THE OCCURRENCE OF GLUTAMATE SYNTHASE IN ALGAE

P.J. Lea and B.J. Miflin

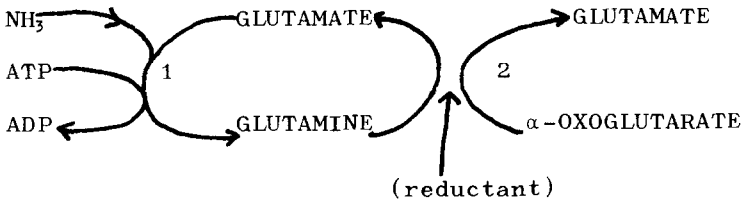
Rothamsted Experimental Station, Harpenden, Herts AL5 2JQ

Received April 1,1975

SUMMARY: The presence of glutamate synthase in the green algae Chlorella fusca var. vacuolata has been demonstrated using a whole cell assay as well as cell free extracts. The assay is complicated by the presence of glutamine(amino):α-oxoglutarate transaminase, but this enzyme can be inhibited by amino oxyacetate. The rates of glutamate synthase activity are sufficient to account for the known rates of nitrate assimilation to occur via the glutamine synthetase/glutamate synthase pathway.

INTRODUCTION

It is now generally accepted (1) that a major route of entry of ammonia into the α-amino group of amino acids in bacteria is through the action of glutamine synthetase (EC 6.3.1.2) (Reaction 1) and glutamine(amide):2-oxoglutarate aminotransferase (glutamate synthase) (EC 2.6.1.53) (Reaction 2).



Until recently glutamate was thought to be formed in green plants, by the direct amination of α-oxoglutarate catalysed by glutamate dehydrogenase (GDH). However, the conversion of nitrite to α-amino nitrogen occurs in the chloroplast (2,3). The level of glutamate dehydrogenase in chloroplasts is low and the K_m for ammonia is high (4), whereas the level of glutamine synthetase is high and its K_m for ammonia is low (5,6). This suggested that the glutamine synthetase/glutamate synthase route of assimilation was

involved; this was verified by the demonstration of a ferredoxin-dependent glutamate synthase in pea chloroplasts (7). The enzyme has subsequently been found in blue-green algae (8) and pea roots (9). The latter enzyme also utilises reduced pyridine nucleotides, as does the enzyme from cells of plant tissue cultures (10).

Although glutamate synthase is widely distributed, it has not so far been reported in green algae. It has been suggested (1) that this alternative route of nitrogen assimilation is absent in Chlorella, since the K_m of GDH for ammonia is low, in the order of 3-5 x 10^{-4}M (11, and unpublished results), and glutamate is the first amino acid formed after the addition of $^{15}NH_3$ to ammonia grown Chlorella (12).

However, Baker and Thompson (13) showed that Chlorella grown on a nitrogen depleted medium, rapidly accumulated $^{15}NH_3$ into the amide group of glutamine; the amino group of glutamate was labelled more slowly. A reappraisal of the work of Kanazawa et al (14,15) also shows that glutamate is formed after the initial synthesis of glutamine when ammonia is fed to nitrogen starved cells of Chlorella.

It was decided therefore to investigate the possibility that glutamate synthase is present in the green algae Chlorella.

MATERIALS AND METHODS

A culture of Chlorella fusca var. vacuolata, Cambridge culture collection strain 211/8P, was a generous gift of Dr I. Morris, Dept of Botany and Microbiology, University College, London.

Cells were grown under sterile conditions in a medium as described by Syrett (16) with 2 g/litre of potassium nitrate as the sole nitrogen source. The cultures were illuminated with 4 'Cryselco' 20W daylight lamps at a distance of 40 cm, and aerated continuously. Cells were harvested by centrifugation and washed once in 50 mM Tris-HCl (pH 7.5) containing 5 mM EDTA and 12.5 mM β-mercaptoethanol. Cells used for the intact cell assay were frozen at -15°C overnight, thawed and dialysed against 50 mM Tris-HCl (pH 7.5) containing 12.5 mM β-mercaptoethanol.

Cell free extracts of Chlorella were prepared by sonication in washing buffer, in a MSE 60W, 20 Kc/sec sonicator for three periods of five minutes. The debris and unbroken cells were removed by centrifugation at 10,000g for 10 min, and the supernatant was brought to 75% saturation with ammonium sulphate. The centrifuged precipitate was resuspended in 50 mM Tris-HCl (pH 7.5) containing 12.5 mM β-mercaptoethanol, dialysed to remove the excess ammonium sulphate and passed over a column of Sephadex G75.

