PARTICULATE NATURE OF GLYCOLATE DEHYDROGENASE IN EUGLENA: POSSIBLE LOCALIZATION IN MICROBODIES

L.B. Graves, R.N. Trelease, and W.M. Becker

Department of Botany
University of Wisconsin
Madison, Wisconsin 53706

Received May 14, 1971

Summary

By using a gentle grinding procedure prior to differential and equilibrium centrifugation, glycolate dehydrogenase was found to be particulate in a streptomycin-bleached strain of Euglena gracilis. Its inhibition by cyanide, ability to oxidize D-lactate, and apparent insensitivity to oxygen indicate it to be the same enzyme previously characterized as a soluble enzyme in several species of green algae, including Euglena. It is speculated that this algal glycolate dehydrogenase may be localized in microbodies as is the glycolate oxidase of higher plants.

All green plants studied thus far, including the algae, have an enzyme capable of oxidizing glycolate to glyoxylate (1-4). The glycolate oxidase of higher plants is known to be housed in microbodies (glyoxysomes, 5; peroxisomes, 6), but the cellular localization of glycolate dehydrogenase, the algal enzyme, is not yet known (2, 7). Substantial glycolate oxidation has been reported to occur in a streptomycin-bleached strain of Euglena gracilis (8, 9). Using the same strain, we have recently shown with the electron microscope that microbodies similar to those seen in higher plants occur in Euglena (10). The investigation reported here was therefore undertaken to determine whether, by analogy to higher plants, the glycolate-metabolizing enzyme of Euglena is located in microbodies.

Materials and Methods

Euglena gracilis var. bacillaris (SM-L1), a nonphotosynthetic (streptomycin-bleached) strain, was grown in a modified Cramer-Myer solution (11, 12), using 0.2 M ethanol as the carbon and energy source. The cells were maintained in the dark at 25 C, and were gently aerated. All steps in the

preparation of cell-free extracts were performed at 0-5 C. The cells were collected in mid-log phase of growth by continuous-flow centrifugation, and washed once in a grinding medium consisting of 0.5 M sucrose in 0.05 M cacodylate, pH 7.2 (6). The washed cells were then resuspended in an equal volume of grinding medium, mixed with enough glass beads ("Superbrite", Minnesota Mining and Manufacturing Company, 0.28 mm average diameter) to make a thick paste, and ground by hand with a mortar and pestle for 30 seconds. The resultant slurry was washed out of the beads with several aliquots of grinding medium and centrifuged at 1,000 x g for 10 minutes. The supernatant from this centrifugation will hereafter be referred to as the homogenate since this fraction is a more reliable estimate of the actual amount of enzyme extracted than would be the washed slurry which contains a large proportion of unbroken cells. The homogenate was subjected to further fractionation by differential centrifugation at the gravitational forces specified in Table I, or by centrifugation to equilibrium on a sucrose density gradient in a zonal rotor (6). Activities of the following enzymes were determined using the assay procedures described in the accompanying references: glycolate dehydrogenase (4), glyoxylate (hydroxypyruvate) reductase (6, with glyoxylate as substrate), malate synthetase (13), and isocitrate lyase (13). Attempts to measure succinate dehydrogenase activity by following $K_3Fe(CN)_6$ reduction (14) were unsuccessful. Protein was determined by the method of Lowry et al. (15).

Results

As shown in Table I for two separate experiments, most of the glycolate dehydrogenase extracted from bleached Euglena by gentle grinding was recovered in the particulate fractions upon differential centrifugation, with specific activities from 3- to 6-fold higher in the pellets than in the starting homogenates. In agreement with reported properties of the glycolate-metabolizing enzyme of algae (2-4), the particulate enzyme isolated from bleached Euglena can oxidize D-lactate in addition to glycolate and L-

lactate, is completely inhibited by cyanide, and apparently does not use oxygen as an electron acceptor, since no significant differences in activity were obtained whether dye reduction was measured aerobically or anaerobically.

