ENZYMATIC CHARACTERIZATION OF SUCROSE-GRADIENT MICROBODIES OF DARK-GROWN, GREENING AND CONTINUOUSLY LIGHT-GROWN *EUGLENA GRACILIS*

James Edward WHITE* and Marcia BRODY**

Department of Biological Sciences, Hunter College of The City University of New York, New York 10021, USA

Received 28 August 1973
Revised version received 15 January 1974

1. Introduction

In earlier works [1, 2] we reported that catalase activity may be detected cytochemically, by the oxidative polymerization of 3,3'-diaminobenzidine (DAB), in the microbodies of *Euglena gracilis* strain Z cultured in aerated, acetate-supplemented media, but not in the microbodies of cells grown in CO_2-free air; catalase activity was additionally demonstrated by application of Lück's photometric assay [3] to cell-free fractions. In these studies [1, 2] we noted that both the number of microbodies in situ and specific catalase activity in cell-free fractions of dark-grown cells begin to increase after about 10–12 hr in the light, and that with further greening (≈24 hr), both microbody population (per cell) and catalase activity double.

Microbodies may be classified as glyoxysomes or peroxisomes on the basis of enzymatic composition. The former contain the enzymes of the glyoxylate cycle [4] and the latter contain the enzymes of the glycolate pathway of metabolism [5]; catalase is found in both types of particles. In the present work, cells from aerated, acetate-supplemented cultures of *Euglena gracilis* strain Z were grown under three light regimes (dark, greening and continuous light), and microbody-containing fractions, isolated by means of discontinuous sucrose gradients, were assayed for catalase, the glyoxysomal marker enzymes malate synthase and isocitrate lyase, as well as for the peroxisomal marker enzymes glycolate dehydrogenase and hydroxypyruvate reductase (glycolate reductase). Enzyme data for these isolated microbodies are presented here, both on the bases of specific activity and activity per cell. It was found with dark-grown cells, that low levels of peroxisomal marker enzymes accompany catalase and the glyoxysomal marker enzymes. Specific activities of glyoxysomal enzymes remain fairly constant for aerated, acetate-supplemented cells grown under the three light regimes; however, the activities of these enzymes approximately double by 24 hr of greening, on a per cell basis. Microbodies isolated from such cells yield catalase-positive DAB reactivity; for reasons not presently apparent, both catalase specific activity and activity per cell increase ≈1.4-fold after 24 hr of greening compared to dark-grown cells. Also by approximately 24 hr of greening, both the levels of hydroxypyruvate reductase and glycolate dehydrogenase increase ≈6-fold on the basis of specific activity and ≈12-fold on a per cell basis, compared to dark-grown cells.

It is noted that with greening, the concentration of protein in the microbody-containing sucrose density fraction doubles after 24 hr of illumination. This doubling undoubtedly has its origin in the 2-fold increase in number of microbodies per cell reported [1, 2] to occur by 24 hr of greening, and is most likely the reason why enzyme activities, when expressed on a per cell basis, are found to be approximately

* Work submitted by J.E.W. in partial fulfillment of the requirements for the Ph.D. at The City University of New York (CUNY).

** This research was partially supported by a Faculty Research Grant to M.B. from The City University of New York (CUNY).

twice those expressed on a per mg protein basis.

We have included an electron micrograph of a microbody which appears to be undergoing an unusual type of 'multilobed division'; such microbodies are noted only in cells between 12–30 hr of greening. It is tentatively suggested that, at about 24 hr of greening, peroxisomal type microbodies – presumably constituting 1/10 to 1/12 the total population of microbodies in dark-grown cells – produce, by multilobed division, 12 to 10 'daughter' microbodies, resulting in both the observed changes in peroxisomal enzyme activities and in the observed doubling of numbers of microbodies per cell.

