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GLYOXYLATE CARBOXY-LYASE ACTIVITY IN THE UNICELLULAR GREEN ALGA GLOEOMONAS SP.

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SUMMARY

Glyoxylate carboxy-lyase (also known as glyoxylate carboligase, systematic name: glyoxylate carboxy-lyase (dimerizing and reducing)) activity has been demonstrated in cell-free extracts prepared from the photoautotrophically grown unicellular green alga Gloeomonas. The enzyme preparation catalysed the anaerobic decarboxylation of [1-^{14}C]glyoxylate as determined by $^{14}CO_2$ evolution with thiamine pyrophosphate and Mg^{2+} as cofactors. The other reaction product was tartronic acid semialdehyde. The stoichiometry was 1 mole of CO_2 from 2 moles of glyoxylate. The K_{app} was 0.2 mM.

Glyoxylate carboxy-lyase activity in algal extracts from cells at stages of cellular division and separation of daughter cells, grown at 1000 ft-candles and 5% CO_2 in air, was associated with substantial activity of isocitrate lyase.

INTRODUCTION

In a previous communication[1] we reported the excretion of an acid semialdehyde by the unicellular green alga Gloeomonas sp. This compound was tentatively identified as mesoxalic acid semialdehyde (ketomalonic acid semialdehyde) and was released into the surrounding medium in light at the stages of maturity and cellular division. Excretion occurred when the algal cells had accumulated carbohydrates during their growth at high light intensity (1000 ft-candles) and high CO_2 tension (5% CO_2, v/v). It was suggested that the excretory product mesoxalic acid semialdehyde was formed from the autoxidation of tartronic acid semialdehyde. The latter could arise by glyoxylate carboxy-lyase (also known as glyoxylate carboligase, systematic name: glyoxylate carboxy-lyase (dimerizing and reducing)) similar to that of bacteria[2,3]. It was also pointed out that glyoxylate required for this reaction would arise *via* isocitrate lyase.

In this paper we have demonstrated glyoxylate carboxy-lyase activity in cell-free preparations from the autotrophic unicellular green alga Gloeomonas sp. which is associated with substantial activity of isocitrate lyase. The presence of these two enzymes provides strong evidence that under the culture conditions described they

catalyse the synthesis of tartronic acid semialdehyde which is oxidized to mesoxalic acid semialdehyde, the major excretory product of Gloeomonas.

MATERIALS AND METHODS

Organism and cultures

The unicellular green alga which has been tentatively identified as Gloeomonas sp. was photoautotrophically grown (1000 ft-candles, 25° and 5% CO_2 in air, v/v) and synchronized as described previously[1]. The algal cells at stage of cellular division (16 h light) were used for preparation of cell-free extracts.

Preparation of cell-free extracts

Cells were harvested from 2-l algal cultures, washed twice in 0.1 M phosphate buffer pH 7.0 and the packed cell volume was diluted to 100 ml suspension by cold phosphate buffer containing 1 mM mercaptoethanol and frozen overnight at −16°. After thawing at room temperature and sonication in ice, the resulting homogenate was centrifuged at 0° for 15 min at 3000 × *g* to remove cell debris. About 5 ml of the green supernatant was used for detection of malate synthase (EC 4.1.3.2) and the remainder was centrifuged for 90 min at 100 000 × *g*. Isocitrate lyase (EC 4.1.3.1) was determined in the greenish-yellow supernatant.

The greenish-yellow supernatant was either frozen at −16° overnight or treated with protamine sulphate and fractionated with $(NH_4)_2SO_4$ according to the procedure of GOTTO AND KORNBERG[4]. All operations were carried out at 4°. The yellowish 40–75% $(NH_4)_2SO_4$ precipitate was collected by centrifugation for 30 min at 35 000 × *g*, dissolved in 8–10 ml of 0.1 M phosphate buffer, pH 7.0 and dialysed for 16 h against 4 l of 0.05 M phosphate–1 mM mercaptoethanol, pH 7.0. The dialysate was centrifuged and the yellowish supernatant used for glyoxylate carboxy-lyase assay. This enzyme preparation retained its activity for about one week at −16°, but gradually lost activity on repeated thawing and freezing.

