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# Location of catalase in crystalline peroxisomes of methanol-grown *Hansenula polymorpha*

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## 1. SUMMARY

We have studied the intraperoxisomal location of catalase in peroxisomes of methanol-grown *Hansenula polymorpha* by (immuno)cytochemical means. In completely crystalline peroxisomes, in which the crystalline matrix is composed of octameric alcohol oxidase (AO) molecules, most of the catalase protein is located in a narrow zone between the crystalloid and the peroxisomal membrane. In non-crystalline organelles the enzyme was present throughout the peroxisomal matrix. Other peroxisomal matrix enzymes studied for comparison, namely dihydroxyacetone synthase, amine oxidase and malate synthase, all were present throughout the AO crystalloid. The advantage of location of catalase at the edges of the AO crystalloids for growth of the organism on methanol is discussed.

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## 2. INTRODUCTION

In eukaryotic cells catalase is of major importance for the decomposition of hydrogen peroxide ($H_2O_2$) produced intracellularly. In yeasts, enhanced levels of this enzyme are synthesized during growth of cells on various carbon and nitrogen sources, the metabolism of which is mediated by peroxisome-borne $H_2O_2$-producing oxidases. Well-known examples of such compounds include oleic acid, methanol, D-amino acids and purines [1]. The induced catalase protein is generally located at the sites of $H_2O_2$ generation, namely inside the peroxisomal matrix [2,3].

In cells of *Hansenula polymorpa* growing in methanol-limited continuous cultures, completely crystalline peroxisomes always develop [1]. These crystalloids, which are composed of octameric alcohol oxidase (AO) molecules, are thought to be very open structures which may accommodate other peroxisomal enzymes, including catalase, probably in a freely diffusable form [4]. Recent 3-D studies, however, suggested that, based on their mature size, catalase molecules may not be

able to move freely through the intracrystalline channels of the AO crystalloids [5]. These findings prompted us to reconsider the location of catalase in vivo in completely crystalline peroxisomes present in methanol-grown *H. polymorpha*. The results of this study are presented in this paper.

## 3. MATERIALS AND METHODS

### *3.1. Growth conditions*

*Hansenula polymorpha* CBS 4732 was grown in methanol-limited chemostat cultures at 37°C at a dilution rate of 0.07 $h^{-1}$ [6] in the presence of 0.25% (w/v) ammonium sulphate or 0.25% (w/v) ethylamine as a nitrogen source. In addition, cells were grown in batch cultures [7] on either glucose (0.5% w/v) or methanol (0.5% w/v) in the presence of the above nitrogen sources.

### *3.2. Electron microscopy*

Spheroplasts were prepared by treatment of suspensions of intact cells with zymolyase [7] and fixed in glutaraldehyde/$OsO_4$ [7]. For freeze substitution, whole cells were frozen in liquid nitrogen, followed by substitution in uranyl acetate/methanol [8] and embedding in HM20.

Cytochemical staining experiments for the location of catalase activity were performed as described previously [9].

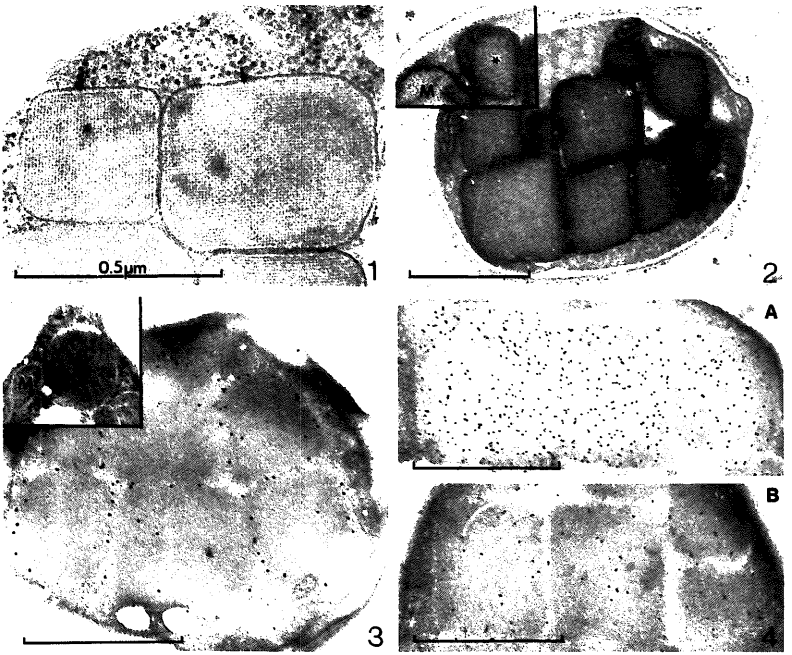
For immunocytochemistry, intact cells were fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 60 min at 0°C, dehydrated in a graded ethanol series and embedded in Lowicryl K4M. Immunolabelling was performed by the protein A/gold method [10], using specific antibodies against catalase, amine oxidase, malate synthase and dihydroxyacetone synthase.

