Effect of catalase-specific inhibitor 3-amino-1,2,4-triazole on yeast peroxisomal catalase in vivo

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Abstract

3-Amino-1,2,4-triazole (3-AT) is known as an inhibitor of catalase to whose active center it specifically and covalently binds. Subcellular fractionation and immunoelectronmicroscopic observation of the yeast *Candida tropicalis* revealed that, in 3-AT-treated cells in which the 3-AT was added to the *n*-alkane medium from the beginning of cultivation, catalase transported into peroxisomes was inactivated and was present as insoluble aggregated forms in the organelle. The aggregation of catalase in peroxisomes occurred only in these 3-AT-treated cells and not in cells in which 3-AT was added at the late exponential growth phase. Furthermore, 3-AT did not affect the transportation of catalase into peroxisomes. The appearance of aggregation only in cells to which 3-AT was added from the beginning of cultivation suggests that, in the process of catalase transportation into yeast peroxisomes, some conformational change may take place and that correct folding may be inhibited by the binding of 3-AT to the active center of catalase. Accordingly, 3-AT will be an interesting compound for investigation of the transport machinery of the peroxisomal tetrameric catalase.

1. Introduction

Catalase is one of the marker enzymes of peroxisomes. After the catalase gene encoded on the nuclear chromosome is transcribed, catalase mRNA is translated on free polysomes in the cytosol [1–3]. The synthesized catalase molecules localize into peroxisomes post-translationally [4].

3-AT inhibits catalase activity by binding covalently to the active center of the active tetrameric form containing hemes [5–7]. Middelkoop et al. report that 3-AT inhibits the peroxisomal transportation of catalase in Zellweger cell lines of human skin fibroblasts [8]; they found that, before the transportation of catalase into peroxisomes, 3-AT bound with it in the cytosol and prevented it from passing across the peroxisomal membrane, and also that catalase, once bound with 3-AT, requires a higher concentration of urea for transition from the folded to the unfolded conformation. These results suggest that for localization into peroxisomes unfolding of catalase is necessary before contact with the peroxisomal membrane and that covalent binding with 3-AT inhibits the transition to the unfolded conformation.

Regarding the transportation mechanism for peroxisomal proteins, specific three amino acid residues at the carboxy-terminus have been thought to act as peroxisomal targeting signals (PTSs) [9]. The sequence serine-lysine-leucine and its variants are known as PTS1. PTS2, which is cleavable in some cases, has been found at the amino-terminus of 3-ketoacyl-CoA thiolases [10,11]. As for the machinery at the peroxisomal membrane, the genes encoding the receptors for PTS1 and PTS2 have been identified and characterized: *PAS10* (*PEX5*) [12–14] and *PAS7* (*PEX7*) [12,15,16] in *Saccharomyces cerevisiae* and *PAS8* (*PEX5*) in the methylotrophic yeast *Pichia pastoris* [17].

Although these typical PTSs have not been found in catalase, it has been shown that a PTS1-like sequence is

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present in some species [18–20]. The difficulty in studying catalase transportation is that the mechanism for catalase subunit transportation and the binding of heme, which is the co-factor of catalase, needs to be considered. The binding of heme with catalase has been postulated to occur inside peroxisomes [21]. This, however, is suspected not only because of the presence of cytosolic catalase in \( S.\ cervevisiae \) but also because of the ability of peroxisomal catalase to bind with heme in the cytosol [22–25]. Our previous studies point to the simultaneous and stoichiometrical transportation of heme and the catalase subunit [26,27], suggesting that catalase is transported from the cytosol into peroxisomes through interaction with its co-factor heme, or that there is an unknown mechanism controlling the stoichiometric relationship between the catalase subunit and heme. Several proteins are known to be transported into peroxisomes as multimeric forms generated in the cytosol [28–30]. Catalase may also be transported into peroxisomes as a tetrameric form. Unlike in human skin fibroblast, in which 3-AT inhibits the transportation of catalase into peroxisomes by retarding unfolding [8], the treatment of yeast cells with 3-AT in the present study did not affect the localization of yeast catalase, and catalase transported into peroxisomes was present in peroxisomes as aggregated forms. These phenomena suggest that some conformational change such as rearrangement or refolding to form a tetramer may occur.

2. Materials and methods

2.1. Cultivation of yeast

\( C.\ tropicalis \) pK 233 (ATCC 20336) was cultivated aerobically at 30°C in a medium containing glucose (16.5 g l\(^{-1}\)) or \( n \)-alkane mixture (\( C_{10} - C_{13} \)) (10 ml \( l^{-1}\)) as the sole source of carbon and energy [26].

