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Purification and some properties of glyoxylate reductase ($NADP^+$) and its functional location in mitochondria in *Euglena gracilis* z*

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Euglena mitochondria contain both glyoxylate reductase ($NADP^+$) and glycollate dehydrogenase to constitute the glycollate–glyoxylate cycle [Yokota & Kitaoka (1979) *Biochem. J.* **184**, 189–192]. *Euglena* glyoxylate reductase ($NADP^+$) was purified and its submitochondrial location was determined in order to elucidate the cycle. The purified glyoxylate reductase was homogeneous on polyacrylamide-gel electrophoresis. Difference spectra of the purified enzyme revealed that the enzyme was a flavin enzyme. The M_r of the enzyme was 82000. The enzyme was specific for NADPH, with an apparent K_m of 3.9 μM, and for glyoxylate, with an apparent K_m of 45 μM. It was 30% as active with oxaloacetate as with glyoxylate. NADH and hydroxypyruvate did not support the activity at all. The optimum pH was 6.45. Submitochondrial fractionation of purified mitochondria showed that the enzyme was located in the intermembrane space and loosely bound to the outer surface of the inner membrane. These properties and the submitochondrial localization of NADPH–glyoxylate reductase facilitate the operation of the glycollate–glyoxylate cycle in combination with glycollate dehydrogenase, which is tightly bound to the inner membrane of *Euglena* mitochondria.

Glyoxylate reductase ($NADP^+$) (EC 1.1.1.79) is distributed widely among photosynthetic organisms (Zelitch, 1971). In higher plants containing glycollate-oxidizing enzyme in peroxisomes, the glyoxylate reductase is located in chloroplasts. It serves to diminish the inhibition of ribulose bisphosphate carboxylase (Lawyer *et al.*, 1983; Cook *et al.*, 1984), of the CO_2-fixing enzyme in photosynthesis and of the photosynthetic carbon-reduction cycle (Mulligan *et al.*, 1983) by glyoxylate, and it removes excess NADPH in chloroplasts (Tolbert *et al.*, 1970; Peterson, 1982). In *Euglena gracilis*, which oxidizes glycollate in mitochondria (Kitaoka *et al.*, 1984), as do green algae (Stabenau, 1974; Bealy *et al.*, 1976) and diatoms (Paul *et al.*, 1975), glyoxylate reductase ($NADP^+$) is located in mitochondria and constitutes the glycollate–glyoxylate cycle in combination with glycollate dehydrogenase (Yokota & Kitaoka, 1979*b*). Since NADPH is oxidized immediately after the addition of glycollate to osmotically broken mitochondria of *Euglena* in the presence of rotenone, it has been suggested that the glyoxylate reductase ($NADP^+$) is located in the vicinity of glycollate dehydrogenase in mitochondria (Yokota & Kitaoka, 1979*b*). However, the lack of enzymological information on glyoxylate reductase ($NADP^+$) purified from photosynthetic organisms has limited understanding of the significance of glyoxylate reduction in *Euglena* mitochondria and in chloroplasts of the plants during their photosynthesis. In the present work we purified glyoxylate reductase ($NADP^+$) from *E. gracilis* and studied some properties of the enzyme. We also determined the submitochondrial localization of the enzyme and discuss the functional glycollate–glyoxylate cycle in *Euglena* mitochondria.

Materials and methods

Organism and culturing

E. gracilis z was grown on Koren & Hutner (1967) medium (initial pH 3.5) under illumination at 2000 lx as reported previously (Yokota *et al.*, 1978). The cells at late-exponential phase of growth were collected by centrifugation at 3000*g* for 5 min.

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Assay of glyoxylate reductase (NADP$^+$)

This was done spectrophotometrically at 30°C (Yokota & Kitaoka, 1979*b*). The reaction mixture contained 50mM-potassium phosphate buffer, pH6.45, 0.15mM-NADPH, 5mM-glyoxylate and enzyme, and the reaction was started by adding glyoxylate. One unit of the enzyme activity is defined as the amount of enzyme that catalyses the oxidation of NADPH at the rate of 1μmol/min.

