also visualized with a confocal laser-scanning microscope (Bio-Rad, München, Germany). Mitochondrial localization of Cta1p-GFP in cells of the $\Delta cta1$ null mutant was determined by co-staining with the mitochondrion-selective probe MitoTracker Orange CMTMRos (Molecular Probes, Leiden, The Netherlands).

RESULTS

Cta1p is responsible for catalase activity in yeast mitochondria

In order to identify which of the two catalase isoenzymes in yeast is responsible for the observed catalase activity in yeast mitochondria, three different catalase mutants ($\Delta cta1$, $\Delta ctt1$, and $cta1-2\ ctt1-1$) and the corresponding catalase wild-type were cultivated in YEPD medium until the cells had entered exponential growth phase and showed maximal mitochondrial catalase activity [9]. Subsequently, cells were harvested, mitochondrial fractions were isolated, and catalase activity was determined. As illustrated in Figure 1(A), mitochondria derived from the catalase wild-type strain BY4741 and from its isogenic $\Delta ctt1$ null mutant MSCTA1 showed significant catalase activities of 4.7 and 6.4 units/mg of protein respectively. In contrast, catalase activity was undetectable in mitochondria either derived from the $\Delta cta1$ null mutant

Figure 1 Cta1p is responsible for catalase activity in yeast mitochondria

(A) Catalase activity in yeast mitochondria isolated from a catalase wild-type (*CTA1*, *CTT1*), a catalase A ($\Delta cta1$) and a catalase T null mutant ($\Delta ctt1$), and a catalase A and T double mutant ($cta1-2\ ctt1-1$) grown in glucose containing SC medium. Assays were performed in triplicate and the results are expressed as the means $\pm$ S.D. (B) Expression of a Cta1p-GFP fusion protein in the $\Delta cta1$ deletion mutant fully restores mitochondrial catalase activity to wild-type level. (C) Purity of the isolated yeast mitochondria was checked by SDS/PAGE and Western blot analysis of the cell extract (E) and the mitochondrial fraction (M) from a catalase wild-type yeast (*CTA1*, *CTT1*). Each lane was loaded with 50 μ g of total mitochondrial protein. Immunoblots were probed with monoclonal antibodies against the indicated marker proteins Cox3p (inner mitochondrial membrane, 29.9 kDa), Pep12p (endosomal compartment, 37.0 kDa) and Pgk1p (cytosol, 44.7 kDa). Arrow heads indicate the position of Cox3p, Pep12p and Pgk1p.

or from the $cta1-2\ ctt1-1$ double mutant (Figure 1A). In the double mutant, chromosomal mutations in *CTA1* and *CTT1* are known to cause a total loss in catalase A and catalase T activity without negatively affecting either Cta1p/Ctt1p protein levels or intracellular catalase A/catalase T targeting [30]. As shown in Figure 1(B), transformation of the $\Delta cta1$ deletion mutant with a plasmid expressing a catalase A-GFP-fusion protein fully restored mitochondrial catalase activity of the null mutant (see also below), indicating that catalase activity in yeast mitochondria can be attributed to Cta1p which normally resides within peroxisomes. Western blot analysis of the organelle fraction additionally confirmed that the preparation was highly enriched in yeast mitochondria, since it predominantly contained the inner mitochondrial membrane marker Cox3p and was not contaminated with significant amounts of either cytosolic (Pgk1p) or endosomal (Pep12p) yeast proteins (Figure 1C).

Co-targeting of peroxisomal catalase A to mitochondria

To further confirm that peroxisomal catalase A can also be co-targeted to yeast mitochondria, a Cta1p-GFP-fusion protein was

constructed in which GFP was used as reporter and immunotag, and had been fused in frame to the C-terminus of Cta1p. Thus the C-terminal peroxisomal targeting signal of Cta1p (SKF) was masked by GFP. However, in contrast to most other peroxisomal proteins, yeast catalase A is known to contain a second internal peroxisomal targeting signal within the N-terminal third of its protein sequence, which has already been shown to be sufficient to independently drive Cta1p import into peroxisomes [31–34].

