CATALASE ACTIVITY IN METHANOL-OXIDIZING *CANDIDA BOIDINII* 11Bh AND ITS CYTOCHEMICAL LOCALIZATION

J. VOŘÍŠEK and O. VOLFOVÁ

Czechoslovak Academy of Sciences, Institute of Microbiology, Budějovická 270, 14220 Praha 4, Czechoslovakia

Received 22 January 1975

1. Introduction

Our previous study of methanol oxidation to formaldehyde, formic acid and CO_2 by *Candida boidinii* 11Bh showed the presence of catalase activity (EC 1.11.1.6) in both cells and cell-free extracts. This enzyme and methanol-oxidase (EC 1.1.3.13) transform methanol to formaldehyde [1]. The simultaneous hydrogen peroxide reduction was previously found in methanol oxidizing bacteria [2] and in methanol oxidizing yeasts [3,4]. This communication summarizes certain kinetic properties of catalase from *C. boidinii* 11Bh cells grown on mineral salt medium containing methanol and describes our attempts at cytochemical localization of this enzyme.

2. Materials and methods

Candida boidinii strain 11Bh was cultivated in 500 ml Erlenmayer flasks containing 50 ml of mineral salt medium with 1% methanol or 1% glucose on a reciprocal shaker at 30°C [5]. For the cytochemical and biochemical assay of catalase activity cells from the middle linear growth phase [5] were employed; these were washed with 0.067 M phosphate buffer pH 7.5 by centrifugation. The cell-free extract was obtained by vigorous stirring of the pellets with ice-cold glass beads and subsequent centrifugation at 4000 *g* for 10 min and 26 000 *g* for 30 min. A modification of the iodometric method [6] was used for the determination of catalase activity. Catalase unit (Cat.U.) equals rate constant k/dry weight of cells in g. Peroxidase activity (EC 1.11.1.7) was detected by a modified method employing *o*-dianisidine [7]; methanol-oxidase activity was calculated from the amount of resulting formaldehyde [8]. Concentration of proteins was measured by a modification of the method described by Lowry et al. [9].

For cytochemical detection of peroxidatic activities whole yeast cells were fixed for 4 hr in ice-cold mixture of glutaraldehyde (UV spectrum checked) and formaldehyde in 0.2 M sodium cacodylate buffer pH 7.6 (MERCK) described by Karnovsky [10]. After three 30 min washings with ice-cold cacodylate buffer and two washings with cold 0.1 M Tris–HCl buffer pH 7.4 (SIGMA) the modification of 3,3-diaminobenzidine (DAB) procedure for the localization of heme-containing enzymes was employed [11,12]. The cells were incubated for 60 min at 37°C in a reaction mixture containing 10 mg 3,3′-diaminobenzidine (SIGMA) in 0.1 M Tris–HCl buffer pH 7.4 or pH 9.0. The reaction was started by adding 0.02% H_2O_2 (LACHEMA) freshly prepared from 30% stock solution [13]. The pelleted cells were washed twice with ice-cold Tris–HCl buffer pH 7.4 and post-fixed in 1% osmium tetroxide in 0.2 M cacodylate buffer pH 7.4 for 2 hr [14]. The cells were dehydrated in the alcohol series and absolute acetone and embedded in Vestopal W. Ultrathin sections were prepared by LKB Ultrotome I and viewed under the electron microscope JEOL JEM-100B without counterstaining.

3. Results

The analytical procedure showed significant catalase activity in *C. boidinii* 11Bh cells harvested at the middle