2. Materials and methods

All details concerning culturing conditions (media, light regimes, gas phase, etc.) were the same as described previously [1, 2]. Microbody fractions were obtained by a slight modification of the procedure given by Tolbert [6]. Cells cultivated under each light regime were harvested during early logarithmic growth by centrifugation at 500 *g* for 5 min, washed once with, and resuspended in, 0.4 M sucrose in 20 mM glycyl–glycine buffer, pH 7.5. Aliquots (10^8 cells) were then broken by careful manual grinding at 4°C, with Ottawa sand (1:3 volume of packed cells:sand) for a total time of ≈30 sec. To preserve the microbodies intact, grinding was halted when cell counts showed ≈25% cell disruption. The broken cell suspension was centrifuged at 250 *g* for 10 min, and the supernatant (8 ml) carefully layered on top of a discontinuous sucrose density gradient column. Such columns were prepared by dissolving sucrose in 20 mM glycyl–glycine buffer, pH 7.5, to yield a stock solution of 2.5 M sucrose; successive dilutions of this stock solution were made with the same buffer. A total of eight, 6-ml layers of the following sucrose solutions were carefully pipetted at 4°C, in succession, into 60-ml polycarbonate centrifuge tubes: 2.5 M sucrose (85.5% w/v), 2.0 M sucrose (68% w/v), 1.75 M sucrose (60% w/v), 1.50 M sucrose (51% w/v), 1.25 M sucrose (43% w/v), 1.0 M sucrose (34% w/v), 0.75 M sucrose (25.5% w/v), and 0.50 M sucrose (17% w/v). After centrifugation at 0–4°C for 4 hr at 65 000 g_{av} (22 500 rev/min) in a swinging bucket rotor, SW 25.2, of a Spinco centrifuge, model L, the tubes were removed, pierced and fractions collected. The fractions containing intact microbodies were found at the interface on top of the 2.0 M sucrose band.

Spectrophotometric assays (with the exception of glycolate dehydrogenase were made using silica cuvettes (1.5 ml volume, 0.5 cm light-path) in a Beckman DB-G Grating Spectrophotometer. Catalase (EC 1.11.1.6) was assayed by the initial disappearance of 12.5 nmoles of H_2O_2 as measured by the decrease of absorbancy at 240 nm [3]. Catalase activity is expressed in units/mg protein or units/cell – a 'unit' being that amount of catalase which decomposes half the H_2O_2 present in the sample in 100 sec at 25°C [3]. Isocitrate lyase (EC 4.1.3.1) was assayed, measuring the formation of glyoxylate phenyl-hydrazone by following the increase of absorbancy at 324 nm [7]. Malate synthase (EC 4.1.3.2) was evaluated by the oxidation of NADH [7]. Glycolate dehydrogenase was assyed in Thunberg tubes, at 600 nm, by the anaerobic reduction of 2,6-dichloroindophenol [8]. Hydroxypyruvate reductase (EC 1.1.1.29) was evaluated by following the oxidation of NADH at 340 nm, after the addition of hydroxypyruvate [6]. Specific activities of enzymes (with the exception of catalase) are expressed in nmoles/min/mg protein. Protein was measured according to Lowry et al. [9], using a calibration curve obtained with crystalline bovine serum albumin, and corrections were made for sucrose buffer interference.

Microbody-containing fractions from the discontinuous sucrose gradient colums were collected also for electron microscopy. To these fractions, 20 mM glycyl–glycine buffer, pH 7.5, was added as diluent to yield final concentrations of 0.4 M sucrose. The fractions were then centrifuged at 25 000 *g* for 15 min, and the pellets suspended in 2% agar to facilitate processing for electron microscopic studies. Cytochemical procedures follow those previously described [1, 2] for catalase-mediated oxidative polymerization of DAB by H_2O_2, and for the inhibition of this reaction by 0.01 M KCN or 0.02 M aminotriazole.

3. Results and discussion

Electron microscopic studies reveal that the mi-

crobody-containing fractions, which are located at the interface between the 2.0 and 1.75 M sucrose bands, contain fewer than 1% intact mitochondria. The measured specific densities (Abbé refractometer) of these sucrose bands was 1.213 and 1.209 g/cm^3, respectively. The microbodies band in a region of sucrose density which corresponds well (1.20 g/cm^3) to that reported by Graves et al. [10] for microbody-containing fractions from continuous sucrose gradients of a streptomycin bleached strain of *Euglena gracilis* var. *bacillaris* (SM-LI).