Enzyme assays

Glyoxylate carboxy-lyase. The enzyme was assayed by measuring the $^{14}CO_2$ produced from [1-^{14}C]glyoxylate and identification of tartronic acid semialdehyde as the other reaction product. A combination of methods described elsewhere by various authors[5–7] was employed and in which conventional double-side-arm Warburg flasks with central well were used. The main compartment contained in μmoles: potassium phosphate buffer, pH 7.5, 100; $MgCl_2$, 3; thiamine pyrophosphate, 0.65, and enzyme in a volume of 1.8 ml. The enzyme assay was carried out under O_2-free nitrogen at 30°, started by tipping in 0.2 ml containing 0.5 μmole unlabelled glyoxylate with 0.16 μmole [1-^{14}C]glyoxylate and stopped after 30 min by the addition of 2 M H_2SO_4 from the other side arm. After shaking for a further 30 min the $^{14}CO_2$ trapped in the central well by 20% KOH was measured with a scintillation counter.

Identification of the reaction product in the main compartment was carried out by thin-layer chromatography using MN-cellulose with binders as follows. At the end of the reaction the protein was removed by centrifugation. 1 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl was added and allowed to stand for 1 h in a water bath at 30°. The 2,4-dinitrophenylhydrazones were extracted into ethyl acetate,

concentrated by evaporation with air and chromatographed using tertiary amyl alcohol–ethanol–water (5:1:4, by vol.). The areas were treated with 4 M NaOH in ethanol to produce the colours characteristic of various 2,4-dinitrophenylhydrazone derivatives. Radioactivity was detected in these spots by exposing the plates to X-ray films.

Synthetic tartronic acid semialdehyde was prepared from dihydroxyfumaric acid according to the procedure of FUKUNAGA[8] and VALENTINE *et al.*[9] and used as the reference for chromatography.

Isocitrate lyase and malate synthase. Isocitrate lyase was assayed spectrophotometrically by measuring the increased absorbance at 324 nm resulting from the formation of glyoxylatephenylhydrazone[10]. Malate synthase was assayed by incubating the algal extract at 30° with 1 μC [2-^{14}C]glyoxylate (0.064 μmole), 0.1 μmole acetate and cofactors as described elsewhere[11]. The radioactive malate produced was chromatographed on MN-cellulose thin-layer chromatography using ethyl acetate–formic acid–water (11:5:3, by vol.) and exposed to X-ray film. The radioactive malate area was scraped off the thin-layer chromatography plate and counted. Protein was determined by the method of LOWRY *et al.*[12].

RESULTS

Demonstration of glyoxylate carboxy-lyase activity

Anaerobic $^{14}CO_2$ evolution from [1-^{14}C]glyoxylate was consistently observed in complete assay systems (Table I). Omission of Mg^{2+} or thiamine pyrophosphate from the reaction mixture led to substantial decreases in $^{14}CO_2$ production which were

TABLE I

ANAEROBIC DECARBOXYLATION OF [1-^{14}C]GLYOXYLATE BY ENZYME PREPARATION FROM GLOEOMONAS SP.

Complete system contained: 100 μmoles potassium phosphate buffer (pH 7.5); 3 μmoles $MgCl_2$; 0.65 μmole thiamine pyrophosphate; 0.16 μmole sodium [1-^{14}C]glyoxylate with 0.5 μmole unlabelled sodium glyoxylate and enzyme (2 mg protein for Expt. 1, and 5.1 mg protein for Expt. 2) in a total volume of 2 ml. Incubation at 30° for 30 min under oxygen-free nitrogen. The enzyme was partially purified and dialysed as indicated in MATERIALS AND METHODS.

Assay System	$^{14}CO_2$ *evolved (disint./min* $\times 10^{-3}$*)*	
	Exp. 1	*Exp. 2*
Complete	95.7	153.7
Minus $MgCl_2$	65.5	88.5
Minus TPP	20.8	36.1
Minus Enzyme	1.7	10.7

most pronounced in absence of thiamine pyrophosphate, as reported for bacterial glyoxylate carboxy-lyase[6]. Non-enzymic decarboxylation of glyoxylate was less than 10%.

The enzymic activity was abolished by boiling, and by a temperature of 60°. In this respect the algal enzyme is different from that of *Escherichia coli*[6] which tolerates 75°. The optimum pH was 7.5 in 0.05 M phosphate buffer and the enzyme

activity was inhibited by Tris buffer which had no inhibitory effect on glyoxylate carboxy-lyase from Hydrogenomonas H16 (ref. 13).

