Occurrence and Operation of the Glycollate–Glyoxylate Shuttle in Mitochondria of *Euglena gracilis* z*

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(Received 24 July 1979)

Both glyoxylate reductase (NADP⁺) and glycollate dehydrogenase were located exclusively in mitochondria in *Euglena gracilis* and constitute the glycollate–glyoxylate shuttle, whose existence in higher plants was thought doubtful, owing to different subcellular locations of the two enzymes. Disrupted *Euglena* mitochondria showed a glycollate-dependent NADPH oxidation, indicating actual operation of the shuttle in this protözoön.

The glycollate–glyoxylate shuttle, first proposed by Tolbert *et al.* (1970) in spinach (*Spinacia oleracea*) and tobacco (*Nicotiana tabacum*), is composed of the two enzymes glycollate oxidase (EC 1.1.3.1) and glyoxylate reductase (EC 1.1.1.79). The shuttle, with the collaboration of catalase as the terminal oxidase, has been claimed to effect consumption of excess NADPH formed in chloroplasts under photorespiratory conditions in higher plants (Tolbert *et al.*, 1970; Tolbert, 1971*a,b*). However, the view apparently prevailing recently is that the shuttle does not actually operate in the plant cells because of the different subcellular locations of the two component enzymes, namely glycollate oxidase in peroxisomes and glyoxylate reductase in chloroplasts (Kelly *et al.*, 1976). Low reductase activity, low cellular glyoxylate concentrations and the lower (by 2 units) optimum pH of the reductase than the pH of chloroplast stroma have also been taken as evidence supporting non-existence of the shuttle (Tolbert, 1971*b*; Schnarrenberger & Flock, 1976).

In *Euglena gracilis*, glycollate is oxidized by glycollate dehydrogenase (Merrett & Lord, 1973, and this enzyme is located in both mitochondria and microbodies (Collins & Merrett, 1975; Yokota *et al.*, 1978*a*). *E. gracilis* does not exhibit typical photorespiration (Cheng & Colman, 1974), and the mitochondrial glycollate dehydrogenase has been shown to be concerned with the metabolism of glycollate, with a physiological function different from that reported for photo-respiring higher plants (Yokota *et al.*, 1978*b*). We have now found that the *Euglena* mitochondria also contain glyoxylate reductase, thus making possible the formation of the glycollate–glyoxylate shuttle in this organism.

* This paper is the fourth in a series on the metabolism of glycollate in *Euglena gracilis*.

Evidence supporting actual operation of this shuttle in *E. gracilis* is reported in the present paper.

Materials and Methods

E. gracilis z was cultured in Koren–Hutner (1967) medium at 27°C with illumination (4000 lux) unless otherwise stated.

Linear sucrose-density-gradient centrifugation was performed as described previously (Yokota *et al.*, 1978*a*), with a cell homogenate obtained by the controlled-digestion method of Tokunaga *et al.* (1976*a*), except that 0.4M-sucrose was used in place of 0.25M-sucrose to obtain undamaged chloroplasts.

Mitochondria were prepared by differential centrifugation of the above cell homogenate (mitochondria were sedimented at 1000g for 5 min and 10000g for 10 min). For enzyme assays, mitochondria were osmotically disrupted by suspending the organelles in sucrose-free reaction mixture.

Enzyme activities were assayed at 30°C throughout the experiments. Glyoxylate reductase (NADP⁺) was assayed spectrophotometrically by measuring the oxidation of NADPH at 340 nm by the method of Zelitch & Gotto (1962) with some modifications as follows: the reaction mixture (3 ml) contained 10 mM-potassium phosphate buffer, pH 7.2, 0.2 mM-NADPH, 3 mM-glyoxylate and enzyme, and the reaction was started by the addition of glyoxylate. Succinate semialdehyde dehydrogenase (EC 1.2.1.16) and glucose 6-phosphatase (EC 3.1.3.9) were assayed as described by Tokunaga *et al.* (1976*b*) and de Duve *et al.* (1966) respectively.

Chlorophyll was determined by the method of MacKinney (1941) and protein by that of Bradford (1976). Sucrose density was determined refractometrically at 20°C.

Fig. 1. Subcellular distribution of glyoxylate reductase in *E. gracilis*

Activities of glyoxylate reductase and succinate semialdehyde dehydrogenase are given in nmol of substrate transformed/min per 0.1 ml of fraction and that of glucose 6-phosphatase in μg of P_i formed/min per 0.1 ml of fraction. Chlorophyll and protein are expressed in mg/ml and sucrose density in g/cm^3 . $\circ$, Glyoxylate reductase; $\square$, succinate semialdehyde dehydrogenase; $\blacksquare$, glucose 6-phosphatase; $\triangle$, chlorophyll; $\blacktriangle$, protein; $\bullet$, sucrose density.

Fig. 2. Time course of glycollate-dependent NADPH oxidation by disrupted mitochondria

The reaction mixture was the same as that for the assay of glyoxylate reductase, except that 5 mM-glycollate was substituted for glyoxylate and 10 μM -rotenone was added. The reaction was started by addition of glycollate at the time indicated in the Figure. Protein concentration in the reaction mixture was 0.55 mg/ml.