Protein determinations [9] on isolated microbody-containing bands from similar size aliquots (2.5 × 10^7 cells) reveal that with greening, microbody-associated protein approximately doubles (325 γ/ml sucrose gradient) in continuously light-grown cells compared to dark-grown cells (160 γ/ml sucrose gradient).

Catalase activity is, for the first time, here demonstrated cytochemically, in microbody-containing sucrose fractions of aerated, acetate-supplemented *Euglena gracilis* strain Z (fig. 1); although this figure is for dark-grown cells, similar positive results were obtained with greening and continuously light-grown cells. This enzymatic oxidative polymerization of DAB fails to manifest itself in the presence of the catalase inhibitor aminotriazole (fig. 2), KCN, or when microbodies were isolated from cells which had been deprived of CO_2 in the gas phase. We wish, additionally, to report that catalase may be detected photometrically, by the method of Lück [3], in sucrose-isolated microbody fractions of such cells (fig. 3A). From fig. 3A it may be seen that the activity of catalase increases with greening in sucrose-isolated microbody fractions. (It should be pointed out, that on the basis of cell-free fractions, a two-fold increase in specific catalase activity was noted [2] with greening; the difference between this value and the ≈1.4 fold increase found with isolated microbodies, may result from the difficulties inherent in determining the very low activity of catalase in dark-grown cells.) If isolated microbody data on catalase are expressed on a per cell basis, one notes that with greening, activity increases from 3.1 × 10^{-7} units/cell in 24-hr greening cells and 4.6 × 10^{-7} units/cell in 72-hr greening cells. Catalase activity could be observed neither cytochemically with DAB, nor photometrically [3], in microbody-containing sucrose fractions of cells aerated in CO_2-free air.

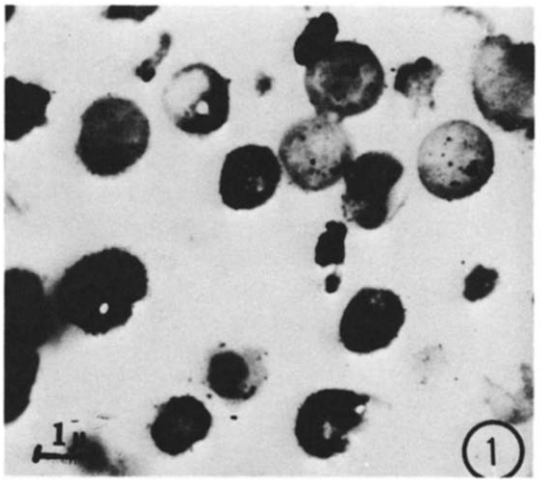

Fig. 1. Electron micrograph of DAB incubated (pH 9.0, 37°C) microbodies, located at the interface between the 2.0 and 1.75 M bands of a discontinuous sucrose gradient. Source of microbodies was log phase, dark-grown, aerated, acetate-supplemented *Euglena gracilis* strain Z.× 8000.

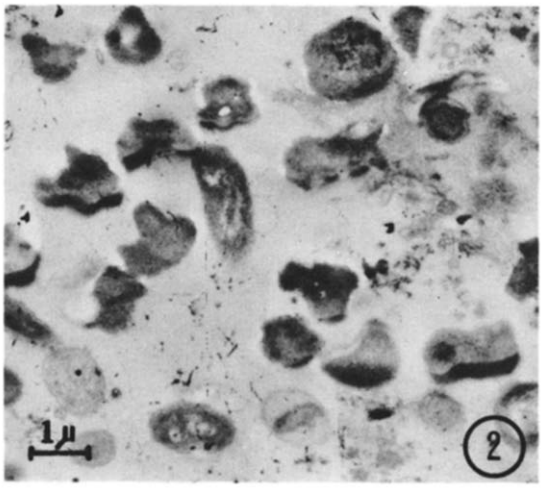

Fig. 2. Electron micrograph of DAB plus aminotriazole incubated (pH 9.0, 37°C) microbodies, located at the interface between the 2.0 and 1.7 M bands of a discontinuous sucrose gradient. Source of microbodies was log phase, dark-grown, aerated, acetate-supplemented *Euglena gracilis* strain Z. × 8000.