The other reaction product formed an orange coloured bis-2,4-dinitrophenylhydrazone which produced a deep blue purple color[14] in alkaline solution showing an absorption maximum at 550–560 nm, similar to that of alkaline bis-2,4-dinitrophenylhydrazone derivative of synthetic tartronic acid semialdehyde and mesoxalic acid semialdehyde[1]. By chromatography, the 2,4-dinitrophenylhydrazone derivative of tartronic acid semialdehyde could be separated from those of hydroxypyruvate, dihydroxyfumarate, glyoxylate, glycolaldehyde and glyoxal. The R_F value and blue purple colour with alkali was identical to that of synthetic tartronic acid semialdehyde and the area was radioactive.

Since the enzyme preparation contained high glyoxylate reductase (EC 1.1.1.26) and utilized the substrate in presence of NAD(P)H, it was not possible to use the latter to reduce tartronic acid semialdehyde quantitatively to glycerate in the complete reaction mixture shown in Table I. However, when this reaction mixture was brought to completion as demonstrated in Fig. 1 and after being deproteinized and neutralised, the product in the clear supernatant could be completely reduced to glycerate in the presence of the same enzyme preparation, buffer, and 1.0 μmole

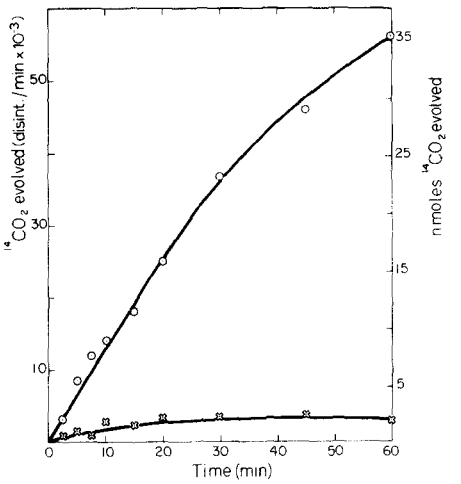

Fig. 1. Progress curve of anaerobic decarboxylation of [^{14}C]glyoxylate by glyoxylate carboxy-lyase. 78 nmoles [1-^{14}C]glyoxylate (117 000 disint./min), 0.25 ml of enzyme solution (1.8 mg protein/ml) were used. Buffer and cofactors as given in Table I. Enzymic reaction (○) and non-enzymic reaction (×).

NADH; a similar assay system as described for bacterial tartronic acid semialdehyde reductase[4] (EC 1.1.1.g). This reaction was followed spectrophotometrically by recording the oxidation rate of NADH and the end product glycerate was identified chromatographically.

In Fig. 1, it is clearly shown that about 35 nmoles of CO_2 are produced from 78 nmoles [1-^{14}C]glyoxylate, a stoichiometry similar to that reported for bacterial enzymes[2,13,14] where the anaerobic condensation of two glyoxylate molecules yielded 1 molecule CO_2 and 1 molecule of tartronic acid semialdehyde.

For determining the effect of substrate concentration on enzyme activity, initial velocities of the reaction were obtained from counting $^{14}CO_2$ evolved and were expressed as nmoles $^{14}CO_2$ produced per 10 min. The enzyme showed typical Michaelis kinetics and a K_m of 0.2 mM was calculated from double-reciprocal plots.

Relation of glyoxylate carboxy-lyase to the glyoxylate cycle

When isocitrate lyase was assayed in cell-free extracts prepared from photoautotrophic cultures of Gloeomonas grown under varied environmental conditions as shown in Table II, the enzyme could be consistently detected in extracts from cultures maintained at 1000 ft-candles aerated with 5% CO_2 in air with NH_4^+ as nitrogen

TABLE II

LEVELS OF ISOCITRATE LYASE ACTIVITY IN GLOEOMONAS GROWN PHOTOAUTOTROPHICALLY IN SYNCHRONOUS CULTURES AS INFLUENCED BY NITROGEN SOURCE OF CULTURE MEDIA (NO_3^- OR NH_4^+), CO_2 CONCENTRATION AND LIGHT INTENSITY

Cell-free extracts were prepared from cells at stages of cellular division and separation of daughter cells (16 h continuous illumination).

