ENVIRONMENTAL FACTORS CONTROLLING ENZYMATIC ACTIVITY IN MICROBODIES AND MITOCHONDRIA OF *EUGLENA GRACILIS*

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1. Introduction

Glyoxylate by-pass enzymes have been demonstrated in *Euglena gracilis* strain Z grown heterotrophically on acetate [1,2]. In the present work, catalase activity in microbodies is observed cytochemically by the enzymatic oxidative polymerization of 3,3'-diaminobenzidine (DAB) in the presnece of H_2O_2 [3] in aerated cultures of *E. gracilis* strain Z grown on inorganic medium supplemented with acetate or glucose. Catalase activity is also detected photometrically in cell-free extracts of acetate-supplemented cells, but cannot be detected by this method in glucose-supplemented cells in which the number of microbodies is an order of magnitude fewer. In contrast with aerated cultures of acetate-supplemented cells, those grown in CO_2-free air fail to exhibit detectable levels of catalase activity.

Although cytochrome oxidase could not be detected using conditions of incubation considered optimal [4], a KCN-sensitive mitochondrial enzyme is revealed under different conditions of DAB incubation. This (unidentified) enzyme is first observed in the mitochondrial matrix after 20–24 hr of greening, attaining a maximum intensity at 40–48 hr, and falling below the limits of detectability sometime before 72 hr of greening. The mitochondrial enzyme seems to be photosynthesis-dependent; in the presence of 3 (3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or in CO_2-free air, the DAB stain is no longer observed in the matrix.

2. Materials and methods

The medium described by Cramer and Myers was supplemented with acetate or glucose [5]. Three light regimes were used: "light-grown" cells were continuously cultured in incandescent light (1.9×10^3 erg/cm^2/sec), "dark-grown" cells (previously depleted of chlorophyll and chloroplasts by being grown in the absence of light for over 20 generations) were cultured in flasks wrapped with aluminum foil, "greening" cells were "dark-grown" cells cultured for indicated periods of time in the light. Cells were either grown in the presence of CO_2 (air) or in the absence of CO_2 (air passed through Ascarite). In some experiments photosynthesis was inhibited by adding 3(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) (Sigma Chemical Co., St. Louis, Mo., USA) at a final concentration of 1×10^{-5} M to the culture flasks. Cell-free extracts were obtained by sonication of cells for 15 sec at a frequency of 20 kilocycles/sec in a Biosonik III (Bronwill Scientific Co., Rochester, N.Y., USA) and assayed for catalase by Luck's photometric method [6]. In our assays, concentrations < 0.03 mg/ml of catalase were below the limit of detection.

Electron microscopic studies followed the procedures given by Vigil [3] for the enzyme-mediated oxidative polymerization of DAB by hydrogen peroxide. Conditions of DAB incubation (pH and temperature) were varied to favor the reactivity of specific enzymes with the DAB reaction mixture. At a final pH of 9.0 and 37°, the DAB product of catalase activity can best be visualized – this reaction is specifically inhibited by the addition of 0.02 M aminotriazole (3-amino-1,2,4-triazole; Aldrich Chemi-

cal Co., Milwaukee, Wisc., USA) to the reaction mixture. At a pH of 7.0 and 25°, the presence of cytochrome oxidase can be detected – this reaction is inhibited by the addition of 0.01 M KCN to the DAB reaction mixture.

3. Results and discussion

3.1. *Dark-grown cells*

Approx. 3–5 microbodies per cell of a given section are found in aerated acetate-supplemented cultures of *E. gracillis* strain Z, with as many as 10 seen in some cells of a section. In contrast, numerous sections of glucose-supplemented cells must be scrutinized to find a single microbody. The DAB-positive reaction of these microbodies at pH 9.0 and 37°, as well as inhibition of the reaction by aminotriazole, indicates the enzymatic activity of catalase. The irregularly shaped, single membrane-bound microbodies are 0.4–1.4 μ in diameter (fig. 1) and are most frequently located in regions near the cell periphery and and gullet.