Purification of glyoxylate reductase (NADP$^+$)

Purification procedures were conducted at 0–4°C: 10mM-potassium phosphate buffer, pH7.2, containing 20% (v/v) ethylene glycol was used throughout the purification. The *Euglena* cells (100g wet wt.) were suspended in the phosphate buffer containing 0.1% Triton X-100 and disintegrated in a mortar and pestle with HCl-washed sea sand. The homogenate was centrifuged at 3000*g* for 5min. To the cell extract was added ethanol cooled with solid CO_2 (final concn. 33%, v/v) with stirring with a magnetic stirrer. The ethanol-precipited protein was collected by centrifugation at 5000*g* for 10min, and resuspended in the cell-homogenizing buffer containing Triton, with a Teflon homogenizer. The suspension was centrifuged at 10000*g* for 5min. The clarified supernatant was applied to a column (4cm × 54cm) of DEAE-cellulose equilibrated with the phosphate buffer. The column was washed with the same buffer until protein could not be detected in the effluent, and the enzyme was eluted with a 2-litre linear gradient of 0–0.5M-KCl in the phosphate buffer at a flow rate of 1.8ml/min. The active fractions (11ml each) were collected, concentrated in dialysis tubing with poly(ethylene glycol) 20000 powder (average M_r 20000) overnight, dissolved in the phosphate buffer, and applied on to a column (1.5cm × 24cm) of CM-cellulose equilibrated with the phosphate buffer. The effluent fractions were pooled and applied on to a column (1cm × 2cm) of Cibacron Blue 3G-A (formerly Cibacron Blue F3G-A)-linked alkali-hydrated paramylon prepared as described by Klein *et al.* (1969) and equilibrated with the phosphate buffer. After extensive washing of the column with the buffer, the enzyme was eluted with 36ml of a 0–2M-KCl linear gradient in the phosphate buffer. The active fractions (2ml each) were collected and stored at −20°C.

Disc electrophoresis in polyacrylamide gel

This was performed by the method of Davis (1964), with 7.5%-acrylamide gel (pH9.4) at 5mA/gel for 4h at 4°C. Protein in the gel was stained with Coomassie Brilliant Blue R-250 (Meyer & Lamberts, 1965).

Subfractionation of purified mitochondria

To avoid contamination by broken chloroplasts of the mitochondrial preparation, a streptomycin-bleached mutant of *E. gracilis*, which had lost chloroplasts without any change in other cellular components (Buetow, 1968; Mego, 1968), was used for experiments. Cell cultivation of the bleached mutant, preparation and purification of mitochondria and submitochondrial fractionation were conducted by the method established by Isegawa *et al.* (1984). Marker enzymes, rotenone-insensitive NADPH–cytochrome *c* reductase (Yokota & Kitaoka, 1979*b*) for the outer membrane, adenylate kinase (Bergmeyer, 1974) for the intermembrane space, lactate dehydrogenase (cytochrome) (Yokota & Kitaoka, 1979*a*) for the inner membrane, and malate dehydrogenase (NAD$^+$) (Ting, 1968) for the matrix, were assayed by the methods in the cited references.