North-Holland Publishing Company – Amsterdam

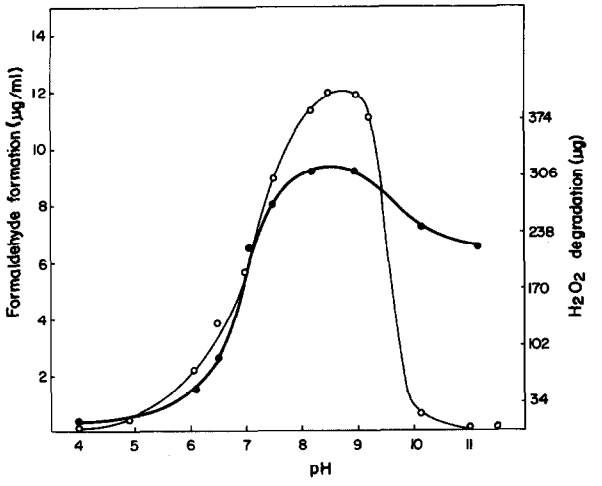

Fig.1. Activity of catalase and methanol-oxidase as a function of pH. Ordinate 1 - Formaldehyde produced in μg per ml of reaction mixture after 30 min incubation at 30°C. 3 ml of the reaction mixture contained 160 μg of protein, 10 μmol of methanol and 200 μmol of Britton-Robinson buffer. Ordinate 2 - H_2O_2 decomposition in μg per 5 ml of reaction mixture after 30 min of incubation at 30°C. 5 ml of the reaction mixture contained 80 μg of proteins, 340 μmol of H_2O_2 and 300 μmol of Britton-Robinson buffer.

linear phase of growth in the medium containing 1% methanol and in corresponding cell free extract. On the other hand, the measured H_2O_2 decomposition by peroxidase was negligible. Catalase activity in cells was not changed when 1% glucose was substituted for methanol as a carbon source (4.4 Cat.U. on methanol, 4.5 Cat.U. on glucose). The last finding shows that, in variance with strain of *C. boidinii* employed by Roggenkamp et al. [4], our strain 11Bh synthesized catalase constitutively.

The catalase and methanol-oxidase activities in cell-free extracts isolated from yeast grown on 1% methanol are plotted in fig.1 as a function of pH. It is obvious that both enzymes have a similar pH optimum at about pH 8.5. At higher pH values only the methanol oxidase activity was substantially reduced. The influence of conventional inhibitors on catalase activity in vitro is shown in table 1. It was not surprising that 0.001 M KCN or NaN_3 inhibited catalase activity by more than 90% after 10-min incubation. However, the specific inhibitor of catalase, 3-amino-1,2,4-triazole (AT) [15], did

Table 1
Catalase activity inhibition in *Candida boidinii* 11Bh cell free extracts

Compound	Concentration, M	% inhibition 10 min	% inhibition 30 min incubation
KCN	10^{-3}	97.2	–
NaN_3	10^{-3}	93.8	–
3-amino-1,2,4-triazole	10^{-2}	0	10
	10^{-1}	37.5	87.5

Reaction mixture contained the cell-free extract (80 μg of protein) preincubated for 10 min (30 min) with an inhibitor, 340 μmol of H_2O_2 and 300 μmol of phosphate buffer pH 7.5. The total vol was 5 ml. Incubation at was 30°C. After 3 min of incubation a 10 ml of 10% H_2SO_4, 0.1 g KI, 1 ml of molybdate were added and titrated with $Na_2S_2O_3$.

not influence catalase activity at a concentration of 0.02 M commonly held to be sufficient for yeast catalase inhibition [13]. As much as 0.1 M AT was necessary for 37% inhibition after 10 min incubation [3]. Residual activity (12%) was detectable even after 30 min of incubation.

This fact necessarily influenced our concept of the following experiments concerned with the cytochemical demonstration of H_2O_2 reducing hemoproteins in methanol-grown *C. boidinii* cells. Because of the low specific inhibition of catalase by AT we had to make use of the difference between the pH optima of peroxidase and catalase in the DAB reaction [16]. When the reaction mixture was buffered to pH 9.0 cytochemical reaction of both peroxidase and catalase could be expected to occur. Under such experimental arrangement the stain was deposited only in the mitochondria (fig.2) where it was uniformly distributed between the cristae and outer membranes (fig.3). When the reaction mixture was buffered to pH 7.4 (fig.4) a significantly lower amount of osmium black was found in mitochondria. Here the cytochemical reaction of peroxidase could be expected to proceed [16]. The amount of electron-dense reaction product in mitochondria membranes was also lower when 1% H_2O_2 was added at pH 9.0 [14]. No reaction product was formed when H_2O_2 was ommitted from the reaction mixture or when 0.01 M KCN was present. The addition of 0.02 M AT did not influence the DAB reaction.