## 4. RESULTS AND DISCUSSION

Cells of *H. polymorpha* grown in methanol-limited chemostat cultures contain completely crystalline peroxisomes, often cuboid in shape when either ammonium sulphate or ethylamine was used as a sole nitrogen source. Independent of the method used (thin-sectioning, freeze-substitution), the intraperoxisomal space between this crystalloid and the peroxisomal membrane was invariably very narrow and measured up to 10–20 nm (Fig. 1). Following incubation of such cells with DAB/$H_2O_2$ for the detection of catalase activity, the whole peroxisomal matrix was positively stained; however, staining was more intense in the small non-crystalline zone (Fig. 2). After immunocytochemical experiments, using specific antibodies against catalase and protein A/gold, labelling was located mostly in the non-crystalline zone with only few particles present in the crystalline matrix (Fig. 3). These results indicate that catalase protein is mainly located at the edges of the AO crystalloids. In partly crystalline peroxisomes, present in cells from batch cultures on methanol, accumulation of the DAB reaction product as well as specific-gold labelling was enhanced in the non-crystalline part of the organellar matrix (Fig. 2, inset). Moreover, in peroxisomes lacking AO, which are present in glucose/ethylamine-grown cells, gold labelling was randomly distributed over the organellar matrix (Fig. 3, inset). Immunocytochemical experiments on methanol/ethylamine-grown cells indicated that other peroxisomal matrix enzymes (amine oxidase, malate synthase and dihydroxyacetone synthase (DHAS)) were also located throughout the crystalline matrix (Fig. 4). Unexpectedly, a similar distribution pattern was frequently observed in partly crystalline peroxisomes in batch-cultured cells (not shown). Therefore, of the enzymes tested, only catalase was mainly outside the AO crystalloid. The preferential location of amine oxidase, DHAS and malate synthase in the peroxisomal crystalloid may reflect a specific affinity between these enzymes and AO. Further evidence for this suggestion was obtained in experiments with peroxisome-deficient mutants of *H. polymorpha* [11]; at high-expression levels of AO, large cytosolic crystalloids developed, the architecture of which was identical to WT AO crystalloids [12]. In these cytosolic crystalloids (lacking a surrounding membrane) other peroxisomal matrix proteins, such as DHAS, amine oxidase and

malate synthase had also accumulated, except for catalase which remained largely soluble [12].

Previous cytochemical experiments on purified peroxisomes, subjected to a mild osmotic shock, suggested that catalase was freely diffusible throughout the intracrystalline spaces of AO crystalloids [4]. However, recent data of Vonck and van Bruggen [5] on the 3-dimensional architec-




Figs. 1–4. M = mitochondrion. The marker represents 1 $\mu$m unless otherwise indicated. **1.** An ultrathin section of a glutaraldehyde/$OsO_4$-fixed spheroplast of *H. polymorpha*, grown in continuous culture on methanol, showing the completely crystalline structure of peroxisomes. Note the narrow space (arrows) between the alcohol oxidase crystalloid and the surrounding membrane. **2.** Cytochemical demonstration of catalase activity (DAB/$H_2O_2$). Peroxisomes (as shown in Fig. 1) are more intensely stained at their edges ($KMnO_4$). In partly crystalline peroxisomes in cells, grown in batch cultures on methanol, the non-crystalline part is more intensely stained (inset; crystalloid indicated by *; magnification 28000×). **3.** Immunocytochemical demonstration, using specific antibodies against catalase and protein A/gold, of catalase protein in peroxisomes of *H. polymorpha* grown in continuous culture on methanol. Labelling is predominant at the edges of the organelles. In non-crystalline peroxisomes in cells grown in batch cultures on glucose/ethylamine, labelling is randomly distributed over the organellar matrix (inset, magnification 24000×). **4.** Intraperoxisomal location of dihydroxyacetone synthase (DHAS; **4A**) and malate synthase (MS; **4B**). The labelling patterns indicate that both enzyme proteins are randomly distributed throughout the crystalloid (specific antibodies against DHAS/– or MS/protein A/gold).