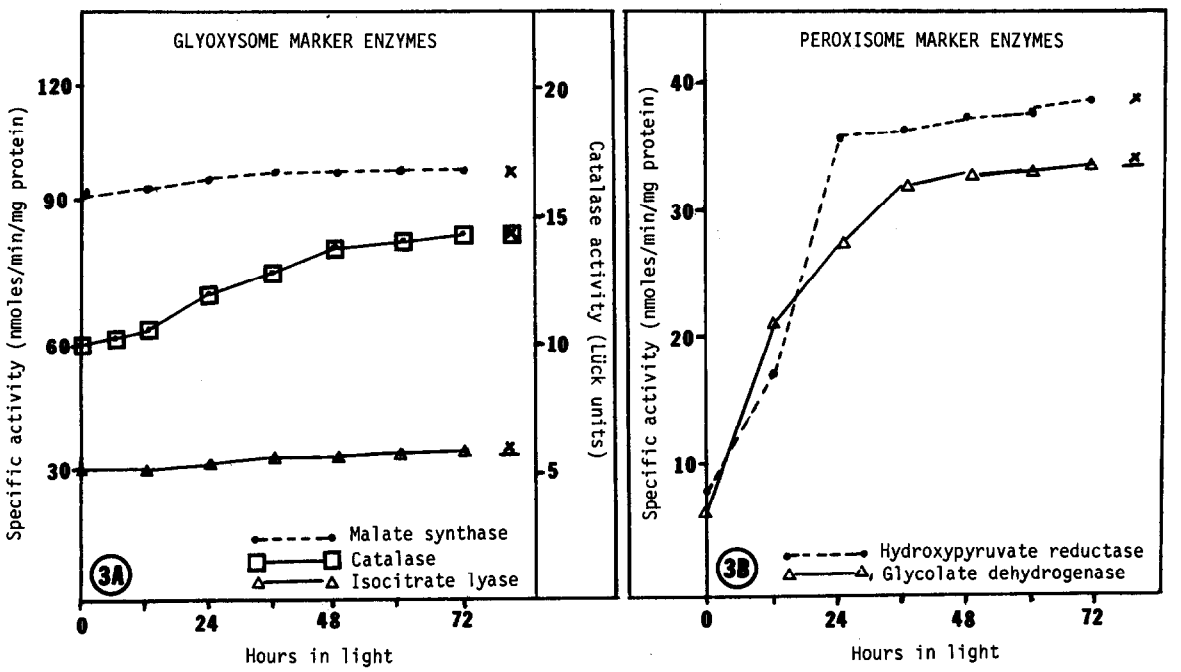

Fig. 3. Glyoxysomal and peroxisomal marker enzyme activities in microbody-containing fractions (isolated from discontinuous sucrose gradients) of dark-grown, greening and continuously light-grown, aerated, acetate-supplemented *Euglena gracilis* strain Z. Data given by 'X's' correspond to values measured with continuously light-grown cells. (Data for catalase has been arbitrarily placed with the glyoxysomal marker enzymes; catalase activity was measured in Lück units/mg protein.)

The specific activities of the glyoxysomal marker enzymes isocitrate lyase and malate synthase remain fairly constant with greening, as judged from data obtained with sucrose-isolated microbody fractions (fig. 3A); on a per cell basis, these enzymes approximately double in activity after 24 hr of greening. Graves et al. [10] reported isocitrate lyase (31 nmoles/min/mg protein) and malate synthase (95 nmoles/min/mg protein) to be present in continuous sucrose gradient fractions of streptomycin-bleached, ethanol-supplemented *Euglena gracilis* var. *bacillaris* (SM-LI).