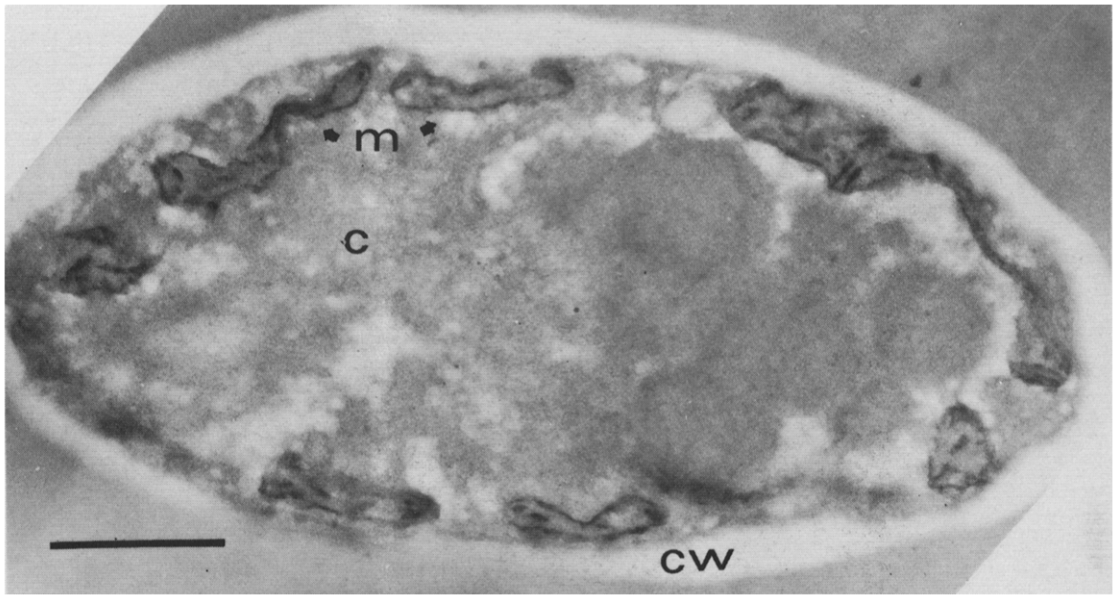

Fig.2. DAB reaction at pH 9.0; cw - cell wall, m - mitochondria, c - cytoplasma. Marker bar indicates 1 μm.

