Effect of L-glutamate on 2-oxoglutarate decarboxylase in *Euglena gracilis*

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The effect of tricarboxylic acid-cycle intermediates and related compounds on 2-oxoglutarate decarboxylase activity was investigated. The addition of L-glutamate to *Euglena* cells grown on glucose/(NH₄)₂SO₄ medium resulted in an increase in 2-oxoglutarate decarboxylase activity, which was abolished by the simultaneous addition of cycloheximide. Immunochemical titration, immunoblot analysis and labelling *in vivo* with antibody raised against 2-oxoglutarate decarboxylase showed that the increase in 2-oxoglutarate decarboxylase activity was due to synthesis of new protein and not to activation of pre-existing protein. The experimental results reported here demonstrate that L-glutamate is assimilated by the pathway, via 2-oxoglutarate, that consists of L-glutamate-oxaloacetate aminotransferase, 2-oxoglutarate decarboxylase and succinate semialdehyde dehydrogenase, rather than by the γ -aminobutyrate shunt, consisting of L-glutamate decarboxylase and γ -aminobutyrate aminotransferase.

INTRODUCTION

We have demonstrated previously that 2-oxoglutarate (2-OG) decarboxylase, which catalyses the conversion of 2-OG into succinate semialdehyde (SSA), occurs in mitochondria of *Euglena gracilis*, which lacks a 2-OG dehydrogenase complex [1]. Tokunaga *et al.* [2] have reported that *Euglena* mitochondria contain two SSA dehydrogenases, one requiring NAD⁺ and the other NADP⁺, which oxidize SSA to succinate. Incubation of 2-OG and NADP⁺ with partially purified 2-OG decarboxylase and SSA dehydrogenase in combination has been found to give strictly stoichiometric consumptions of 2-OG and NADP⁺, evolution of CO₂ and formation of NADPH and succinate [1]. Therefore it is clear that *Euglena* contains an alternative route to the tricarboxylic acid (TCA) cycle, which consists of 2-OG decarboxylase and SSA dehydrogenase. We have reported recently on the enzymological and molecular properties of purified 2-OG decarboxylase from *Euglena gracilis* and the occurrence of a precursor 2-OG decarboxylase which is about 3 kDa larger than the subunit of the mature enzyme, which has a molecular mass of 62 kDa [3]. We also suggested that this enzyme is a key regulatory enzyme in the TCA cycle of *Euglena* because of the following properties. (1) The conversion of 2-OG into SSA is the only irreversible reaction in the *Euglena* TCA cycle. (2) 2-OG decarboxylase is competitively inhibited by SSA, the reaction product. (3) The presence of NADP⁺ and its analogues in the reaction mixture causes 2-OG decarboxylase activity to increase significantly.

In order to elucidate in detail the regulatory system of 2-OG decarboxylase in the TCA cycle, we investigated the effect of several TCA-cycle-intermediates and related compounds on 2-OG decarboxylase activity. In the present paper we report on the molecular mechanism of the increase in 2-OG decarboxylase activity caused by addition of L-glutamate and on the physiological function of 2-OG decarboxylase in the assimilation of L-glutamate in *Euglena gracilis*.

EXPERIMENTAL

Culture

Euglena gracilis SM-ZK, a streptomycin-bleached mutant of

Euglena gracilis z, was grown heterotrophically at 26 °C for 5 days under illumination (2000 lux) in modified Koren–Hunter medium [4] containing D-glucose (12 g/l) and (NH₄)₂SO₄ (3.95 g/l) (final ratio 6:1) as the sole carbon and nitrogen sources: this is termed glucose/(NH₄)₂SO₄ medium. Growth was measured by counting the cell number with a haemocytometer.

Preparation of crude homogenate

The harvested cells were washed twice, resuspended in 50 mM-potassium phosphate buffer, pH 7.0, containing 20 % (w/v) ethylene glycol, and disrupted by ultrasonic treatment (10 kHz) for 1 min. The resulting lysate was centrifuged at 12000 *g* for 15 min and the supernatant was used as a crude homogenate.

Enzyme assay

2-OG decarboxylase was assayed as described previously [1].