Sucrose-isolated microbody fractions reveal (fig. 3B) that after 24 hr of greening, the specific activities of glycolate dehydrogenase and hydroxypyruvate reductase increase ≈6-fold, compared to dark-grown cells; a 12-fold increase is noted when the data are expressed on a per cell basis. Lord and Merrett [11] demonstrated, with discontinuous sucrose gradients of cell-free extracts of *Euglena gracilis* strain Z, grown phototrophically, that glycolate: DCPIP (glycolate dehydrogenase) is a particulate enzyme. Graves et al. [10] found that co-banding with (presumably) microbody particles, which showed glyoxysomal type enzyme activity, were some weakly active peroxisomal enzymes – inculding glycolate dehydrogenase and hydroxypyruvate reductase (they used glyoxylate as substrate and, therefore refer to this enzyme as glyoxylate reductase). They, furthermore, noted that the activities of these two enzymes increase upon illumination in both the streptomycin-bleached and normal variety *bacillaris*, despite the fact that the former organism does not green upon exposure to light.

In fig. 4 is observed an electron micrograph of an in situ 'microbody-like' organelle, made up of several lobes, in a 24 hr greening, aerated, acetate-supplemented *Euglena*. Such structures, not observed until ≈12 hr of greening nor after ≈30 hr of greening, exhibit catalase-mediated DAB reactivity. At higher magnifications than those given here, long (up to ≈0.5 μm), 25–30 A° thick fibrils are observed within

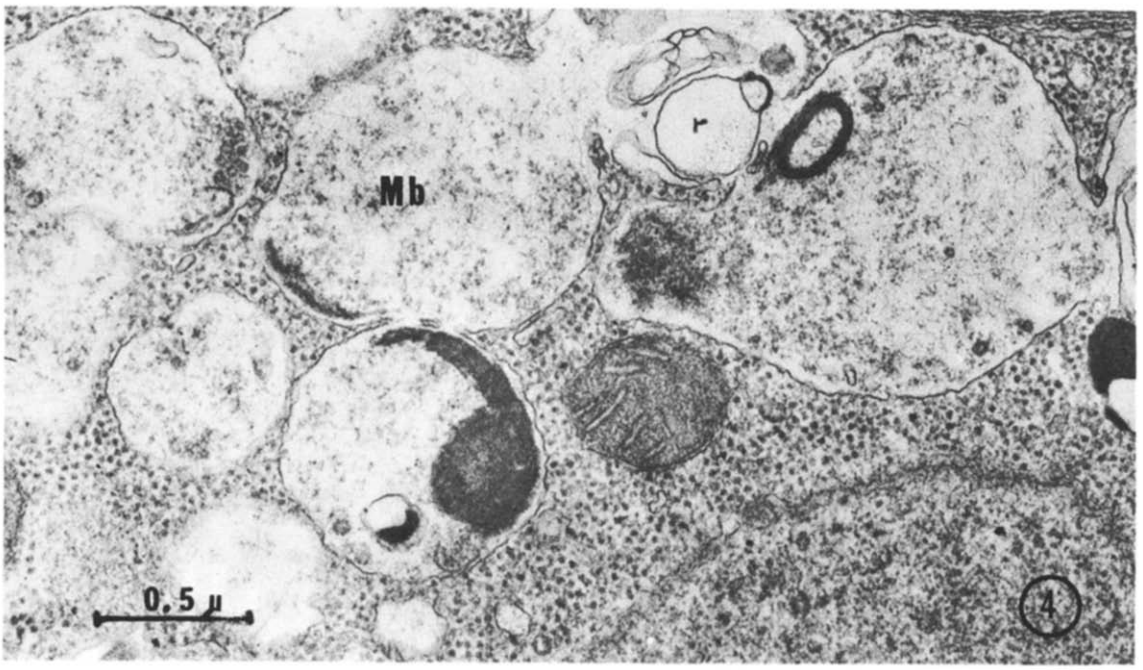

Fig. 4 Electron micrograph of multilobed microbody (Mb) in a cell from a 24 hr greening, aerated, acetate-supplemented culture of *Euglena gracilis* strain Z.× 40 000.

these multilobed microbodies. Additionally, DNA [12] and RNA [13] were detected photometrically in microbody-containing sucrose fractions from discontinuous sucrose gradients of dark-grown, greening and continuously light-grown *Euglena*. Fine structure observations of such fibrils, and photometric detection of DNA and RNA suggest the possible presence of genetic material in the microbodies of *Euglena*.