Results and Discussion

Fig. 1 shows the subcellular distribution of glyoxylate reductase in *E. gracilis* together with those of marker enzymes for various organelles. Succinate semialdehyde dehydrogenase, a marker enzyme for mitochondria (Tokunaga *et al.*, 1979) showed a peak of activity at an equilibrium density of $1.19 \text{ g}/\text{cm}^3$ (fraction 12). Chloroplasts, with chlorophyll as a marker, showed a peak at an equilibrium density of $1.17 \text{ g}/\text{cm}^3$ (fraction 14). The activity of glucose 6-phosphatase, a marker enzyme for the microsomal fraction (de Duve *et al.*, 1966), gave a peak at an equilibrium density of $1.13 \text{ g}/\text{cm}^3$ (fraction 17). Fractions 20–26 represent cytosol fractions. The activity of glyoxylate reductase showed only one peak in the mitochondrial fraction. More than 90% of the total activity of this enzyme in the cell homogenate was recovered in this fraction. Neither peak nor shoulder of the activity of glyoxylate reductase was found in the chloroplast fraction. Low activity of the reductase found in the cytosol fractions was attributable to the mitochondrial enzyme leaking out during the experimental process, since some activity

of succinate semialdehyde dehydrogenase was also recovered in the cytosol fractions. A similar mitochondrial location of glyoxylate reductase was also observed with *E. gracilis* grown photoautotrophically.

The apparent K_m values of the glyoxylate reductase from *Euglena* mitochondria for glyoxylate and for NADPH were $160\mu\text{M}$ and $12\mu\text{M}$ respectively. The values reported for glyoxylate of the enzyme from spinach chloroplasts (Tolbert *et al.*, 1970) and from *Pseudomonas* (Cartwright & Hullin, 1966) are $130\mu\text{M}$ and 14mM respectively. The K_m for NADPH has not been reported for either enzyme. The *Euglena* enzyme showed a pH optimum at 7.2, whereas those for the spinach and *Pseudomonas* enzymes were at 5.8–6.5 and 6.0–6.8 respectively.

Occurrence of both glycollate dehydrogenase and glyoxylate reductase in mitochondria in *E. gracilis* strongly suggests the possibility of the existence of the glycollate–glyoxylate shuttle in this organism. The activities of the two enzymes were 0.4 nmol of glycollate oxidized/min per 10^6 cells and 20.4 nmol of NADPH oxidized/min per 10^6 cells respectively. The relatively high activity of glyoxylate reductase compared with that of glycollate dehydrogenase, and the low K_m value of the former enzyme toward glyoxylate, suggest that the cyclic pathway from glycollate to glyoxylate can proceed via glyoxylate when NADPH is supplied abundantly.

Actual operation of the glycollate–glyoxylate shuttle in *E. gracilis* was observed by a glycollate-dependent NADPH oxidation in a reaction mixture consisting of osmotically broken mitochondria, glycollate, NADPH and rotenone. Oxidation of NADPH was linear with incubation time (Fig. 2), and the oxidation rate was dependent on the protein concentration in the reaction mixture. The specific activity of the oxidation was 10 nmol of NADPH oxidized/min per mg of protein and was close to the rate of the glycollate oxidation in the same mitochondrial preparation. The glycollate-dependent NADPH oxidation was inhibited 100 and 50% by 0.1 mM-KCN and 1 mM- NaN_3 respectively; the activities of both glycollate dehydrogenase and glyoxylate reductase were not affected by these concentrations of the inhibitors. Accordingly, the results show that glycollate is oxidized to glyoxylate in the *Euglena* mitochondria by coupling with the electron-transport chain, as reported by Collins *et al.* (1975) and Yokota *et al.* (1978b). The inhibition of electron transport stops the glycollate oxidation, and the cessation of the glycollate oxidation in turn stops the NADPH oxidation coupled to reduction of glyoxylate.

It should be noted that NADPH was oxidized at nearly the rate of the glycollate oxidation without any lag, as shown in Fig. 2. The glycollate-dependent

NADPH oxidation had been expected to show the lag phase for about 2 min according to the theory developed by McClure (1969) and Bergmeyer (1974), since the activity of glyoxylate reductase (NADP^+) was high enough to satisfy the conditions of Bergmeyer (1974) as described above, and V and K_m for glyoxylate of the enzyme were 0.33 mM/min and 0.16 mM respectively, in our reaction system. The results shown in Fig. 2 may suggest that glyoxylate reductase is located in the vicinity of glycollate dehydrogenase in *Euglena* mitochondria.

The above results clearly indicate that, unlike in higher plants, the glycollate–glyoxylate shuttle works in the mitochondria of *E. gracilis* and the operation of the shuttle results in elimination of the excess energy formed under the so-called photorespiratory conditions in *E. gracilis*. NADPH formed in chloroplast has been known to be readily transferred to other cellular compartments by the malate–oxaloacetate shuttle (Heber, 1974; Walker, 1976; Halliwell, 1978). In *Euglena*, the terminal oxidase participating in the glycollate–glyoxylate shuttle is cytochrome oxidase, but not catalase, and two electrons from glycollate are transferred to oxygen through cytochromes *b*, *c* and *a* of mitochondria to give water, but not hydrogen peroxide (Yokota *et al.*, 1978b). Glyoxylate reductase in mitochondria should also work to protect the tricarboxylic acid cycle from the inhibiting action of glyoxylate (Ruffo *et al.*, 1967; Slabas & Whatley, 1977).

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