Immunoblot analysis

Western immunoblot experiments were performed by the method described previously [5]. The crude homogenate from *Euglena* cells as above was subjected to SDS/PAGE on 7.5–10 % (w/v) acrylamide slab gels. The precursor (65 kDa) and mature (62 kDa) proteins of 2-OG decarboxylase on the nitrocellulose sheet were detected immunologically by using an antibody raised against *Euglena* 2-OG decarboxylase and goat anti-rabbit IgG serum conjugated with alkaline phosphatase as described previously [3].

Pulse-labelling of cells

Labelling of *Euglena* cells was carried out by the method of Ohashi *et al.* [6]. L-[³⁵S]Methionine was administered for 2 h to *Euglena* cells grown for 5 days on glucose/(NH₄)₂SO₄ medium, together with the addition of L-glutamate. The labelled cells were harvested by centrifugation at 500 *g* for 5 min, washed twice with 50 mM-potassium phosphate buffer, pH 7.0, containing 20 % (w/v) ethylene glycol, 0.1 % (v/v) Nonidet P-40, 10 μ M-cycloheximide and 20 mM-L-methionine, resuspended with the same buffer and disrupted by sonication (10 kHz) for 1 min. The cell homogenate was centrifuged at 12000 *g* for 20 min. For the detection of 2-OG decarboxylase protein, a crude extract with a

Abbreviations used: SSA, succinate semialdehyde; 2-OG, 2-oxoglutarate; TCA, tricarboxylic acid.

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protein content of 2.03 mg/ml was incubated with 1.1 mg of purified anti-(2-OG decarboxylase) antibody for 60 min at 4 °C, followed by addition of 40 µl of Protein A–Sepharose in 10 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl and 1 % (v/v) Nonidet P-40. The mixture was continuously shaken at 4 °C for 60 min, left overnight at 4 °C and then centrifuged at 5000 *g* for 5 min to obtain the pellet, which was washed with 500 µl of 10 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl, 0.5 % (v/v) Nonidet P-40 and 0.1 % (w/v) SDS. The antigen–antibody complex was then dissociated by boiling for 3 min in 200 µl of 5 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-dithiothreitol, 2 % (v/v) 2-mercaptoethanol and 5 % (w/v) SDS, followed by centrifugation at 5000 *g* for 10 min. The radioactivity in the supernatant obtained (antigen) was measured by using a scintillation photometer (Aloka, model LSC-903, Tokyo, Japan).

For the measurement of total protein radioactivity, 500 µl of 5 % (w/v) trichloroacetic acid was added to the crude labelled extract. The mixture was left for 10 min at room temperature and centrifuged at 5000 *g* for 5 min. The resulting pellet was washed with 20 % (w/v) trichloroacetic acid and dissolved by boiling for 3 min in 200 µl of 5 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-dithiothreitol, 2 % (v/v) 2-mercaptoethanol and 5 % (w/v) SDS, followed by centrifugation at 5000 *g* for 5 min. The radioactivity in the supernatant obtained was assumed to represent total protein synthesis.

Immunochemical titration

This was performed by the method of Cannons & Merrett [7]. Cell-free extracts were prepared as described above from cells grown on glucose/(NH₄)₂SO₄ medium for 5 days and from cells 2 h (*t*₂) after the addition of L-glutamate. Different amounts of both extracts were incubated with 5 µl of anti-(2-OG decarboxylase) antibody (615 µg of protein) for 60 min at 30 °C. The antigen–antibody complexes were removed by centrifugation at 10000 *g* for 15 min and the 2-OG decarboxylase activity remaining in the supernatant was determined.

Extraction and translation *in vitro* of mRNA

Extraction of total RNA and isolation of polyadenylated RNA by using oligo(dT)–cellulose from *Euglena* cells were carried out as described previously [3]. Elution of RNA was monitored by using a u.v. spectrophotometer (model UV 120-02; Shimadzu, Japan) and total RNA was determined by measuring *A*₂₆₀ [7]. Polyadenylated RNA (0.3 µg) was used for translation *in vitro* in rabbit reticulocyte lysates, using L-[³⁵S]methionine as a label [3]. The products from polyadenylated RNA were obtained as trichloroacetic acid precipitates. Immunoprecipitation of 2-OG decarboxylase formed by translation was performed by using a specific antibody and Protein A–Sepharose. Radioactivity in the proteins of the total products and 2-OG decarboxylase was measured by the method described above.

Other methods

Antibody to purified 2-OG decarboxylase was raised in rabbits as described previously [3]. Protein was determined by the method of Bradford [8], with BSA as a standard.