Catalase activity can not be detected photometrically with cell-free extracts of aerated glucose-supplemented cells despite positive histochemical observations. Failure to detect this enzyme photometrically is probably due to the paucity of microbodies in glucose-supplemented cells. Application of Luck's photometric assay to cell-free extracts of aerated acetate-supplemented cells (2×10^6 cells/ml) reveals 0.32 mg/ml of catalase. Catalase activity could not be detected in cells grown in air depleted of CO_2 by passage through Ascarite. Conditions of incubation which are cytochemically optimal for detecting cytochrome oxidase (pH 7.0, 25°C) fail to reveal its presence in *E. gracilis* strain Z under all conditions in the present study.

3.2. *Greening cells*

After approx. 10 hr of greening, catalase activity and the number of microbodies begins to increase in each cell. The number of microbodies approximately doubles within 24 hr of greening and then remains fairly constant. In cell-free preparations of aerated acetate-supplemented cells, the amount of catalase detected photometrically on cell-free extracts (2×10^6 cells/ml) is 0.50 mg/ml in 12-hr greening cells, 0.80 mg/ml in 24-hr greening cells, and 0.90 mg/ml in 48-hr greening cells. Catalase activity could not be detected photometrically or cytochemically in cells allowed to green in CO_2-free air. We are presently unable to ascertain whether this increase in microbodies represents a proliferation of those already

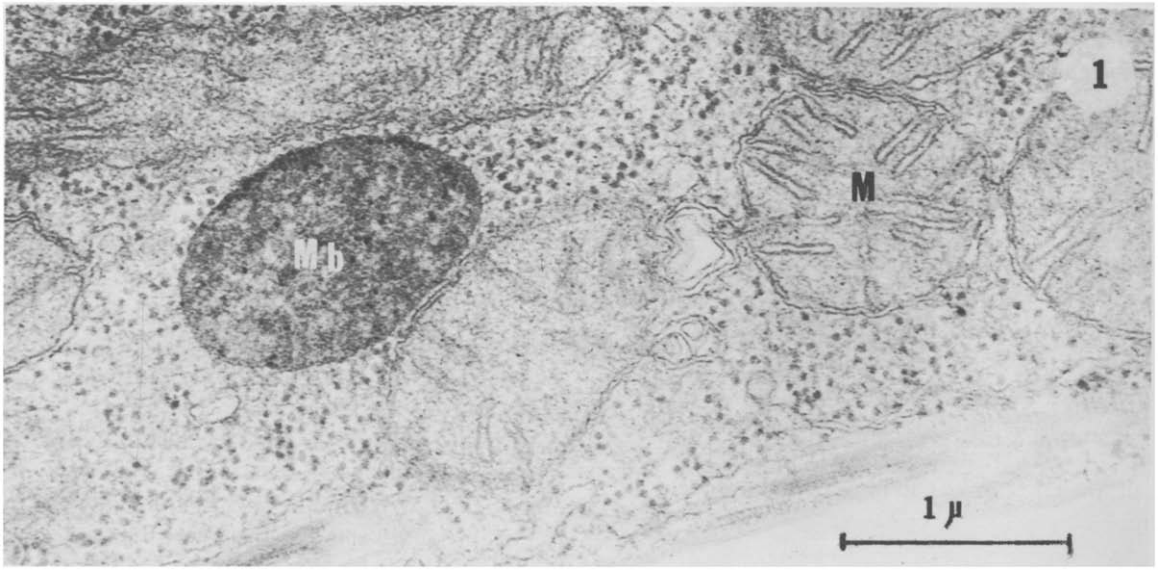

Fig. 1. Electron micrograph of DAB/H_2O_2 incubated (pH 9.0, 37°) microbody (Mb) surrounded by mitochondria (M) in dark-grown, aerated, acetate-supplemented *E. gracilis* strain Z. × 30,000.