The Cta1p–GFP-fusion protein was constitutively expressed in cells of the $\Delta cta1$ mutant, and the corresponding transformants were cultivated in SC medium with glucose as carbon source. To confirm that the GFP-tagged version of catalase A is functional in the $\Delta cta1$ null mutant, mitochondrial catalase activity was determined in cell fractions that were enriched for yeast mitochondria, as judged from the presence of Cox3p and the absence of cytosolic and/or endosomal cross-contaminants (see Figure 1C). Interestingly, catalase activity in mitochondrial fractions of the Cta1p–GFP-expressing mutant was even higher than in the corresponding wild-type catalase (Figure 1A), most likely because catalase A expression was driven by the strong and constitutive *PGK1* promoter. Furthermore, expression of Cta1p–GFP also complemented the known growth defect of a $\Delta cta1$ mutant [30] in the presence of oleic acid as peroxisome-inducing carbon source (results not shown), again confirming that the Cta1p–GFP-fusion protein is indeed functional. Localization *in vivo* of Cta1p–GFP in cells of the $\Delta cta1$ mutant was further analysed by fluorescence microscopy. This showed a punctuate fluorescence pattern that is characteristic for distinct organelles, such as peroxisomes and mitochondria, in part overlapping with the peroxisomal targeting of the C-terminally SKL-extended GFP variant GFP^{SKL}, which had been included as positive control (Figure 2A). Comparison of the GFP fluorescence pattern with the peroxisomal targeting seen in the same cell after co-expression of DsRed^{SKL} identified partial overlap of the fluorescent signal in yeast peroxisomes, but additional staining of organelles which most likely represent yeast mitochondria (Figure 2B). Mitochondrial localization of Cta1p–GFP in the $\Delta cta1$ deletion mutant could be demonstrated by simultaneous co-staining with the mitochondrion-selective probe MitoTracker Orange, resulting in an identical staining pattern *in vivo* of Cta1p–GFP and yeast mitochondria (Figure 2C).

In order to obtain additional evidence for the dual targeting of catalase A into both peroxisomes and mitochondria, yeast cells that had been cultivated in SC medium supplemented with 2% glucose were subjected to further cell fractionation. The resulting subcellular fractions were purified by ultracentrifugation through a Nycodenz step gradient and subsequently analysed for protein content, D-amino acid oxidase and glutamate dehydrogenase activity. Aliquots of each gradient fraction were also used for Western blot analysis probed with monoclonal antibodies against the marker proteins Cox3p, CPY and the endosomal yeast protein Pep12p. The results presented in Figure 3 clearly showed that there were two peaks in the protein distribution in fractions 7 and 13. As the maxima of D-amino acid oxidase activity and the presence of the endosomal marker protein Pep12p co-localized in fraction 7, it can be concluded that this fraction represents the bulk fraction of yeast peroxisomes (labelled P in Figure 3). In contrast, glutamate dehydrogenase activity was mainly present in fraction 13, which also contained the inner mitochondrial membrane marker enzyme Cox3p, as shown in the corresponding immunoblot (labelled M in Figure 3). All together, these results indicate that fractions 7 and 13 are enriched in yeast peroxisomes and mitochondria respectively. As expected, the vacuolar marker protein CPY was mainly present in the last three fractions, because vacuoles possess specific floating properties that prevent entry

Figure 2 Targeting of catalase A to yeast peroxisomes and mitochondria

Fluorescence microscopy and confocal laser-scanning microscopy of a yeast $\Delta cta1$ null mutant expressing either Cta1p–GFP or GFP^{SKL} (A) or co-expressing Cta1p–GFP and DsRed^{SKL} (B) after growth in synthetic complete (SC) medium on glucose as carbon source. (C) Mitochondrial localization of Cta1p–GFP was determined by co-staining with the mitochondrion-selective probe MitoTracker Orange CMTMRos. Scale bar, 2 μ m.

and deeper penetration into the gradient [26]. The observed sedimentation profile of Cta1p–GFP in Nycodenz gradient fractions strongly indicates that yeast catalase A can be efficiently co-targeted to two different organelles, peroxisomes and mitochondria, and also nicely confirmed the enzyme assays in which Cta1p was shown to be responsible for catalase activity in yeast mitochondria (see Figure 1).