We present the following model which correlates microbody data in regard to number, fine structure, protein content, and enzyme activity (for aerated, acetate-supplemented *Euglena gracilis* strain Z). There exist in each dark-grown cell ≈300 microbodies (unpublished data). It will be assumed 1/10 to 1/12 of these microbodies contain low levels of peroxisomal-type enzymes and catalase, and that the remainder of the microbodies contain glyoxysomal-type enzymes and catalase. It will further be assumed that the observed doubling [2] in number of microbodies (at 24 hr of greening) results because of multilobed (12 to 10 lobes) division of the peroxisomal-type microbodies. The consequences of this division would be:

a) A doubling in number of microbodies, from ≈300 to ≈600 per cell. This is in agreement with our (unpublished) microscopy observations. b) A doubling in microbody-associated protein – as observed in the present work. c) A ⩽ 2-fold increase in catalase activity per cell by 24 hr of greening. (If in dark-grown cells both peroxisomal and glyoxysomal microbodies possess the same catalase activity, a 2-fold increase would obtain; if there were lower levels of catalase in the peroxisomal-type microbodies, a less than 2-fold increase would obtain.) Data from cell-free fractions [2] indicate a 2-fold increase, whereas data from isolated microbodies indicate a 1.4-fold increase. d) Constant glyoxysomal enzyme activity on a per cell basis, and a halving of activity on a per mg protein basis, as a function of greening. Although with these enzymes, we observed a doubling on a per cell basis, and a constancy in specific activity, these findings may be attributed to the enhanced utilization of acetate in the light compared to the dark [14]. e) A 12- to 10-fold increase, with greening, in activities of peroxisomal enzymes, on a per cell basis, and

a 6- to 5-fold increase on a specific activity basis. These predictions are in agreement with the findings.

Acknowledgements

We wish to thank Dr. K. Lyser and Dr. E. Balboni for their gracious cooperation.

References

[1] Brody, M. and White, J.E. (1972) FEBS Letters 23, 149.
[2] Brody, M. and White, J.E. (1973) Develop. Biol. 31, 348.
[3] Lück, H. (1963) in: Methods in Enzymatic Analysis, Bergmeyer, H.U., (ed.), p. 885, Academic Press, New York and London.
[4] Breidenbach, R.W. and Beevers, H. (1967) Biochem. Biophys. Res. Commun., 27, 462.
[5] Frederick, S.E. and Newcomb, E.H. (1969) J. Cell. Biol. 43, 343.
[6] Tolbert, N.E., (1971) in: Methods of Enzymology, San Pietro, T. (ed.), Vol. 23, part A, p. 665, Academic Press, New York and London.
[7] Dixon, G.H. and Kornberg, H.L. (1959) Biochem. J. 72, 3P.
[8] Tolbert, N.E., Oeser, A., Kisaki, T., Hageman, R.H. and Yamazaki, R.K. (1968) J. Biol. Chem., 243, 5179.
[9] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., 193, 265.
[10] Graves, Jr., L.B., Trelease, R.N., Grill, A. and Becker, W.M. (1972) J. Protozool., 19, 527.
[11] Lord, J.M. and Merrett, M.J. (1971) Biochem. J. 124, 275.
[12] Burton, K. (1965) Biochem. J., 62, 315.
[13] Lin, R.I. and Schjeide, O.A. (1969) Anal. Biochem. 27, 473.
[14] Cook, J.R. (1965) Plant and Cell Physiol., 6, 301.