Materials

L-[³⁵S]Methionine (1050 Ci/mmol) and rabbit reticulocyte lysate were obtained from Amersham, Tokyo, Japan. Oligo(dT)–cellulose and protein A–Sepharose were from Sigma Chemical Co. All other chemicals were of reagent grade and obtained from commercial sources.

RESULTS

Effect of TCA-cycle intermediates and related compounds on 2-OG decarboxylase activity

TCA-cycle intermediates and related compounds were added at a final concentration of 110 mM to *Euglena* cells grown on glucose/(NH₄)₂SO₄ for 5 days. As shown in Table 1, 2-OG decarboxylase activity was measured after incubation with each compound for 24 h. Similar increases in enzyme activity were found for 2-OG, succinate, DL-malate, L-alanine and L-aspartate. The addition of L-glutamate and L-lactate to the culture medium resulted in 3.4- and 2.3-fold increases in 2-OG decarboxylase activity respectively.

Effect of L-glutamate on 2-OG decarboxylase activity

When L-glutamate (110 mM) was added to the cells in the stationary phase grown on glucose/(NH₄)₂SO₄ medium, there was about a 2-fold increase in enzyme activity over a period of 2 h: this enhanced activity continued when the incubation was prolonged to 24 h (Fig. 1). When incubated for 2 h, 2-OG decarboxylase activity increased with increasing L-glutamate concentration up to 8.1 g/l (55 mM), beyond which the activity remained constant up to 30 g/l (204 mM) (results not shown). The addition of cycloheximide, an inhibitor of protein synthesis on 87 S cytoplasmic ribosomes, together with L-glutamate resulted in abolition of the increase in enzyme activity.

Effect of L-glutamate on the synthesis of 2-OG decarboxylase protein

In order to elucidate whether the increase in 2-OG decarboxylase is due to synthesis *de novo* of 2-OG decarboxylase protein or activation of pre-existing protein, an immunochemical titration was carried out on the crude extract prepared from the cell cultures at *t*₀ (0 h) and *t*₂ (2 h) after the addition of L-glutamate. When the activity of 2-OG decarboxylase at *t*₀ and *t*₂ was inhibited by a fixed amount of anti-(2-OG decarboxylase) antibody, the amount of 2-OG decarboxylase activity was identical for both enzyme preparations (Fig. 2). This means that

Table 1. Effect of TCA-cycle intermediates and related compounds on 2-OG decarboxylase activity

Euglena cells (15 × 10⁶ cells/ml) grown on glucose/(NH₄)₂SO₄ medium for 5 days were incubated with 110 mM of each compound. Each individual incubation was conducted under illumination (2000 lux) at 26 °C for 24 h and stopped by centrifugation at 3000 *g* for 5 min. The preparation of the crude homogenate from the harvested cells and the assay of 2-OG decarboxylase were carried out as described in the Experimental section. Values are means of three assays ± S.D. The enzyme activity in the control to which no compound had been added is shown as 100 % relative activity.

Compound	Enzyme activity (nmol/min per 10 ⁶ cells)	Relative activity (%)
None	1.29 ± 0.06	100
L-Glutamate	4.36 ± 0.12	338
Pyruvate	1.53 ± 0.08	119
L-Lactate	2.93 ± 0.07	227
L-Alanine	1.91 ± 0.07	148
Citrate	1.69 ± 0.05	131
Isocitrate	0.37 ± 0.02	29
2-Oxoglutarate	2.26 ± 0.08	175
Succinate	2.17 ± 0.10	168
Propionate	1.61 ± 0.04	125
Fumarate	1.56 ± 0.04	121
DL-Malate	2.19 ± 0.07	170
L-Aspartate	2.05 ± 0.07	159

Fig. 1. Effect of L-glutamate and cycloheximide on 2-OG decarboxylase activity

L-Glutamate (110 mM) was added to the cell cultures grown on glucose/ $(\text{NH}_4)_2\text{SO}_4$ medium for 5 days (○). Cycloheximide (0.1 mM), an inhibitor of protein synthesis, was fed together with L-glutamate (●). 2-OG decarboxylase activity was assayed as described in the Experimental section. The data are means of values obtained from four experiments (coefficient of variation < 5%).