Import of catalase A into mitochondria and peroxisomes depends on the growth conditions

Next, we studied if carbon source and growth conditions do have any effect on the co-import of Cta1p–GFP into peroxisomes and mitochondria. For this purpose, Cta1p–GFP-expressing cells of the $\Delta cta1$ mutant were grown to mid-log phase in either complex YEPD medium or synthetic minimal medium with either raffinose or oleic acid as non-fermentable carbon source. Control cultures with glucose as fermentable carbon source were routinely included in the experiments. Yeast biomass was harvested, cells were fractionated and the resulting subcellular fractions were probed with a monoclonal anti-GFP antibody as described above. Immunoblot analysis indicated that after cultivation of Cta1p–GFP-expressing yeast cells under peroxisome-inducing conditions in the presence of oleic acid as carbon source, catalase activity was not only present in peroxisomes, but also in

Figure 3 Subcellular localization of a Cta1p-GFP in the yeast *S. cerevisiae*

An organelle pellet was isolated from a yeast $\Delta cta1$ null mutant carrying a catalase A-GFP expression vector (pCTA1-GFP) and further purified and fractionated by Nycodenz equilibrium density gradient centrifugation. Fractions 1 and 18 represent the bottom and top fractions of the gradient. Activities of D-amino acid oxidase (D-AAO, $\square$) and glutamate dehydrogenase (GDH, $\blacksquare$) were determined as peroxisomal and mitochondrial marker enzymes respectively. Protein concentration in each gradient fraction is shown by a grey box. Aliquots of each gradient fraction were also separated by SDS/PAGE and probed with monoclonal antibodies against Cox3p (inner mitochondrial membrane protein), Pep12p (endosomal protein) and CPY (vacuolar protein). Cta1p-GFP was detected with monoclonal anti-GFP antibody. Note that protein signals of Cta1p-GFP and Cox3p present in the lighter gradient fractions 16–18 are caused by organelle damage during cell lysis and subsequent leakage of matrix and/or membrane-associated proteins.

Figure 4 Enhanced targeting of Cta1p-GFP to mitochondria under respiratory growth conditions

Organelle fractions were isolated from the $\Delta cta1$ null mutant [carrying a catalase A-GFP expression vector (pCTA1-GFP)] after growth in SC medium containing either glucose, raffinose or oleic acid as carbon source, and peroxisomes and mitochondria were separated by density gradient centrifugation on Nycodenz (as described in the legend to Figure 3). Aliquots from each gradient fraction were used to determine the activity of marker enzymes [D-amino acid oxidase (D-AAO, $\square$) and glutamate dehydrogenase (GDH, $\blacksquare$)], and after SDS/PAGE and Western blot analysis, Cta1p-GFP was probed with a monoclonal anti-GFP antibody. Protein content in each gradient fraction is shown by the grey boxes.

significant amounts in yeast mitochondria (Figure 4), indicating that Cta1p-GFP is being constitutively targeted to both organelles. The same was true when yeast was grown in complex YEPD medium until glucose was consumed and cells had entered a second growth phase by utilizing amino acid compounds of the complex medium. Interestingly, the exact opposite phenomenon

Figure 5 Activity and distribution of peroxisomal and mitochondrial catalase A is greatly affected by the nature of the carbon source

(A) Mitochondrial catalase A co-targeting and activity in a yeast $\Delta cta1$ deletion mutant expressing a Cta1p-GFP-fusion protein is significantly enhanced when cells are grown on raffinose as non-fermentable carbon source. In contrast, growth on the peroxisome-inducing carbon source oleic acid targets Cta1p-GFP mainly to yeast peroxisomes. Subcellular fractionation and organelle purification through Nycodenz equilibrium density gradient centrifugation was performed as described in the legends to Figures 3 and 4. (B) Nycodenz gradient fractions from cells of the *CTA1* wild-type strain BY4741 and the $\Delta cta1$ null mutant served as positive and negative controls respectively, and showed Cta1p activity in peroxisomes and mitochondria in the *CTA1* wild-type, but no organelle-associated catalase activity in the $\Delta cta1$ mutant. Results are presented for a single representative experiment.

could be observed when cells were cultivated under respiratory conditions on the non-fermentable carbon source raffinose. Under such growth conditions, catalase A was predominantly targeted to mitochondria, whereas only low amounts of Cta1p-GFP were present in peroxisomes. When cells were grown in SC medium with glucose as carbon source, Cta1p-GFP was equally distributed in peroxisomes and mitochondria, again indicating that under fermentative growth conditions catalase is constitutively targeted to both organelles (Figure 4).