TABLE 1

Distribution of Glycolate Dehydrogenase Activity by Differential Centrifugation[1]

Fraction[2]	Substrate (Activity in nmoles·min^{-1})			Cyanide inhibition (% inhibition)	
	Glycolate	D-lactate	L-lactate	Glycolate	D-lactate
Experiment a:					
Homogenate	1419 (2.7)[3]	7050 (34.1)	3217 (15.6)	-	-
23,000 x g Pellet	958 (17.7)	6703 (123.7)	1761 (32.5)	100	58
23,000 x g Supernatant	523 (3.1)	1262 (7.5)	336 (2.0)	-	-
Experiment b:					
Homogenate	505 (6.3)	4020 (50.3)	- -	48	40
3,000 x g Pellet	125 (10.5)	1322 (110.4)	- -	100	53
10,000 x g Pellet	171 (20.1)	1288 (151.5)	- -	100	53
23,000 x g Pellet	14 (8.2)	150 (90.5)	- -	100	38
23,000 x g Supernatant	67 (0.9)	439 (5.9)	- -	100[4]	50

[1]Activity measured by DCIP reduction. Cyanide at 2 mM final concentration was added directly to the assay.

[2]Centrifugations at 23,000 x g were for 30 minutes. All others were for 10 min.

[3]Specific activity in nmoles·min^{-1} ·mg protein^{-1}.

[4]20 mM cyanide; 2 mM cyanide resulted in a 25% stimulation.

The fractions from experiment a of Table I were also assayed for malate synthetase and glyoxylate reductase. About 20% of the activity of each enzyme was associated with the particulate fraction.

Upon centrifugation of the homogenate to equilibrium on a 35 to 60% (w/w) sucrose gradient in a zonal rotor, activities of glycolate dehydrogenase, isocitrate lyase, malate synthetase, and glyoxylate reductase co-banded at a density of about 1.22 g/cm^3 (Fig. 1). The proportions of applied activities of these enzymes recovered in the density range 1.215 - 1.240 g/cm^3 were 29.9, 12.7, 12.4, and 56.5%, respectively. Most of the protein on the gradient was also found in this region of peak enzyme activity in five different experiments.

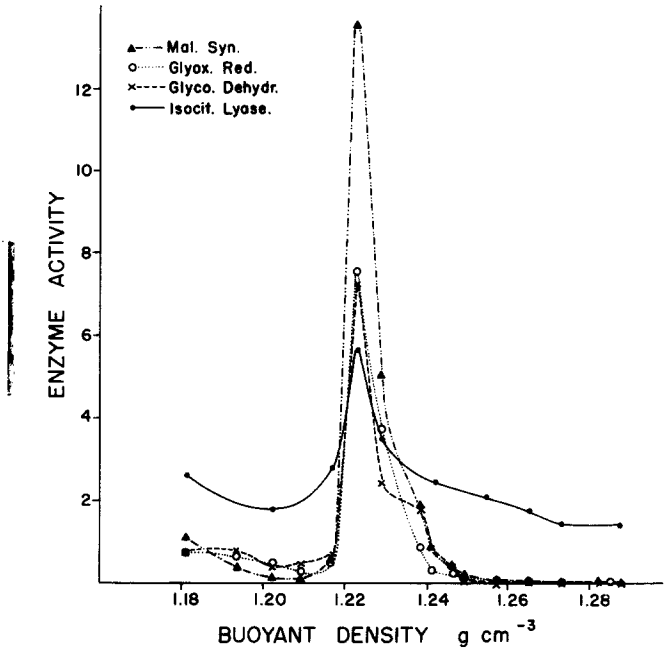

Figure 1. Distribution of glycolate dehydrogenase, glyoxylate reductase, malate synthetase, and isocitrate lyase activities on the entire sucrose gradient, 35% (1.18 g/cm^3) to 60% (1.29 g/cm^3) w/w sucrose, after sedimentation to equilibrium in a zonal rotor. The 1,000 x g supernatant fraction was used as the applied sample. Enzyme activity (per 20 ml fraction): glyoxylate reductase and malate synthetase, µmoles substrate consumed/min; glycolate dehydrogenase, 10 x µmoles substrate consumed/min; and isocitrate lyase, 100 x µmoles substrate consumed/min.

Discussion

In higher plants, many of the enzymes of both the glyoxylate cycle and the glycolate pathway are located in microbodies (glyoxysomes and peroxisomes). In algae, the key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthetase, are known to be particulate in several species (16, 17, 18), including Euglena, but the enzymes involved in glycolate metabolism have thus far been characterized only as soluble enzymes. By using a gentle grinding procedure prior to differential and equilibrium centrifugation, we have now established that two enzymes of the algal glycolate pathway, glycolate dehydrogenase and glyoxylate (hydroxypyruvate) reductase are also particulate in Euglena.