Fig. 2. Immunochemical titration of 2-OG decarboxylase from *Euglena* cells 0 h (t_0) and 2 h (t_2) after addition of L-glutamate

Cell-free extracts of t_0 (○) and t_2 (●) samples were prepared and immunochemical titration with anti-(2-OG decarboxylase) antibody was carried out as described in the Experimental section. The abscissa represents the 2-OG decarboxylase activity before immunochemical reaction and the ordinate represents the activity remaining after removal of any antigen-antibody complex. Each experimental point represents the mean of three assays (coefficient of variation < 8%).

the observed increase in 2-OG decarboxylase activity over the time t_0 – t_2 is attributable to an increase in enzyme protein and not to activation of pre-existing protein.

On immunoblot analysis of the crude extract of *Euglena*, we discovered that the anti-(2-OG decarboxylase) antibody had reacted with two polypeptides with molecular masses of 62 and 65 kDa [3]. Two radiolabelled proteins with molecular masses identical with those of the precursor and mature subunit were immunoprecipitated from cell-free extracts prepared from L-[^{35}S]methionine-labelled *Euglena* cells which had been allowed to incorporate the labelled L-methionine for 2 h. Over this time period, the incorporation of L-methionine into 2-OG decarboxylase protein was linear with time (Fig. 3), indicating that the synthesis of 2-OG decarboxylase protein is similar to the increase in 2-OG decarboxylase activity. The incorporation of L-meth-

Fig. 3. Incorporation of L-[^{35}S]methionine into 2-OG decarboxylase and total protein after the addition of L-glutamate

Details are given in the Experimental section. Each point represents the mean of three assays (coefficient of variation < 5%). ○, 2-OG decarboxylase protein; ●, total protein.

Fig. 4. Effect of L-glutamate on conversion of the precursor of 2-OG decarboxylase into the mature form

The cell homogenate was subjected to slab-gel electrophoresis and transferred to a nitrocellulose sheet by Western blotting as described previously [3]. Lanes: 1, crude cell extracts before addition of L-glutamate; 2 and 3, those 1 and 2 h after addition of L-glutamate respectively; 4 and 5, those 1 and 2 h respectively after addition of cycloheximide together with L-glutamate.

ionine into trichloroacetic acid-precipitable protein, i.e. total protein, increased linearly over 2 h with a lag phase immediately after the addition of L-glutamate.

Immunoblot analysis of the crude homogenate from *Euglena* cells at t_0 , t_1 and t_2 showed increases in the precursor and mature enzyme proteins (Fig. 4). The addition of cycloheximide together with L-glutamate inhibited the increase in those proteins. These results indicate that the increase in 2-OG decarboxylase activity over 2 h in the presence of L-glutamate is the result of enzyme synthesis.

Effect of L-glutamate on 2-OG decarboxylase mRNA content

At each time point after the addition of L-glutamate, 2-OG decarboxylase mRNA was measured by immunoprecipitation using a specific antibody to 2-OG decarboxylase from lysate translations directed by total polyadenylated RNA. Translatability of the polyadenylated RNA (0.3 μg) after addition of L-glutamate did not alter, with each time point giving similar

Fig. 5. Effect of L-glutamate on 2-OG decarboxylase mRNA and total mRNA activities

Extraction of total RNA and isolation of polyadenylated RNA, by using oligo(dT)-cellulose, from *Euglena* cells were performed as described previously [3]. The amount of total RNA (■) was determined as described in the Experimental section. The polyadenylated RNA (0.3 μg) was used for translation in lysates and the L-[³⁵S]methionine incorporated into proteins was determined. The products from polyadenylated RNA (●) were obtained as trichloroacetic acid precipitates. 2-OG decarboxylase mRNA (○) was determined by using a specific antibody [3]. Each point, expressed per 10⁶ cells, represents the mean of three assays (coefficient of variation < 5%).

incorporations per lysate (results not shown). As shown in Fig. 5, the amount of total RNA and radioactivity obtained *in vitro* for translation products from polyadenylated RNA, expressed per 10⁶ cells, increased linearly with the incubation time up to 120 min after addition of L-glutamate. The amount of 2-OG decarboxylase mRNA increased significantly over 20 min to reach a peak, and after that remained constant.

Effect of an antibiotic and some inhibitors on 2-OG decarboxylase activity

The effects of some compounds fed to *Euglena* cell culture concurrently with the addition of L-glutamate on the increase in 2-OG decarboxylase activity after 4 h are shown in Table 2. Cycloheximide, 1,10-phenanthroline, 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone markedly inhibited the increase in 2-OG decarboxylase activity.