Mitochondrial catalase activity was also studied after growth of Cta1p-GFP-expressing cells of the $\Delta cta1$ null mutant on different carbon sources. As illustrated in Figure 5(A), catalase activity in subcellular fractions was in perfect agreement with the results from the immunoblot analysis: highest peroxisomal catalase activity was detected when Cta1p-GFP-expressing cells were cultivated either on SC medium supplemented with oleic acid or on complex YEPD medium. Under peroxisome-repressing conditions in the absence of oleic acid, maximal mitochondrial catalase activity was present when cells were grown in the presence of the non-fermentable carbon source raffinose (Figure 5A). Significantly lower levels of catalase activity were detected in glucose-grown yeast cultures; however, under such conditions catalase activity was equally distributed between both compartments, peroxisomes and mitochondria. As expected, catalase activity in Nycodenz gradient-purified cell fractions of

Figure 6 Cta1p-GFP localizes to the mitochondrial matrix

(A) Enzyme activities of catalase, adenylate kinase and glutamate dehydrogenase in submitochondrial fractions of a yeast $\Delta cta1$ null mutant expressing Cta1p-GFP and grown in glucose containing SC medium. Isolated and Nycodenz gradient-purified yeast mitochondria were fractionated into mitoplasts (M) and intermembrane space (IMS) by a hypotonic treatment and subsequently analysed for the indicated marker enzymes. All assays were performed in triplicate and the results are expressed as the means $\pm$ S.D. (B) Submitochondrial localization of the Cta1p-GFP-fusion protein was performed by Western blotting using a monoclonal anti-GFP antibody. Each lane was loaded with 40 μ g of total mitochondrial protein. The protein bands marked with an asterisk in lane M are most likely to represent N-terminal degradation products of Cta1p-GFP that were not present in mitochondrial subfractions of negative control cells (results not shown). The low-molecular-mass protein signal seen in lanes IMS and M represent unspecific cross-hybridization products of the anti-GFP antibody.

the $\Delta cta1$ null mutant (negative control) was neither detectable in peroxisomes nor in mitochondria, whereas in the catalase wild-type strain BY4741 distribution of Cta1p activity exactly portrayed that of its tagged derivative, Cta1p-GFP, expressed in the $\Delta cta1$ null mutant (Figure 5B). Thus catalase mistargeting eventually caused by its C-terminal fusion to GFP can be excluded.

Submitochondrial localization of yeast catalase A

To further define the submitochondrial localization of catalase A, mitochondria were isolated from Cta1p-GFP-expressing cells of the $\Delta cta1$ null mutant and purified by Nycodenz equilibrium density gradient centrifugation as described above. The outer mitochondrial membrane was ruptured by a hypotonic treatment, and the intermembrane space was separated from the mitoplast fraction by differential centrifugation. Mitoplasts were additionally ruptured by a swelling-shrinking procedure and both fractions (intermembrane space and mitoplast) were subsequently used for enzyme activity assays, as well as for SDS/PAGE and Western blot analysis (Figure 6). A strong Cta1p-GFP signal was only detectable within the mitochondrial matrix fraction, which also contained 99 % of total mitochondrial catalase activity. Furthermore, activity of yeast adenylate kinase (as marker for the mitochondrial intermembrane space) and glutamate dehydrogenase (as mitochondrial matrix marker) likewise confirmed the matrix localization of catalase A in yeast; Cta1p-GFP activity always co-fractionated with the mitochondrial matrix enzyme glutamate dehydrogenase, again indicating that yeast catalase A is present within the mitochondrial matrix (Figure 6).

Co-targeting of yeast catalase A is not negatively affected by fusion of GFP to the C-terminus

To further exclude the possibility that the GFP moiety in Cta1p-GFP might have caused mitochondrial mistargeting of catalase A, two additional Cta1p derivatives were constructed in which GFP was replaced either by the 10-amino-acid epitope c-Myc

Figure 7 Peroxisomal and mitochondrial co-targeting of Cta1p^{myc} and Cta1p^{myc-SKF}

Organelle fractions from either Cta1p^{myc} or Cta1p^{myc-SKF} expressing yeast cells ($\Delta cta1$) after growth in synthetic complete (SC) medium on glucose as carbon source were purified by Nycodenz density centrifugation, and aliquots from each gradient fraction were probed with a monoclonal anti-c-Myc antibody; a monoclonal anti-porin antibody served as yeast mitochondrial outer membrane marker. Nycodenz gradient fractions enriched for yeast peroxisomes and mitochondria (as judged by the activity of peroxisomal and mitochondrial marker enzymes) are indicated by P and M respectively.