2.2. Preparation of cell-free extract and subcellular fractionation

Cell-free extract was prepared by disintegrating the cells through sonication (2.4 A, 20 kHz, 2.5 min) in 50 mM potassium phosphate buffer (pH 7.2) at 0°C [26]. Subcellular fractionation of \( n \)-alkane-grown \( C.\ tropicalis \) was performed using a procedure described previously [26]. The cell wall was lysed with zymolyase 20T, the protoplasts obtained homogenized with a Teflon homogenizer, and the homogenate fractionated by differential centrifugation. The fractions obtained were as follows: \( S_1 \) fraction (5000 \( \times g \) supernatant); \( S_2 \) fraction (20000 \( \times g \) supernatant composed of cytosol and microsomes); \( P_2 \) fraction (20000 \( \times g \) pellets consisting of mitochondria and peroxisomes); \( S_1 \) fraction (139000 \( \times g \) supernatant consisting of cytosol); \( P_3 \) fraction (139000 \( \times g \) pellet consisting of microsomes). The \( P_2 \) fraction was further subjected to discontinuous sucrose density gradient centrifugation (sucrose concentration from top to bottom, 20, 30, 40, 41.3, 42.5, 50% (w/v), each 2.5 ml; 49600 \( \times g \); 2 h, 0°C) to separate peroxisomes and mitochondria [26]. To separate soluble matrix proteins and insoluble proteins in the \( P_2 \) fraction, it was subjected to hypotonic shock for 1 h on ice in 10 mM Tris–HCl buffer (pH 7.4) and frozen overnight [31]. After thawing, membrane and matrix fractions were separated by ultracentrifugation at 127000 \( \times g \) for 1 h. The soluble fraction obtained was concentrated with Centriplus-30 (Amicon Co., Beverly, MA, USA).

2.3. Measurement of cellular and subcellular heme content

The amount of cellular and subcellular heme, which contained both protein-associated and free heme, was determined by the pyridine hemochrome method [26] as follows: 1 M NaOH (0.5 ml) and pyridine (0.5 ml) were added to 3 ml of a protein solution or cell suspension. The difference spectrum of the reduced minus-oxidized pyridine hemochrome was recorded and heme content quantified from the molar extinction coefficient \( \epsilon_{578}^{\text{M}} = 20.7 \).

2.4. Enzyme and protein assay

Cytochrome \( c \) oxidase, acyl-CoA oxidase, and protein were assayed using methods previously described [32]. Catalase activity was measured spectrophotometrically by following the decrease of \( H_2O_2 \) at 240 nm [26].

![Distribution of catalase in peroxisomes from yeast cells treated with 3-AT.](https://example.com/fmsle102062-03.png)
2.5. Western blot analysis

Western blot analysis using anti-*C. tropicalis* peroxisomal catalase antibodies was carried out as reported previously [27].

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Catalase activity (nmol min(^{-1}) mg(^{-1}) protein)</th>
<th>Heme content (nmol mg(^{-1}) protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>3.224</td>
<td>0.652</td>
</tr>
<tr>
<td>3-AT-added cells</td>
<td>0.130</td>
<td>0.708</td>
</tr>
<tr>
<td>3-AT-treated cells</td>
<td>0.089</td>
<td>0.660</td>
</tr>
</tbody>
</table>

Fig. 2. Electronmicrographs and immunoelectronmicrographs with anti-*C. tropicalis* catalase antibodies of *C. tropicalis* cells and P\(_2\) fractions. a\(_1\), b\(_1\), c\(_1\): electronmicrographs of cells; a\(_2\), b\(_2\), c\(_2\): immunoelectronmicrographs of cells; a\(_3\), b\(_3\), c\(_3\): P\(_2\) fractions. a\(_1\), a\(_2\), and a\(_3\): control cells; b\(_1\), b\(_2\), and b\(_3\): 3-AT-treated cells; c\(_1\), c\(_2\), and c\(_3\): 3-AT-added cells. a\(_3\), b\(_3\), and c\(_3\) are the P\(_2\) fractions of each cell. Arrowheads in b\(_3\) show aggregated portion of catalase proteins. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole.
Fig. 3. Subcellular fractionation of cells cultivated in presence or absence of 3-AT. A: Particulate localization of enzymes in P2 fractions. P2 fractions were subjected to discontinuous sucrose density gradient centrifugation to separate peroxisomes and mitochondria. Acyl-CoA oxidase and cytochrome c oxidase were the marker enzymes of peroxisomes and mitochondria, respectively. The volume of each fraction was as follows: F1, 3.75 ml; F2–F5, 2.5 ml each; F6, 1.25 ml. a: control cells; b: 3-AT-treated cells. B: Localization of catalase detected by Western blot analysis (10 μg protein per lane). a, b, and F1–F6: the same as in A.
2.6. Electronmicroscopic studies

The subcellular structure was observed after yeast cells were pre-fixed with 5% (w/v) glutaraldehyde and post-fixed with osmium tetroxide. For immunocytochemical labelling, the cells were fixed with 0.5% glutaraldehyde and 3% paraformaldehyde. A gold particle-labelled goat anti-rabbit IgG was used as the secondary antibody [27,32].