DISCUSSION

Euglena gracilis is known to grow on L-glutamate as the sole source of carbon, nitrogen and energy [9]. Tokunaga *et al.* [10] have reported that the γ -aminobutyrate shunt of *Euglena*, which involves the enzymes L-glutamate decarboxylase, γ -aminobutyrate aminotransferase and SSA dehydrogenase, occurs in *Euglena* mitochondria, suggesting that this shunt may contribute to the assimilation of L-glutamate and may be operative as a bypass pathway of the TCA cycle. However, the actual significance of the γ -aminobutyrate shunt in cell physiology is not clear. *Euglena* mitochondria have been shown to lack the 2-OG dehydrogenase complex [1]. Our previous results on the occurrence of 2-OG decarboxylase in *Euglena* mitochondria and the stoichiometric conversion of 2-OG into succinate in the presence of 2-OG decarboxylase and SSA dehydrogenase [1] led us to the conclusion that SSA dehydrogenase coupled to 2-OG decarboxylase is one of the constituent enzymes in the unique TCA cycle rather than that of the γ -aminobutyrate shunt.

Table 2. Effects of some compounds on the increase in 2-OG decarboxylase activity

Each compound was added to the cell suspensions grown on glucose/(NH₄)₂SO₄ medium for 5 days to the final concentration shown, which did not affect cell viability. The effect is represented as percentage inhibition against the increase in 2-OG decarboxylase activity in the respective control to which no compound was added. Values are means of three assays $\pm$ s.d.

Compounds	Concn. (M)	Enzyme activity (nmol/min per 10 ⁶ cells)	Inhibition (%)
None			
0 h		1.30 $\times$ 0.05	
4 h		4.29 $\pm$ 0.11	0
1,10-Phenanthroline	5 $\times$ 10 ⁻⁵	3.93 $\pm$ 0.09	12
	1 $\times$ 10 ⁻⁴	1.72 $\pm$ 0.06	86
2,4-Dinitrophenol	1 $\times$ 10 ⁻⁴	1.33 $\pm$ 0.04	99
	5 $\times$ 10 ⁻⁴	1.29 $\pm$ 0.04	100
	1 $\times$ 10 ⁻⁵	2.29 $\pm$ 0.07	67
Carbonyl cyanide <i>m</i> -chlorophenylhydrazone	5 $\times$ 10 ⁻⁵	1.32 $\pm$ 0.04	99

Addition of L-glutamate to *Euglena* cells grown on glucose/(NH₄)₂SO₄ medium for 5 days significantly increased the activity of 2-OG decarboxylase compared with other compounds related to the TCA cycle (Table 1 and Fig. 1). It has been reported that SSA dehydrogenase exists as two isoenzymes, one requiring NADP⁺ and the other NAD⁺ as electron acceptor, and that NADP⁺-linked SSA dehydrogenase is induced by L-glutamate, whereas the NAD⁺-linked enzyme is not [2]. When *Euglena* cells were grown on glucose/(NH₄)₂SO₄ medium for 5 days, the activities of 2-OG decarboxylase, NADP⁺-linked SSA dehydrogenase and L-glutamate-oxaloacetate aminotransferase were 1.37, 2.03 and 34.3 μmol/min per 10⁹ cells respectively (S. Shigeoka & K. Nakamura, unpublished work). The activity of NADP⁺-linked SSA dehydrogenase has been reported to be about 1220- and 570-fold greater than those of L-glutamate decarboxylase and γ -aminobutyrate aminotransferase which constitute the γ -aminobutyrate shunt [10]. Therefore it is conceivable that L-glutamate metabolism in *Euglena* mainly occurs via 2-OG decarboxylase, which is coupled to L-glutamate-oxaloacetate aminotransferase and SSA dehydrogenase, rather than via the γ -aminobutyrate shunt. 2-OG decarboxylase and SSA dehydrogenase are located only in mitochondria [1,10], whereas L-glutamate-oxaloacetate aminotransferase is present in mitochondria, chloroplasts, microbodies and cytosol [11,12]. *Euglena* cells have high NADPH-dependent L-glutamate dehydrogenase activity when grown on L-glutamate, but the activity is repressed completely in cells grown on (NH₄)₂SO₄ as nitrogen source [13,14]. However, this enzyme is exclusively located in the cytosol of *Euglena* [3].