(Cta1p^{myc}) or by a c-Myc tag that had been C-terminally extended by the natural PTS1 (-SKF) of Cta1p (Cta1p^{myc-SKF}). Both Cta1p variants, Cta1p^{myc} and Cta1p^{myc-SKF}, were biologically active and restored specific catalase activity in isolated yeast mitochondria of the $\Delta cta1$ null mutant to 5.3 and 5.6 units/mg respectively. As shown above for the catalase A-GFP fusion, both c-Myc-tagged Cta1p derivatives likewise co-targeted to peroxisomes and mitochondria after expression in a $\Delta cta1$ null mutant (Figure 7), and, again, mitochondrial co-targeting was most prominent under respiratory growth conditions when yeast cells were cultivated on the non-fermentable carbon source raffinose (results not shown). Since catalase activity in a yeast Cta1⁺ wild type showed exactly the same subcellular distribution as either of the tagged Cta1p derivatives Cta1p-GFP, Cta1p^{myc} or Cta1p^{myc-SKF} (see Figure 5B), we concluded that co-targeting of Cta1p to peroxisomes and mitochondria *in vivo* is not negatively affected by the addition of an epitope tag to the natural C-terminus of catalase A or by a C-terminal fusion to GFP. The observed peroxisomal and mitochondrial co-localization of both derivatives, Cta1p^{myc} and Cta1p^{myc-SKF}, further suggests that catalase A co-targeting is equally effective in the presence or absence of its natural SKF motif at the C-terminus, confirming previous studies by Kragler et al. [33] who showed that the C-terminal PTS1 in Cta1p can either be removed or masked without affecting catalase A targeting *in vivo*.

DISCUSSION

In contrast to mammalian cells which only express a single type of catalase in peroxisomes, yeast cells (*S. cerevisiae*) possess two distinct catalase isoenzymes, an atypical peroxisomal catalase A and a typical cytosolic catalase T, which both play an essential role in protecting yeast cells against oxidative stress [35]. However besides peroxisomes, mitochondria represent another compartment where most cellular ROS are being produced, and recently we presented evidence indicating that yeast mitochondria might indeed be the third compartment in which catalase activity can be found [9]. In the present study, we show that peroxisomal catalase A is responsible for mitochondrial catalase activity in *S. cerevisiae*, and provide additional evidence that peroxisomal

catalase A can be co-targeted to mitochondria, in particular when yeast is cultivated under respiratory growth conditions that favour ROS accumulation in this organelle.

In addition to its C-terminal PTS1, yeast catalase A contains a second internal targeting signal within the N-terminal part of the protein which can independently direct Cta1p to the peroxisome, even in the complete absence of PTS1 or even when the C-terminal PTS1 is masked by GFP [33]. We now extend these findings by expressing a Cta1p–GFP-fusion protein, as well as two different C-terminally Myc-tagged derivatives of Cta1p (Cta1p^{myc} and Cta1p^{myc-SKF}) in a yeast $\Delta cta1$ null mutant. We thereby demonstrate that the N-terminal signal in Cta1p harbours more than just a single organelle targeting signal, since we show that it can function in both peroxisomal and mitochondrial targeting. Cta1p–GFP, in which GFP was fused in frame to the C-terminus of catalase A, was efficiently targeted to peroxisomes, in particular when cells were grown on oleic acid as carbon source. The increase in peroxisomal catalase activity observed in cells grown in YEPD after glucose consumption was due to the availability of amino acid compounds present in the medium which are metabolized in yeast and higher fungi through the action of H₂O₂-producing oxidases [36]. Oleic acid is the major carbon source that induces peroxisome formation in yeast [30,37], resulting in maximal activation of β -oxidation and, in turn, leading to endogenous H₂O₂ production and high-level expression of peroxisomal catalase A [38]. Our results confirm that catalase A is predominantly targeted to yeast peroxisomes under conditions that enhance H₂O₂ formation in this organelle. However, even under peroxisome-inducing conditions, about 25 % of total catalase activity was also present in mitochondria where respiration causes rapid accumulation of ROS. In yeast cells that had been cultivated on the fermentable carbon source glucose, catalase A activity in peroxisomes and mitochondria was significantly lower, but Cta1p protein level was equally distributed in both organelles. When yeast cells were cultivated on raffinose, formation of peroxisomes is depressed and intensive mitochondrial respiration occurs [39]. In this case, a remarkable shift was observed in the subcellular distribution of yeast peroxisomal catalase A, and 60 % of the Cta1p activity could be localized to the mitochondrial matrix. Since no difference in the targeting of Cta1p–GFP *in vivo* or its C-terminally c-Myc-tagged derivatives Cta1p^{myc} and Cta1p^{myc-SKF} was observed, it is unlikely that the addition of either GFP or a Myc tag to CTA1 might have caused mitochondrial mistargeting of Cta1p. We therefore conclude that yeast catalase A can be targeted independently to two distinct compartments, peroxisomes and mitochondria, and that the distribution of Cta1p between both organelles is greatly affected by the cell physiology, the growth conditions and by the nature of the carbon source. Although Cta1p expression was driven from a constitutive yeast promoter (*PGK1*), the same subcellular distribution was observed when enzymic catalase activity was measured in cell fractions of a catalase wild-type yeast in which Cta1p expression is driven from its natural promoter. Based on these observations it can be predicted that catalase co-targeting is regulated at the post-translational level; whether transcriptional regulation might also play an additional role in Cta1p *in vivo* co-targeting, especially under different growth conditions in the presence of a non-fermentable carbon source, could be addressed in further studies by using a CTA1–GFP-fusion gene that has been integrated into its natural chromosomal location and expressed from its own promoter; such kind of experiments are planned for the near future.