Evidence for the particulate nature of glycolate dehydrogenase is twofold: more than 60% of the activity was reproducibly recovered in pellet form upon differential centrifugation, and a relatively high percentage of the applied activity was recovered at a density of about 1.22 g/cm^3 upon centrifugation to equilibrium on a sucrose gradient. That this is the same enzyme as the glycolate dehydrogenase previously characterized as a soluble enzyme by other workers (4) is indicated by its inhibition by cyanide, its ability to oxidize D-lactate, and its apparent insensitivity to oxygen.

Recovery of glycolate dehydrogenase from Euglena in particulate form required a very gentle grinding procedure; increasing the length of grinding time beyond 30 sec severely reduced the amount of sedimentable enzyme. A similar observation was made by Grill (9). Although disruption of Euglena by sonication (7) or by passage of cells through a French pressure cell (2-4) breaks a much higher percentage of the cells and extracts more of the glycolate dehydrogenase than did our procedure, these techniques are also more deleterious to organelles (19), and may preclude isolation of glycolate pathway enzymes in particulate form.

Since we have previously identified microbodies with the electron microscope in the strain of Euglena used in the present study (10), it is

tempting to speculate that glycolate dehydrogenase occurs in these microbodies, together with glyoxylate reductase, malate synthetase, and isocitrate lyase. Microbodies were in fact seen upon electron microscopic examination of the particulate fractions obtained by differential centrifugation (Table I), but in no greater abundance relative to mitochondria than in whole cells. All four enzymes co-banded on a sucrose gradient at about 1.22 g/cm^3, the same density reported for glyoxysome-like organelles from Neurospora (20) and for peroxisomes from guinea pig intestinal epithelium (21). Since most of the protein on the gradient was also recovered at this density, however, it is clear that these enzymes, although particulate, cannot yet be assigned a specific intracellular location. Crucial to the definitive localization of these enzymes will be the resolution of microbodies from mitochondria. As extensive aggregation of the particulate material was seen both with the phase microscope in homogenates and with the electron microscope in the peak fractions from the density gradients, it appears that microbodies are as intimately associated with mitochondria in these fractions as they are in vivo (10). This may make separation difficult, but current attempts to eliminate this aggregation suggest it may be possible to effect at least a partial separation of Euglena microbodies from other organelles.

Acknowledgement

This work was supported by NSF grant GB-15246.

References

1. Tolbert, N.E., Annual Rev. Plant Physiol. 22, in press (1971).
2. Nelson, E.B., and Tolbert, N.E., Biochem. Biophys. Acta 184, 263 (1969).
3. Codd, G.H., Lord, J.M., and Merrett, M.J., FEBS Letters 5, 341 (1969).
4. Nelson, E.B., and Tolbert, N.E., Arch. Biochem. Biophys. 141, 102 (1970).
5. Breidenbach, R.W., Kahn, A., and Beevers, H., Plant Physiol. 43, 705 (1968).
6. Trelease, R.N., Becker, W.M., Gruber, P.J., and Newcomb, E.H., Plant Physiol., in press (1971).
7. Codd, G.A., and Merrett, M.J., Planta 95, 127 (1970).
8. Ross, M.R., and Jahn, T.L., J. Protozool. 16 (Suppl.), 11 (1969).
9. Grill, A., M.S. Thesis, Northern Illinois University (1971).
10. Graves, L.B., Hanzely, L., and Trelease, R.N., Protoplasma, in press (1971).

11. Cramer, M. and Myers, M., Arch. Mikrobiol. 17, 384 (1952).
12. Graves, L.B., J. Protozool., in press (1971).
13. Cooper, T.G., and Beevers, H., J. Biol. Chem. 244, 3507 (1969).
14. Veeger, C., DerVartanian, D.V., and Zeylemaker, W.P., in "Methods in Enzymology" (S.P. Colowick and N.O. Kaplan, eds.), Vol. 13. Academic Press, N.Y. (1969).
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem. 193, 265 (1951).
16. Haigh, W.G., and Beevers, H., Arch. Biochem. Biophys. 107, 152 (1964).
17. Harrop, L.C., and Kornberg, H.L., Proc. Royal Soc. Ser. B 166, 11 (1966).
18. Heinrich, B., and Cook, J.R., J. Protozool. 14, 548 (1967).
19. Buetow, D.E., in "Methods in Cell Physiology" (D.M. Prescott, ed.), Vol. 4, Academic Press, N.Y. (1970).
20. Kobr, M.J., Vanderhaeghe, F., and Combepine, G., Biochem. Biophys. Res. Commun. 37, 640 (1969).
21. Connock, M., and Pover, W., Histochem. J. 2, 371 (1970).