The same assay procedure was used for both preparations. Approximately 5 mg dry weight of intact cells and 0.5 mg of extract protein were employed. The assay system contained 3.5 μmoles glutamine, 3.5 μmoles α-oxoglutarate, 5.0 μmoles aminooxyacetate, and 0.05 mg methyl viologen (MV) or 0.05 mg Spirulina ferredoxin (Fd). Methyl viologen or ferredoxin was reduced by the addition of 100 μl of a freshly prepared solution of sodium dithionite and sodium bicarbonate, both 8 mg/ml (DIT). The final volume of the assay medium was 0.75 ml, and after incubation at 30°C for 30 min, the reaction was terminated by the addition of 1 ml of ethanol. The reaction tubes were shaken vigorously to ensure that the contents of the cells were liberated, centrifuged and the glutamate content of the supernatant determined by the method previously described (7).

Spirulina ferredoxin was prepared by the method of Hall et al (17), and protein determined by the method of Lowry et al (18).

RESULTS AND DISCUSSION

Frozen and thawed cells catalyse a reaction between glutamine and α-oxoglutarate which is independent of added reductant, but which is prevented by the addition of a transaminase inhibitor aminooxyacetate (Table 1). Using aminooxyacetate to inhibit this

Table 1 Glutamate formation by frozen cells of Chlorella

Substrates added to the reaction medium	Glutamate formed nmoles/min/mg dry wt
Glutamine (Gln)	1.54
Gln, α-oxoglutarate	7.32
Gln, α-oxoglutarate, aminooxyacetate (AOA)	1.67
Gln, AOA, DIT	0
α-oxoglutarate, AOA, DIT	0
Gln, α-oxoglutarate, AOA, DIT	3.05
Gln, α-oxoglutarate, AOA, DIT, MV	21.65
Gln, α-oxoglutarate, AOA, DIT, Fd	2.61
NH_3, α-oxoglutarate, AOA, DIT, MV	0

Assays were carried out as described in methods section

reaction, it is then possible to demonstrate a dithionite-dependent formation of glutamate. Presumably, the dithionite reduces the internal ferredoxin, however the rate is greatly stimulated by the addition of methyl viologen. Added ferredoxin has little effect and probably does not penetrate the cell membrane. The reaction is dependent on the presence of α-oxoglutarate, and is not caused by the breakdown of glutamine to ammonia, as ammonia will not act as a substrate for the reaction.

Syrett (16) incubated cells in malate and glucose-6-phosphate to reduce the internal NAD and NADP respectively. However we were unable to detect glutamate formation with added malate, but there was a slight production with glucose-6-phosphate which could have been due to a reduction of internal ferredoxin by NADPH.

Although considerable difficulty was encountered in breaking the cells, it was possible to demonstrate the same reaction in cell free extracts of Chlorella (Table 2). Again there is a reductant independent transamination reaction between glutamine and α-oxoglutarate, which is inhibited by aminooxyacetate. Dithionite apparently inhibits the action of glutaminase, and

Table 2 Glutamate formation by cell free extracts of Chlorella

Substrates added to the reaction medium	Glutamate formed nmoles/min/mg protein
Glutamine (Gln)	27.6
Gln, α-oxoglutarate	63.5
Gln, α-oxoglutarate, aminooxyacetate (AOA)	26.2
Gln, AOA, DIT	0
α-oxoglutarate, AOA, DIT	0
Gln, α-oxoglutarate, AOA, DIT	1.7
Gln, α-oxoglutarate, AOA, DIT, MV	26.9
Gln, α-oxoglutarate, AOA, DIT, Fd	156.2
NH_3, α-oxoglutarate, AOA, DIT, Fd	0

Assays were carried out as described in methods section

brings the endogenous rate of glutamate formation almost to zero. Having inhibited the glutaminases and transaminases, it is then possible to demonstrate a reductant-dependent glutamate synthesis. In contrast to frozen cells, the cell free extract exhibits a higher rate of glutamate formation with ferredoxin, the likely physiological substrate, than with methyl viologen: thus confirming that ferredoxin is unable to penetrate the membrane of frozen Chlorella cells. No glutamate synthesis was detected when either NADH or NADPH was used as a source of reductant. However, if all the ammonia was not removed from the extracts, glutamate formation (which was independent of glutamine) could be detected due to the action of glutamate dehydrogenase.