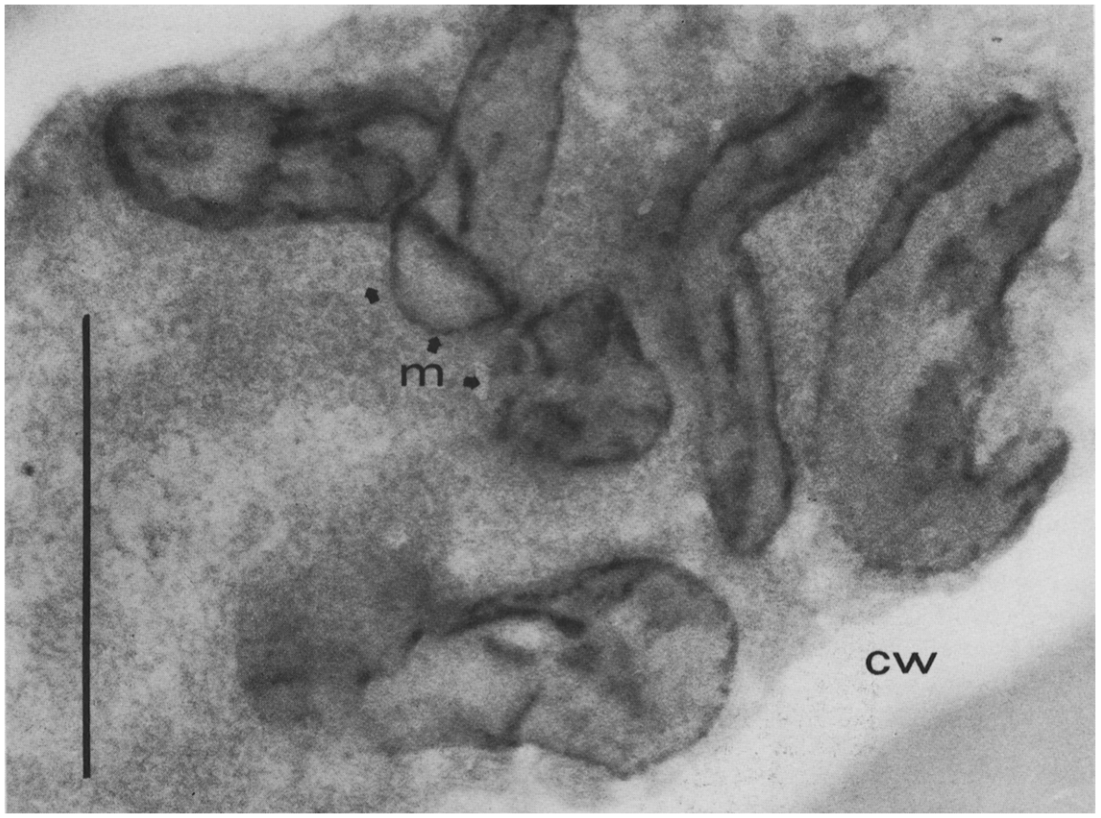

Fig.3. DAB reaction at pH 9.0; cw - cell wall, m - mitochondria. Marker bar indicates 1 μm.

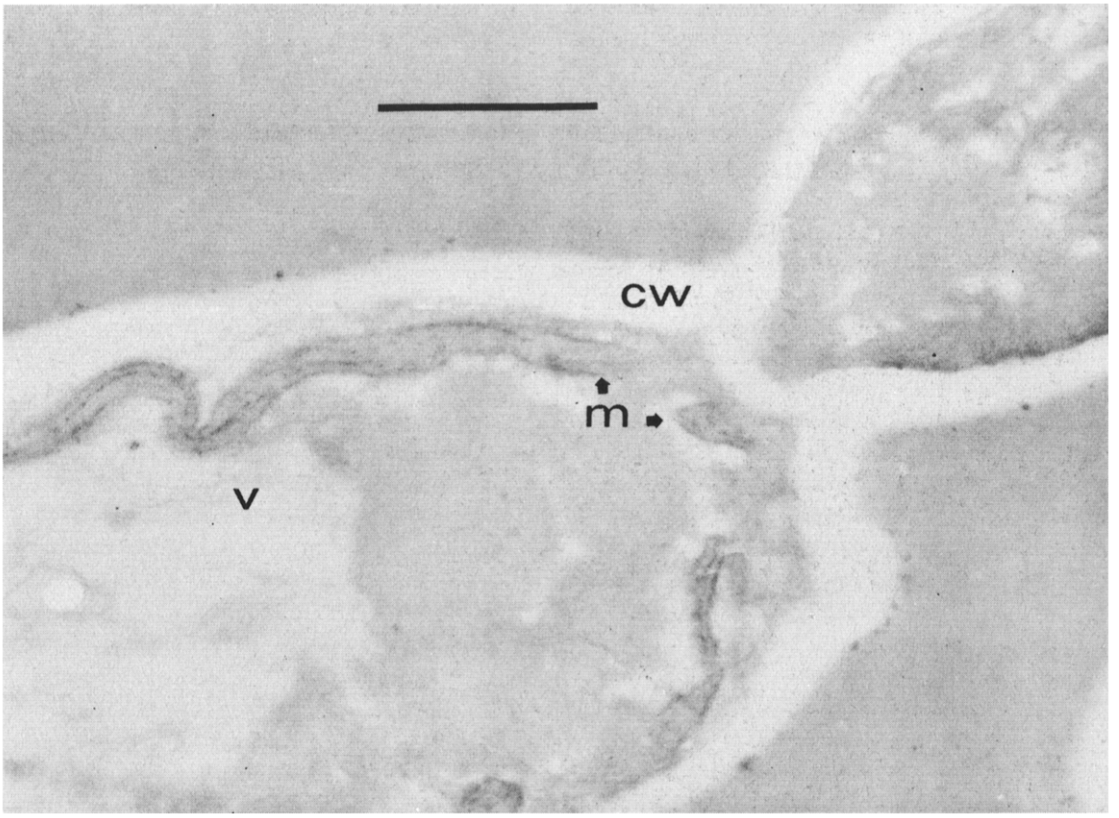

Fig.4. DAB reaction at pH 7.4; cw - cell wall, v - vacuole, m - mitochondria. Marker bar indicates 1 μm.

The cytochemical staining of mitochondria was distinct in more than 50% of cells from the yeast cultures employed. As the mitochondria were stained during the budding process (fig.4) we could assume that the cytochemical stain appeared only in the metabolizing cells.