Protein determination

Protein was quantified by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results and discussion

Euglena glyoxylate reductase (NADP$^+$) was labile in the crude cell extract in the presence of 50mM-potassium phosphate buffer, pH7.2, and lost its activity within 24h in ice, but addition of 20% ethylene glycol to the buffer significantly prevented the inactivation of the enzyme. Table 1 summarizes the purification of glyoxylate reductase (NADP$^+$) from *E. gracilis*. The enzyme in the crude cell extract was precipitated or membrane-associated in the presence of 33% ethanol, like the enzyme in spinach leaf extract (Zelitch & Gotto, 1962), and was collected by centrifugation at slow speed. The precipitated enzyme could be recovered in the phosphate buffer by suspension in the buffer containing 0.1% Triton X-100, with a Teflon homogenizer. The glyoxylate reductase was eluted with 0.2–0.25M-KCl from the DEAE-cellulose column as a sharp single peak. No activity was found in any other fraction, indicating that *Euglena* contains only one kind of glyoxylate reductase (NADP$^+$). The glyoxylate reductase was not adsorbed by CM-cellulose at pH7.2 at all. Affinity chromatography of the enzyme on an adsorbent prepared with Cibacron Blue 3G-A and alkali-hydrated paramylon (β-1,3-glucan from *Euglena*) was used as the final purification step. The enzyme was eluted from the column with the phosphate buffer containing 0.5M-KCl. Disc electrophoresis in polyacrylamide gel of the eluted active fractions revealed a single protein band

Table 1. *Purification of NADPH–glyoxylate reductase from Euglena gracilis*
Details of the purification are described in the text.

Purification step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Purification (fold)
Crude extract	885	9735	597.6	0.061	100	1
Precipitation by ethanol	940	9033	569.7	0.063	95.3	1.0
Extraction from ethanol precipitate	895	1127	474.8	0.421	79.5	6.9
DEAE-cellulose	220	66.0	466.9	7.074	78.1	115.2
CM-cellulose	34.2	33.9	375.3	11.071	62.4	180.3
Affinity chromatography	34.0	16.9	285.6	17.102	47.8	278.5

Fig. 1. *Polyacrylamide-gel electrophoresis of glyoxylate reductase (NADP+) from the final purification step*
For details see the text.

(Fig. 1). After these purification steps, the specific activity of the purified enzyme was 17.1 units/mg of protein, 280-fold that in the crude cell extract.

The purified glyoxylate reductase (NADP+) had a pale-yellow tint. The yellow colour disappeared on addition of 0.1 mM-NADPH and was recovered by subsequent addition of an excess amount of glyoxylate, as shown in Fig. 2. The inset shows the difference spectra of the NADPH-reduced minus glyoxylate-oxidized and NADPH-reduced minus purified enzyme. The bleaching around 450 nm on reduction of NADPH indicates that the *Euglena* mitochondrial glyoxylate reductase (NADP+) is a flavin enzyme.

Table 2 shows some properties of the purified glyoxylate reductase (NADP+). The M_r of the enzyme was estimated to be 82000 by gel filtration on calibrated Sephadex G-150. Optimum pH was at pH 6.45, and the enzymic activity was decreased by 40% and 50% at pH 5.5 and 8.0 respectively. The

Table 2. *Summary of some properties of Euglena glyoxylate reductase (NADP+)*
M_r was determined with a Sephadex G-150 column (1.5 cm × 90 cm) calibrated with catalase from bovine liver (M_r 232000) (Luck, 1963), alcohol dehydrogenase from yeast (M_r 140000) (Schimpfessel, 1968), glucose-6-phosphate dehydrogenase (M_r 128000) (Kornberg & Horecker, 1955), malate dehydrogenase (NAD+) from pig heart (M_r 70000) (Ting, 1968) and ovalbumin (M_r 43000). The activities of the enzymes were assayed by the methods in the cited references. Ovalbumin was determined by the method of Lowry *et al.* (1955). For experiments on substrate specificity, concentrations of nicotinamide nucleotides and of sodium salts of keto acids and aldehydes were 0.15 mM and 1 mM respectively. The pH optimum was determined by using 10 mM McIlvaine (1921) buffer for the reaction buffer between pH 5.0 and 7.0, 10 mM-potassium phosphate buffer between pH 6.0 and 7.8, and 10 mM-Tris/HCl buffer between pH 7.2 and 8.7. Enzyme concentration for these assays was 2.6 μg per reaction mixture (2 ml). Refer to the text and Fig. 3 legend for other details.