It can be seen from Table 3 that two molecules of glutamate are formed from one molecule of glutamine lost in the presence of methyl viologen and dithionite: this is as predicted by the glutamate synthase reaction in Reaction 2. The reductant independent production of glutamate involves the production of only one molecule of glutamate for each glutamine lost, and is probably due to transamination of the α-amino group of glutamine to α-oxoglutarate (Reaction 3)

Glutamine + α-oxoglutarate ⟶ α-oxoglutaramate + glutamate 3

If the glutamine synthetase/glutamate synthase pathway is involved in nitrate assimilation, it is important that its activity should be of the same order of magnitude as that quoted for *in vivo* nitrate uptake in Chlorella. From the experiments of Grant and Turner (19), it is possible to calculate that the rates for nitrate and nitrite assimilation are of the order of 5.5 nmoles/min/mg dry weight. Syrett (16), using the frozen cell assay, recorded values for nitrite reductase at levels of

Table 3 Stoichiometry of the two glutamine:α-oxoglutarate reactions catalysed by Chlorella frozen cells

Reaction	Glutamine metabolised nmoles/30 min	Glutamate formed nmoles/30 min	Ratio Glu:Gln
Glutamine:α-oxoglutarate transaminase	1485	1410	0.95
Glutamate synthase	2840	5260	1.85

Assays were carried out as described in methods section

1.25 nmoles/min/mg dry weight. We have measured rates of 3 nmoles/min/mg dry weight. Thus, it would seem that there is sufficient glutamate synthase in nitrate grown cells for the enzyme to be involved in nitrate assimilation.

However, although Chlorella has an active glutamine synthetase (13) with rates greater than those of glutamate synthase (Lea and Miflin unpublished results), to our knowledge the enzyme has not been characterised nor its K_m for ammonia compared with that of GDH. It is not at all clear how the glutamine(amino):α-oxoglutarate transaminase is involved in glutamine metabolism. The relative distribution of the various enzymes in the subcellular compartments is also unknown, but relevant to the eventual understanding of nitrogen assimilation in Chlorella.

ACKNOWLEDGEMENTS

We thank Mrs S. Burgess for technical assistance. PJL is grateful to the Royal Society for the receipt of a Pickering Research Fellowship.

REFERENCES

1 Brown, C.M., MacDonald-Brown, D.S. and Meers, J.L. (1974) Advan. Microbiol. Physiol. 11, 1-52
2 Miflin, B.J. (1974) Planta 116, 187-196
3 Magalhaes, A.C., Neyra, C.A. and Hageman, R.H. (1974) Plant Physiol. 53, 411-415
4 Lea, P.J. and Thurman, D.A. (1972) J. exp. Bot. 23, 440-449
5 Miflin, B.J. (1974) Plant Physiol. 54, 550-555
6 O'Neal, D. and Joy, K.W. (1974) Plant Physiol. 54, 773-779
7 Lea, P.J. and Miflin, B.J. (1974) Nature, 251, 614-616
8 Lea, P.J. and Miflin, B.J. (1975) Biochem. Soc. Trans. 3, 381-384
9 Miflin, B.J. and Lea, P.J. (1975) Biochem. J. (in press)
10 Dougall, D.K. (1974) Biochem. Biophys. Res. Commun. 58, 639-646
11 Morris, I. and Syrett, P.J. (1965) J. Gen. Microbiol. 38, 21-29
12 Kirk, M.R. and Bassham, J.A. (1964) Biochim. Biophys. Acta 90, 553-562
13 Baker, J.E. and Thompson, J.K. (1961) Plant Physiol. 36, 208-212
14 Kanazawa, T. Kirk, M.R. and Bassham, J.A. (1970) Biochim. Biophys. Acta 205, 401-408
15 Kanazawa, T. Kanazawa, K., Kirk, M.R. and Bassham, J.A. (1972) Biochim. Biophys. Acta 256, 656-669
16 Syrett, P.J. (1973) New Phytol. 72, 37-46
17 Hall, D.O., Rao, K.K. and Cammack, R. (1972) Biochem. Biophys. Res. Commun. 47, 798-802
18 Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
19 Grant, B.R. and Turner, I.M. (1969) Comp. Biochem. Physiol. 29, 995-1004