So far, co-targeting to different organelles *in vivo* has only been demonstrated for a few proteins; examples for such a co-targeting are the enzyme carnitine acetyltransferase of *S.*

cerevisiae [40] and yeast citrate synthase Cit2p, a peroxisomal enzyme whose 20-amino-acid N-terminal sequence resembles a cryptic targeting signal for both peroxisomes and mitochondria [1]. Because peroxisomal import of Cit2p is functional even in the absence of Pex5p and Pex7p (the cellular receptors for PTS1 and PTS2 respectively), it has been suggested that peroxisomal targeting mediated by the N-terminal segment of Cit2p might utilize a novel receptor that also catalyses Cit2p import into mitochondria [1]. However, the precise *in vivo* function of such a co-targeting is not at all evident, since yeast possess two additional citrate synthase isoenzymes (Cit1p and Cit3p) which both are exclusively located in mitochondria [38], making it difficult to explain why peroxisomal Cit2p would also be needed in mitochondria. For yeast catalase A, this situation is different. Although peroxisomal and mitochondrial co-targeting of yeast Cta1p and Cit2p might be mechanistically similar, the presence of catalase A in yeast mitochondria makes sense from a physiological point of view. Under respiratory growth conditions in the presence of a non-fermentable carbon source (like raffinose), mitochondrial respiration is maximally induced, ROS are rapidly accumulating and detoxification of H₂O₂ *in vivo* catalysed by Cta1p would be an advantage for the cell. In the present study we show that this is indeed the case: under respiratory growth conditions, yeast Cta1p is efficiently co-targeted to mitochondria where it presumably co-operates with Mn-SOD and Cu,Zn-SOD in the enzymic detoxification of H₂O₂. Our finding that peroxisomal catalase can co-target mitochondria is nicely supplemented and further supported by more recent studies in which catalase was identified within the human heart mitochondrial proteome [41], as well as in rat heart mitochondria [42]. Most interestingly, catalase levels were 6-fold increased in mitochondria derived from diabetic rats [42], indicating that catalase co-targeting to mitochondria, as described in the present study, might represent an important antioxidant defence response to the increased oxidative stress in diabetes. To study the physiological relevance of this interesting observation in more detail, yeast will be an attractive system to identify a distinct phenotype under conditions when mitochondrial co-targeting of catalase is blocked; such studies will be done for future work.

Another intriguing question arising from the observed co-targeting of yeast Cta1p addresses a more general aspect in eukaryotic cell biology by asking how this transport is being realized *in vivo*. While most peroxisomal proteins can be translocated into the peroxisomal matrix in a folded state, as has been confirmed for the *Candida boidinii* peroxisomal catalase A [32], protein import into mitochondria usually occurs on a transport-competent protein in an unfolded state and additionally requires various cytosolic, as well as mitochondrial, chaperones [43–46]. In this respect, yeast catalase A is particularly interesting, because it resembles the prime example of a protein that can be simultaneously co-targeted to peroxisomes and mitochondria, although it lacks a classical N-terminal mitochondrial import sequence. In future studies we will focus, therefore, on the molecular basis of catalase A co-import into peroxisomes and mitochondria, and we will investigate Cta1p targeting in a yeast $\Delta cta1$ deletion mutant that is simultaneously deficient in various Tim, Tom and/or Pex proteins, which are known to be required for proper protein import into both mitochondria and peroxisomes.

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