M_r	82000
Apparent K_m for	
NADPH	3.9 μM
Glyoxylate	45 μM
Substrate specificity	
Keto acids or aldehydes	
Glyoxylate	100
Oxaloacetate	28.6
2-Oxoglutarate	13.9
Acetaldehyde	10.4
Hydroxypyruvate, pyruvate and formaldehyde	0
Nicotinamide nucleotides	
NADPH	100
NADH	0
Optimum pH	6.45

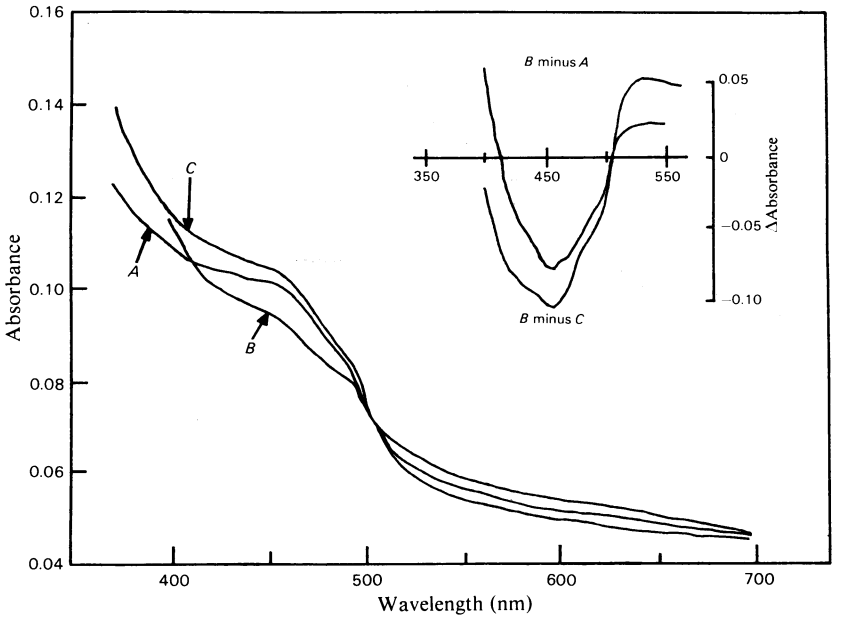

Fig. 2. *Light-absorbance spectra of the purified glyoxylate reductase (NADP$^+$)*
Spectrum *A* is of the purified native enzyme, spectrum *B* is of it reduced by 0.1mM-NADPH, and spectrum *C* is of it oxidized by 5mM-glyoxylate. The inset shows the difference spectra of *B* minus *A* and of *B* minus *C*. Protein concentration was 50μg/ml.