4. Discussion

The peroxidatic activity in yeast mitochondria was first cytochemically demonstrated by Hoffmann et al. [13] who attributed it to the presence of peroxidase activity. They detected peroxidatic activity also in *S. cerevisiae* microsomes where the inhibition of cytochemical staining by AT showed the reaction to be due to catalase. Todd and Vigil [14] studied another strain of *S. cerevisiae* where microbody-like organelles were not found. Here the DAB reaction in cell mitochondria was insensitive to 0.02 M AT and was blocked by high hydrogen peroxide concentration. They discussed the in vitro inhibition of cytochrome *c* peroxidase (EC 1.11.1.5) by increased H_2O_2 concentrations [17] and concluded that this enzyme was most probably present in mitochondrial cristae. The authors failed to localize the catalase although its presence was clearly demonstrated by the evolution of O_2 from the reaction mixture containing H_2O_2.

Catalase-containing microbodies were found in a small fraction of hydrocarbon-utilizing *C. tropicalis* population (10% at least) [18,19]. However, the authors did not explain why a substantial portion of DAB oxidative polymerization product was present in mitochondria. The results and interpretation of the next paper published by the same group about *Candida* grown on *n*-alkanes were similar [20].

Our cytochemical experiments on *C. boidinii* 11Bh showed the DAB reaction to be confined

solely to mitochondria. Simultaneously we have demonstrated that in this yeast catalase is the principal H_2O_2-reducing enzyme and has low sensitivity to AT [3]. When compared with experiments employing *S. cerevisiae* [14], our system possessed two distinctive features. Firstly, the results of biochemical analysis showed mere traces of peroxidase activity which could hardly correspond to electron-dense cytochemical stain observed in mitochondria. Secondly, the DAB reaction was substantially reduced at pH 7.4. We assumed therefore that most probably both heme proteins with peroxidatic activity, catalase plus peroxidase, were present in *C. boidinii* 11Bh mitochondrial membranes. The partial suppression of cytochemical staining at pH 7.4 might indicate that the catalase reaction was inhibited while the analogous effect of 1% H_2O_2 might represent the inhibition of DAB reaction catalysed by cytochrome *c* peroxidase [14,21].

References

[1] Volfová, O. (1974) Proc. 4th Internat. Symp. Yeasts, Vienna, Austria, Part I, A22, p. 43.
[2] Harrington, A. A. and Kallio, R. E. (1960) Can. J. Microbiol. 6, 1–7.
[3] Fujii, T. and Tonomura, K. (1972) Agr. Biol. Chem. 36, 2297–2306.
[4] Roggenkamp, R., Sahm, H. and Wagner, R. (1974) FEBS Lett. 41, 283–286.
[5] Volfová, O. and Pilát, P. (1974) Fol. Microbiol. 19, 249–256.
[6] Kaprálek, F. (1962) J. Gen. Microbiol. 29, 403–419.
[7] Huggett, A. G. and Nixon, D. A. (1957) Biochem. J. 66, 12P.
[8] MacFadien, D. A. (1945) J. Biol. Chem. 158, 107–133.
[9] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[10] Karnovsky, M. (1965) J. Cell. Biol. 27, 137A–138A.
[11] Seligman, A., Karnovsky, M., Wasserkrug, H. and Hanker, J. (1968) J. Cell Biol. 38, 1–14.
[12] Novikoff, A. and Goldfischer, S. (1969) J. Hist. Cyt. 17, 675–685.
[13] Hoffmann, H. P., Szabo, A. and Avers, C. J. (1970) J. Bacteriol. 104, 581–584.
[14] Todd, M. M. and Vigil, E. L. (1972) J. Hist. Cyt. 20, 344–349.
[15] Heim, W. G., Appleman, D. and Pyfrom, H. T. (1955) Science 122, 693.
[16] Schneeberger, E. (1972) J. Hist. Cyt. 20, 180–191.
[17] Yonetani, T. and Ray, G. (1966) J. Biol. Chem. 241, 700–706.
[18] Teranishi, Y., Tanaka, A., Osumi, M. and Fukuji, S. (1974) Agr. Biol. Chem. 38, 1213–1220.
[19] Teranishi, Y., Kawamoto, S., Tanaka, A., Osumi, M. and Fukui, S. (1974) Agr. Biol. Chem. 38, 1221–1225.
[20] Osumi, M., Miwa, N., Teranishi, Y., Tanaka, A. and Fukuji, S. (1974) Arch. Microbiol. 99, 181–201.
[21] Avers, C. (1967) J. Bacteriol. 94, 1225–1230.