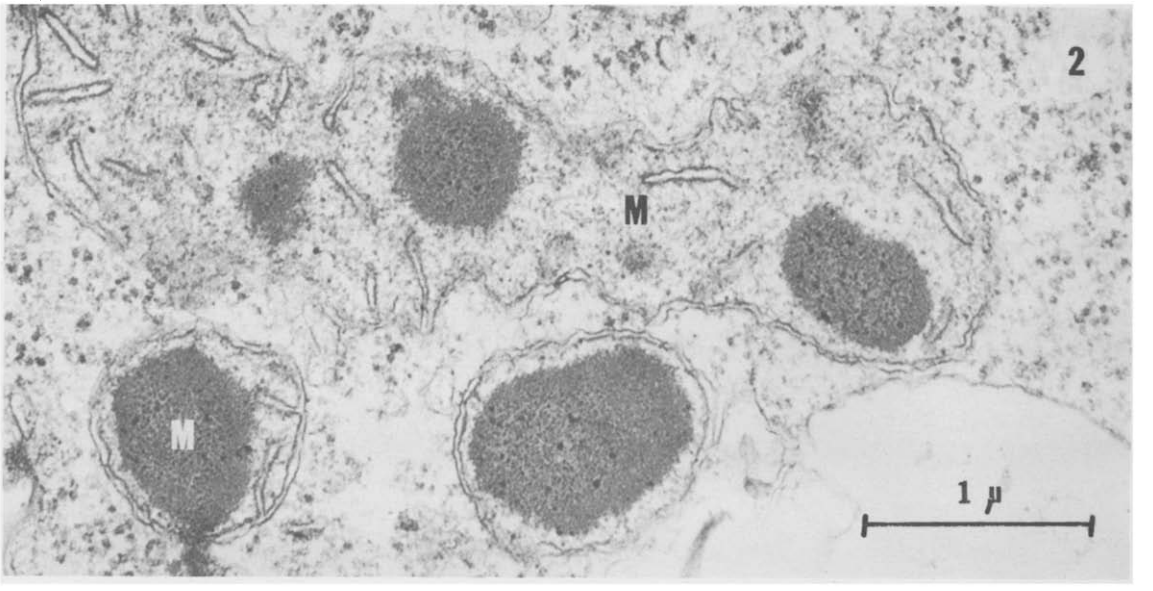

Fig. 2. Electron micrograph of DAB/H_2O_2 incubated (pH 9.0, 37°) mitochondria (M) of *E. gracilis* strain Z after 48 hr of greening, under conditions of aeration and acetate supplementation. × 30,000.

present in dark-grown cells (glyoxysomes) or if they represent a new type of microbody (peroxisomes).

After 20–24 hr of greening, slight DAB reactivity is observed in the mitochondrial matrix of cells incubated at pH 9.0 and 37° (conditions which are not optimal for visualization of cytochrome oxidase [4]). The electron dense DAB-positive material is most opaque after 40–48 hr of greening (fig. 2), and becomes again undetectable in cells allowed to green for 72 hr. Aminotriazole (0.02 M) inhibits the DBA stain only in microbodies, and not in the mitochondrial matrix of 20–48 hr greening cells; however, 0.01 M KCN inhibits the stain in both organelles.

When DCMU is added to cultures at various times of greening, the DAB stain does not develop in the mitochondrial matrix. Therefore, we propose that the activity (or presence?) of this mitochondrial enzyme is photosynthesis-dependent. (The DAB stain is also absent in the mitochondria of cells which are allowed to grow and green in CO_2-free air).

3.3. *Light-grown cells*

Cell-free preparations of continuously light-grown, three-day old cultures of aerated, acetate-supplemented cells disclosed the presence of 0.95 mg/ml (2×10^6 cells/ml) of catalase; again the removal of CO_2 from the gas phase made it impossible to detect this enzyme.

We speculate that CO_2 exerts a regulatory influence on the activity or presence of catalase as well as the unidentified mitochondrial enzyme in acetate-supplemented *E. gracilis* strain Z.

Hanzely et al. [7] and Graves et al. [8] observed microbodies in a streptomycin-bleached strain of *E. gracilis* var. *bacillaris*, but were unable to detect the presence of catalase either histochemically utilizing DAB/H_2O_2 or photometrically. We believe their results can be explained by the assumption that streptomycin bleaching has not only inhibited the enzymes of chlorophyll synthesis and chloroplast development, but other enzymes as well, including those of catalase synthesis. Lord and Merrett [9] were also unable to detect catalase activity in *E. gracilis* strain Z grown on inorganic media supplemented with glucose. However, as shown in the present work and by Graves et al. [8], glucose-supplemented cells have so few microbodies as to render catalase activity undetectable by photometric techniques.

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