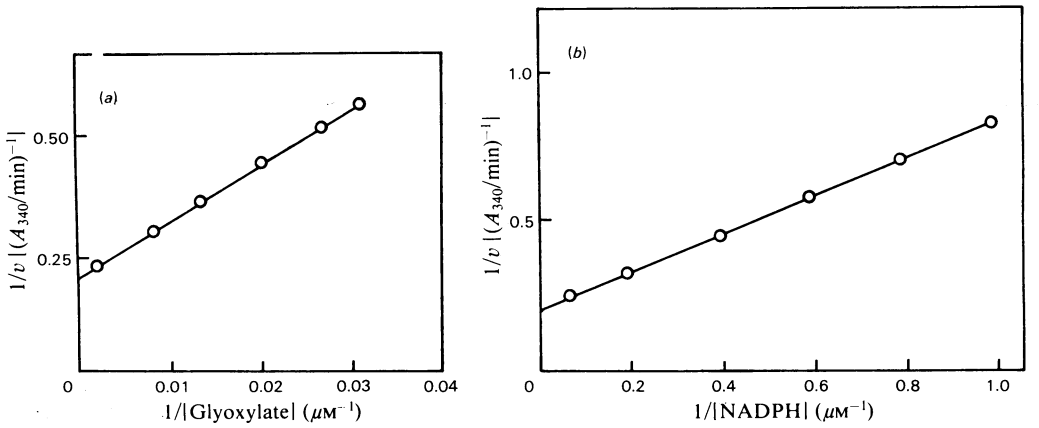

Fig. 3. *Lineweaver–Burk plots of the relationships between the activity of glyoxylate reductase (NADP$^+$) and concentrations of NADPH and glyoxylate*
In (*a*) NADPH was 0.15mM and in (*b*) glyoxylate was 5mM. Protein concentration was 2.57μg/reaction mixture.

apparent K_m values of the enzyme were 3.9μM for NADPH in the presence of 5mM-glyoxylate and 45μM for glyoxylate in the presence of 0.15mM-NADPH (Fig. 3). NADH could not substitute for NADPH at all. The enzyme was specific for glyoxylate, and oxaloacetate, 2-oxoglutarate and acetaldehyde supported only weak activities. It was inactive with hydroxypyruvate, pyruvate and formaldehyde. Although glyoxylate reductase (NADP$^+$) studied so far in non-photosynthetic organisms either reduces hydroxypyruvate at a rate near to or more than the rate of reduction of glyoxylate, or has a K_m for glyoxylate more than 10mM (Cartwright & Hullin, 1966; Tochikura *et al.*, 1979; Fukuda *et al.*, 1980, 1981), the *Euglena* enzyme catalysed the reduction of glyoxylate relatively specifically, with an apparent K_m value 100-fold lower than those of other sources. The

Euglena enzyme was inhibited by 55% after preincubation for 5min with 0.1mM-*p*-chloromercuribenzoate and by 75% with 10mM-*N*-ethylmaleimide. Adenine also inhibited the activity by 70% at 10μM concentration, whereas adenine nucleoside and nucleotide were much less inhibitory (20% inhibition at 10mM).

Table 3 shows the submitochondrial distribution of glyoxylate reductase (NADP+) in *Euglena* mitochondria. In these experiments, we used a bleached mutant of *Euglena*, obtained by removing chloroplasts from the wild-type cells with streptomycin (Buetow, 1968; Mego, 1968) to remove interference by broken chloroplasts with accurate subfractionation of mitochondria. The mutant gives cell functions quite similar to those of the wild-type cells in the enzymic constitution of the glycollate pathway (Kitaoka *et al.*, 1985; Yokota & Kitaoka, 1981). Glyoxylate reductase (NADP+) and glycollate dehydrogenase are localized in mitochondria to constitute the functional glycollate–glyoxylate cycle, as in the wild-type cells (Yokota & Kitaoka, 1981). Rotenone-insensitive NADPH–cytochrome *c* reductase, adenylate kinase, lactate dehydrogenase (cytochrome) and malate dehydrogenase (NAD+) were used as marker enzymes for outer membrane, intermembrane space, inner membrane and matrix respectively (Isegawa *et al.*, 1984). These marker enzymes were well separated from each other and recovered in the appropriate submitochondrial fractions, although the reason for the recovery of a large amount of protein in the intermembrane-space fraction could not be elucidated. Most of the glyoxylate reductase (NADP+) was recovered in the intermembrane-space and inner-membrane fractions. Since the specific activity of purified glyoxylate reductase (NADP+) was 17.1 units/mg of protein (Table 1) and the activity of the enzyme in purified mitochondria was 0.36 unit/mg of mitochondrial protein, the glyoxylate reductase was calculated to constitute 2.1% of total mitochondrial protein. Mitochondria, in general, contain several hundred kinds of proteins, and the percentage of the reductase in mitochondrial proteins suggests that this enzyme is one of the major components in the organelles. The high specific activity of glyoxylate reductase (NADP+) observed in the outer-membrane fraction is due to contamination of this fraction, which constitutes only a very small proportion of total mitochondria, by a major constituent of this organelle. In addition, both inner-membrane and intermembrane-space fractions contained most of the glyoxylate reductase activity, and therefore it can be concluded either that the glyoxylate reductase (NADP+) is located both in the intermembrane space and on the outer surface of the inner membrane or that the enzyme loosely binds to the inner membrane from the side of the intermembrane space, and the activity in the intermembrane-space fraction is due to exfoliation of the enzyme from the inner membrane during the subcellular-fractionation procedures. Since glycollate dehydrogenase of *E. gracilis* is an enzyme tightly bound to the mitochondrial inner membrane (Kitaoka *et al.*, 1985), the inner-membrane-bound glyoxylate reductase (NADP+) is a constituent of the glycollate–glyoxylate cycle.