The results from immunochemical titration (Fig. 2), labelling *in vivo* (Fig. 3) and immunoblot analysis (Fig. 4) indicate that the increase in 2-OG decarboxylase activity results from an increase in protein, as is the case for the increase in citrate synthase in re-greening [7] and division-synchronized *Euglena* cells [15]. The increase in 2-OG decarboxylase and NADP⁺-linked SSA dehydrogenase activities after the addition of L-glutamate indicates that, as L-glutamate is accumulated in *Euglena* cells, both NADP⁺-linked SSA dehydrogenase and 2-OG decarboxylase are induced to assimilate the amino acid. The synthesis of 2-OG decarboxylase protein was parallel to the increase in enzyme

activity (Figs. 1 and 3). However, synthesis of total protein increased over 2 h with a lag phase at the beginning, which was distinct from that of 2-OG decarboxylase protein. The difference in the synthesis of 2-OG decarboxylase and total protein suggests that 2-OG decarboxylase protein is immediately induced by addition of L-glutamate in preference to other proteins, in order to deal with the increased flow of substrate through the TCA cycle.

As shown in Fig. 5, mRNA determination, by translation and immunoprecipitation, revealed that the rate of increase in 2-OG decarboxylase mRNA is faster than the rate of increase in total polyadenylated RNA (Fig. 5), indicating the high availability of mRNA for 2-OG decarboxylase synthesis after addition of L-glutamate to *Euglena* cell cultures grown on glucose/(NH₄)₂SO₄ medium.

On immunoblot analysis, the extract of *Euglena gracilis* was shown to contain two polypeptides, with molecular masses of 62 and 65 kDa, which reacted with the anti-(2-OG decarboxylase) antibody [3]. In addition, translation of *Euglena* polyadenylated RNA in a cell-free rabbit reticulocyte lysate system revealed only a product with a molecular mass of 65 kDa. These results suggest that *Euglena* 2-OG decarboxylase is initially synthesized as a precursor molecule, which is about 3 kDa larger than the mature subunit, and that the precursor from the cytosol is imported into mitochondria as the mature form, which has a molecular mass of 62 kDa. The inhibitory effect (demonstrated in Table 2) of uncouplers and proteinase inhibitors supports this view.

We are grateful to the late Professor Y. Iizuka for his stimulating advice and discussion. This work was supported in part by a research grant from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Shigeoka, S., Onishi, T., Maeda, K., Nakano, Y. & Kitaoka, S. (1976) *FEBS Lett.* **195**, 43–47
2. Tokunaga, M., Nakano, Y. & Kitaoka, S. (1976) *Biochim. Biophys. Acta* **429**, 55–62
3. Shigeoka, S. & Nakano, Y. (1991) *Arch. Biochem. Biophys.* **288**, 22–28
4. Koren, L. E. & Hunter, S. H. (1967) *J. Protozool.* **14**, (Suppl.) 17
5. Shigeoka, S., Takeda, T. & Hanaoka, T. (1991) *Biochem. J.* **275**, 623–627
6. Ohashi, A., Gibson, J., Gregor, I. & Schatz, G. (1982) *J. Biol. Chem.* **257**, 13042–13047
7. Cannons, A. C. & Merrett, M. J. (1984) *Eur. J. Biochem.* **142**, 597–602
8. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
9. Kempner, E. S. (1982) in *The Biology of Euglena* (Buetow, D. E., ed.), vol. 3, pp. 197–252, Academic Press, New York
10. Tokunaga, M., Nakano, Y. & Kitaoka, S. (1979) *J. Protozool.* **26**, 471–473
11. Oda, Y., Miyatake, K., Nakano, Y. & Kitaoka, S. (1981) *Agric. Biol. Chem.* **45**, 2619–2621
12. Collins, N. & Merrett, M. J. (1975) *Biochem. J.* **148**, 321–328
13. Fayyaz-Chaudhary, M., Javed, Q. & Merrett, M. J. (1985) *New Phytol.* **101**, 367–376
14. Parker, J. E., Javed, Q. & Merrett, M. J. (1985) *Eur. J. Biochem.* **153**, 573–578
15. Cannons, A. & Merrett, M. J. (1985) *Planta* **164**, 529–533

Received 30 April 1991/25 July 1991; accepted 15 August 1991