ture of crystalloids of purified AO, formed in vitro from *H. polymorpha*, are in conflict with this view. A detailed 3-D analysis of such crystalloids showed that they contained large holes of $\pm 15$ nm$^2$, connected by channels. Based on the dimensions of these channels (4–5 nm) they concluded that relatively small-matrix enzymes such as DHAS (dimer of 154 kDa) may be mobile in these channels [5]; a similar reasoning may be true for amine oxidase (dimer of 156 kDa) and malate synthase (trimer of 186 kDa). In contrast, the size of mature catalase protein (a tetramer of 240 kDa) does not allow free passage through the channels but, on the other hand, may fit in the intracrystalline holes. Therefore, trapping of catalase during AO crystalloid formation may account for the catalase-protein contents of these structures in vivo, demonstrated in this study. Additional evidence for the intracrystalline location of catalase was obtained from a series of kinetic studies using methanol as the $H_2O_2$ generating system in the DAB reaction [4]. However, the amount of entrapped catalase is probably low as indicated by our immunocytochemical results. Hence, the relatively high-electron density of complete crystalline peroxisomes, after incubations with DAB/$H_2O_2$, may be at least partly explained by diffusion of DAB reaction products into the matrix. A similar mechanism, namely leakage of reaction products, rather than of enzyme protein, may account for the absence of DAB-reaction products in isolated peroxisomal AO crystalloids subjected to osmotic shock [4]. Our present data may also explain another general finding not yet understood, namely the preferential leakage of catalase from peroxisomes of methanol-grown *H. polymorpha* upon their isolation by convential subcellular fractionation methods [13].

The intriguing question remains why catalase, which constitutes only a relatively minor fraction of the total peroxisomal protein contents, is not fully entrapped in the crystalloids, as may have been expected from the fact that peroxisomes in chemostat-cultured *H. polymorpa* also are completely crystalline during their developmental stage. Is it possible that the observed location at the edges of the AO crystalloids may have distinct metabolic advantages during methylotrophic growth? That methanol can be used effectively as additional energy source during growth of catalase-negative mutants of *H. polymorpha* on glucose [12] suggests that the activities of peroxisomal enzymes are probably not affected by the $H_2O_2$ generated from methanol under these conditions; hence, the main function of catalase probably does not include protection of the matrix enzymes from inhibition by $H_2O_2$.

On the other hand, the presence of intact, catalase-containing peroxisomes is essential for growth of *H. polymorpha* on methanol as a sole carbon source [12,14]. Experimental evidence that peroxisomal catalase is indispensible for this purpose was obtained after expression of the two *Saccharomyces cerevisiae* catalase genes in catalase-deficient mutants of *H. polymorpha*; functional complementation of the methanol negative phenotype was only achieved in transformants of *H. polymorpha*, which expressed peroxisomal catalase A, but was not mediated by its cytosolic counterpart, catalase T, even when expressed at high levels [14]. The essential function of catalase during methylotrophic growth of *H. polymorpha* became clear from physiological studies conducted by van der Klei et al. [12] on peroxisome-deficient mutants of this organism. These studies showed that intact peroxisomes fulfill two essential requirements to support growth on methanol as sole-carbon source. Firstly, intact peroxisomes are essential to control partitioning of formaldehyde fluxes over assimilatory and dissimilatory pathways. Secondly, in the non-compartmented state, catalase could not act as scavenger of the $H_2O_2$ generated from methanol, due to the low affinity of the enzyme for its substrate, compared with alternative $H_2O_2$-decomposing mechanisms present in the cells [15]. However, bypass of the catalase reaction was associated with severe metabolic and energetic disadvantages, leading to yield reduction on glucose and methanol mixtures in chemostat cultures and inability to grow on methanol when present as the sole carbon source. The presence of high concentrations of catalase between the sites of $H_2O_2$ generation and the peroxisomal membrane may function as a barrier and prevent leakage of $H_2O_2$ into the cytosol.

One additional advantage of a peroxisome-bound $H_2O_2$-decomposition reaction may be a stimulation of the rate of methanol oxidation by AO, as a result of enhanced $O_2$ concentrations, due to the catalytic decomposition of $H_2O_2$. Such a stimulation is readily envisaged from the low affinity of the enzyme for $O_2$ [16].

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