In a previous paper (Yokota & Kitaoka, 1979*b*), we found immediate oxidation of NADPH by adding glycollate to osmotically broken mitochondria in the presence of rotenone. This reaction is due to re-reduction of glyoxylate, formed from glycollate by glycollate dehydrogenase on the inner membrane, by glyoxylate reductase (NADP+). In this reaction, there is no lag of the oxidation of NADPH, which is ordinarily encountered in two-step consecutive enzymic reactions. The K_m value of purified glyoxylate reductase (NADP+) for

Table 3. *Subcellular location of glyoxylate reductase (NADP+) in purified mitochondria*
For details see the text. Numbers in parentheses represent percentage of the activity recovered in the fraction.

		Specific activity (μmol/min per mg of protein)				
Submitochondrial fraction	Protein (mg)	Lactate dehydrogenase (cytochrome)	Rotenone-insensitive NADPH–cytochrome *c* reductase	Malate dehydrogenase (NAD+)	Adenylate kinase	Glyoxylate reductase (NADP+)
Outer membrane	0.32	0	32.656	0	0	4.433
	(2.9)	(0)	(93.7)	(0)	(0)	(18.55)
Intermembrane space	4.59	0	0.061	0.05	0.18	0.573
	(44.6)	(0)	(2.7)	(1.4)	(100)	(37.0)
Inner membrane	1.33	0.188	0.203	0	0	1.29
	(12.0)	(100)	(2.4)	(0)	(0)	(33.0)
Matrix	4.50	0	0.029	3.84	0	0.189
	(40.5)	(0)	(1.2)	(98.6)	(0)	(11.1)

glyoxylate was 45 μM (Table 2), about one-third that obtained with broken mitochondria (Yokota & Kitaoka, 1979*b*). However, this K_m value is still too high to explain the immediate oxidation of NADPH after addition of glycollate to broken mitochondria; the lag phase in the oxidation was calculated to be 0.64 min by using this K_m value and the theory of Storer & Cornish-Brown (1974) on two-step consecutive enzymic reactions. This suggests the very vicinal compartmentalization of both enzymes in mitochondria. The present studies have provided evidence of the effective glycollate–glyoxylate cycle from enzymological and subcellular-fractionation experiments. Glyoxylate reductase ($NADP^+$) is loosely attached to the outer surface of the inner membrane, probably in the vicinity of glycollate dehydrogenase, and is specific for glyoxylate, with a low apparent K_m of 45 μM. These properties of the enzyme are advantageous to the function of the cycle. Reducing equivalents can be supplied as malate from chloroplasts, since the mitochondrial intermembrane space of *Euglena* also contains $NADP^+$-specific malate dehydrogenase (Isegawa *et al.*, 1984). In addition, since the intracellular pH of *Euglena* is 6–6.5 (Lane & Burris, 1981), and active metabolism of glycollate during photorespiration (Yokota & Kitaoka, 1982) may cause acidification of the outside of mitochondria, the circumstances of the glyoxylate reductase-($NADP^+$)-localizing compartment guarantee the operation of the glycollate–glyoxylate cycle.

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