3. Results and discussion

To investigate the effect of 3-AT on catalase transportation into the peroxisomes of n-alkane-grown C. tropicalis, we used three kinds of cells cultivated under different conditions: (1) control cells cultivated till the late exponential growth phase (16 h) without 3-AT; (2) ‘3-AT-treated cells’ cultivated for 16 h with 3-AT (10 mM) present from the beginning of cultivation and in which 3-AT (10 mM) was thus present during the expression of the catalase gene and transportation of both catalase subunits and heme molecules into peroxisomes; (3) ‘3-AT-added cells’ cultivated without 3-AT (10 mM) for 14 h, by which time the expression of the catalase gene and transportation of both catalase subunits and heme molecules into peroxisomes is thought to be finished, and then incubated for a further 2 h with 3-AT (10 mM) [26]. In the 3-AT-treated cells, 10 mM of 3-AT was enough to decrease the catalase activity to less than one-tenth that of the control cells. The cellular heme content, which was high relative to catalase activity [26], was almost the same as the control cells, when measuring at each harvesting time (Table 1).

Differential fractionation of cells and further fractionation of the soluble and insoluble portions after disruption of the particulate fractions by osmotic shock revealed that catalase transported into the peroxisome-containing particulate fraction aggregated as insoluble forms in the organelle in the case of 3-AT-treated cells (Fig. 1, lanes 5 and 6). Although the amounts of each soluble protein or each insoluble protein in Fig. 1, lanes 1–6 were almost the same, catalase from the control and 3-AT-added cells was recovered as a soluble protein from the peroxisome-containing particulate fraction after osmotic treatment (Fig. 1, lanes 1–4). Immunoelectronmicroscopy with anti-C. tropicalis catalase antibodies was used to confirm these phenomena. Fig. 2 shows that even in the presence of 3-AT (10 mM) for 16 h, catalase localized clearly in the yeast peroxisomes, unlike in human fibroblasts. The mode of catalase localization in peroxisomes, however, was quite different from that of the control cells. The peroxisomes of the 3-AT-treated cells displayed condensing signs of catalase (Fig. 2, b2), indicating that catalase was in the aggregated form. These results clarified that, in C. tropicalis, unlike human fibroblast, 3-AT has no effect on the localization of catalase into peroxisomes, but does influence the assembly of catalase into the native form in the organelle, and suggest that some change of conformation in the native or 3-AT-bound catalase protein may occur during transportation into peroxisomes.

Immunoelectronmicroscopy revealed the above-mentioned signs of aggregated catalase proteins only in the peroxisomes of the 3-AT-treated cells, and not in the control and 3-AT-added cells. Sucrose density fractionation was therefore carried out to investigate the transportation of catalase in the 3-AT-treated cells. As indicated in Fig. 3, acyl-CoA oxidase, the marker enzyme of peroxisomes, was found in the F2 (mitochondrial) fraction of the control cells. Catalase also localized in the F5 fraction of the control cells, but almost no catalase activity was detected in the 3-AT-treated cells. Western blot analysis with anti-C. tropicalis catalase antibodies, in contrast, showed that catalase subunits clearly localized in the F3 fraction of the 3-AT-treated cells, and further showed co-factor heme present in the F2 (mitochondrial) fraction and F3 fraction as in the control cells. These results suggest that both catalase subunit and heme are transported into peroxisomes whether 3-AT is present or not.

It has not been clarified whether the binding of 3-AT with native catalase occurs before or after the latter passes through the peroxisomal membrane. The fact that the aggregated form of catalase was observed only in the 3-AT-treated cells and not in the 3-AT-added cells suggests the possibility that a conformational rearrangement involving transition between the folded and unfolded conformations occurs. The intermediate situation at the peroxisomal membrane in the tetrameric assembly and covalent binding with 3-AT of C. tropicalis catalase needs to be further clarified. Further study of mutants of catalase which cannot assemble into tetramer nor bind with heme may reveal the details of the transportation system.

References