Nitrogen source	*Light intensity (ft-candles)*	*CO_2 (% in air)*	*Specific activity (nmoles glyoxylate formed/min per mg protein)*
NO_3^-	1000	5.0	50
		0.1	0
	300	5.0	8
		0.1	0
NH_4^+	1000	5.0	78
		0.1	0
	300	5.0	0
		0.1	0

source. From such cultures, glyoxylate carboxy-lyase activity was consistently present in the extracts.

If the glyoxylate cycle were operating in photoautotrophically grown Gloeomonas as indicated by isocitrate lyase activity, the second enzyme of the cycle, malate synthase, should also be present. Using the spectrophotometric method[15] in which enzymically produced malate is oxidized to oxaloacetate by NAD and malate dehydrogenase, it was not possible to detect any activity of malate synthase. However, when [2-^{14}C]glyoxylate was included in the reaction mixture as given in MATERIALS AND METHODS, about 10% of the radioactivity was recovered in malate corresponding to a specific activity of 0.1 nmole/min per mg protein. On the other hand, glyoxylate carboxy-lyase assayed in the crude soluble protein fraction of the same algal extract was 10-fold more active.

The low activity of malate synthase is not attributable to slow rate of acetate activation, since the algal extracts contained substantial activity (17 nmoles/min per mg protein) of acetyl-CoA synthetase (EC 6.2.1.1) as determined spectrophotometrically[16] at 540 nm. The low malate synthase activity in Gloeomonas is in agreement with other observations made in autotrophic green algae as Euglena[17] and Chlorella[18].

DISCUSSION

As shown in the present study, detection of glyoxylate carboxy-lyase in Gloeomonas was made possible only by using [1-^{14}C]glyoxylate to trace the evolved $^{14}CO_2$ from glyoxylate. Similar to Chlorella[18], the enzyme activity in Gloeomonas was undetectable in crude or partially purified extracts when the conventional manometric method for measuring anaerobic CO_2 evolution[6] was employed. In the present work, glyoxylate carboxy-lyase activity has been detected in cells exposed to high light intensity and 5% CO_2 at the stages of cellular division and separation of daughter cells. Since these developmental stages are light independent, and apparently the algal cells are forced to utilize unnecessary energy, it seems plausible to postulate the diversion of some isocitrate from the tricarboxylic acid pathway *via* isocitrate lyase and glyoxylate carboxy-lyase. By this additional CO_2 production and diversion of tartronic acid semialdehyde to excretory products the alga would be able to dispose of some of its stored energy if it has to photosynthesize under these environmental conditions[19].

Though isocitrate lyase has been reported by some authors to be induced only when algae are forced to assimilate acetate[20,21], substantial activity (Table II) of this enzyme has been determined in Gloeomonas cultures aerated with 5% CO_2 in air and exposed to relatively high light intensity. Similar activity of isocitrate lyase was observed in photoautotrophic mass cultures of Chlamydomonas and Chlamydobotrys[22].

Actually, the key enzyme of the glyoxylate cycle is known to operate with malate synthase in order to account for the assimilation of acetate by photoheterotrophically grown algae[11]. The very low activity of malate synthase in Gloeomonas cells loaded with plentiful carbohydrates[1] may indicate another role of isocitrate lyase different from the well known anabolic pathway leading to malate synthesis with acetyl-CoA. It is most likely in this organism that isocitrate lyase furnishes glyoxylate as a substrate for glyoxylate carboxy-lyase and thereby functions in a "degradative pathway" and replenishment of C_4 compounds is normally achieved by β-carboxylation of phosphoenolpyruvate in photosynthesis (S. S. BADOUR AND E. R. WAYGOOD, unpublished results).

Detection of glyoxylate carboxy-lyase activity in autotrophic green algae or plants has not been reported previously to our knowledge. The presence of such activity in Gloeomonas may support the opinion that this enzyme would be implicated in the decarboxylation of glyoxylate formed from glycolate or glycine oxidation[23,24] and might be reponsible for the blue light-enhanced respiration in algae[24] as well as for part of the CO_2 loss during photorespiration[25,26].

Studies concerning further purification of this enzyme for kinetic studies are in progress.

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