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OCCURRENCE OF TWO FORMS OF GLUTAMATE SYNTHASE IN *CHLAMYDOMONAS REINHARDII*

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Key Word Index—*Chlamydomonas reinhardii*; Chlorophyceae; nitrogen metabolism; NADH-dependent glutamate synthase; ferredoxin-dependent glutamate synthase; rapid radiochemical assay.

Abstract—A simple and rapid radiochemical assay for measuring glutamate synthase activity in permeable cells and cell free extracts of *Chlamydomonas* is described. Using this method, separate NADH- and ferredoxin-specific enzymes were detected and these were resolved by gel filtration. The properties of the two enzymes differed markedly with respect to MW and affinity for 2-oxoglutarate and glutamine. Activity measurements on cells grown under different physiological conditions revealed a strict coordinate synthesis of the two enzymes. The possible significance of these observations in relation to light and dark NH_3 assimilation in the alga is discussed.

INTRODUCTION

Recent studies with *Chlamydomonas reinhardii* have revealed the diverse role played by the glutamate synthase cycle in the nitrogen metabolism of this unicell. It has been shown that the cycle functions as the sole pathway of NH_3 assimilation both in the light and the dark [1], and moreover that it is directly implicated in the reassimilation of NH_3 released during photorespiration [2]. In this paper we report the occurrence in the alga of NADH- and ferredoxin-specific forms of glutamate synthase and comment briefly on their possible roles in relation to these three functions.

RESULTS

Results which have established the presence of both NADH-dependent glutamate synthase activity (EC 1.4.1.14) and ferredoxin-specific glutamate synthase activity (EC 1.4.7.1) in partially purified extracts of *Chlamydomonas* are presented in Table 1. The two activities showed almost complete dependence on glutamine and 2-oxoglutarate and were extensively inhibited by the glutamine analogue azaserine. Very little glutamine transaminase activity was apparent, in contrast to extracts from *Chlorella* [3]. The pyridine nucleotide activity was specific for NADH; no significant NADPH azaserine sensitive activity has been detected in *Chlamydomonas* grown under a wide range of cultural conditions.

A striking property of the NADH enzyme is that it is cold-labile and sensitive to oxygen. Its partial stabilization has been achieved by addition of mercaptoethanol and high levels of sucrose to enzyme extracts and by purging buffers with N_2. Two discrete peaks of activity corresponding to the NADH and ferredoxin enzymes were observed when freshly prepared extracts were fractionated by gel filtration on Sephacryl S-200 (Fig. 1). Some of the kinetic and hydrodynamic characteristics of the two enzymes are summarized in Table 2. Of particular note are the marked differences in the affinity of the two enzymes for glutamine and 2-oxoglutarate. Measurements of the activity of the enzymes in algae grown on NH_3 under phototrophic and mixotrophic conditions and in those subjected to nitrogen starvation (Table 3) established under these varied conditions of growth, that each form is present in sufficient amount to account for the total rate of nitrogen assimilation.

Table 1. Substrate requirements of ferredoxin-dependent (a) and NADH-dependent (b) glutamate synthase (AZA = azaserine, DT = sodium dithionite, MV = methyl viologen)

	Glutamate formation (pkat/mg dry wt)
(a) Ferredoxin assay	
Complete	281
−2-Oxoglutarate	24
−Glutamine	16
−Ferredoxin	61
−DT	28
+AZA	6
(b) NADH assay	
Complete	390
−2-Oxoglutarate	6
−Glutamine	38
−NADH	14
+AZA	16
−NADH + NADPH	75
−NADH + NADPH + AZA	73
−NADH + MV + DT	203

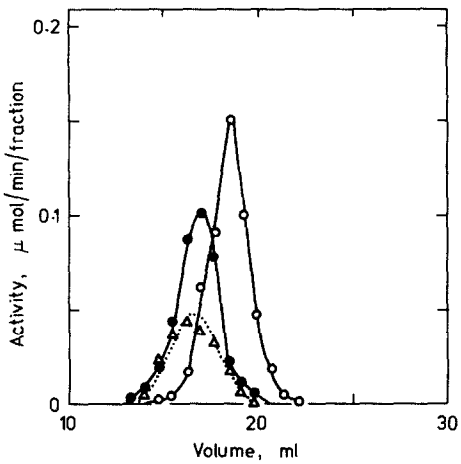

Fig. 1. Elution profile of glutamate synthases from *Chlamydomonas*. A partially purified extract (30–60% satn $(NH_4)_2SO_4$ fraction) was applied to a Sephacryl S200 column. Fractions were assayed for NADH (●), MV (△) and ferredoxin (○) glutamate synthase activities.

DISCUSSION

Evidence presented in this paper has established that photosynthetic cells of *Chlamydomonas reinhardii* possess distinctive NADH- and ferredoxin-specific glutamate synthases. It is striking that the properties of the algal enzymes are similar to those already described for higher plants, the labile NADH form closely resembling the enzyme isolated from soybean cotyledons [4] and lupin nodules [5] and the ferredoxin enzyme possessing characteristics similar to that found in bean leaves [6]. The differences in reductant specificity and affinity of the enzymes for glutamine and 2-oxoglutarate indicate that two spatially separate glutamate synthase cycles might operate within the alga. Activity measurements using methyl viologen as reductant revealed (Fig. 1) that this activity was almost totally associated with the NADH rather than, as hitherto found [7], the ferredoxin enzyme. Additional evidence suggesting that the methyl viologen and NADH activities may be associated with the same enzyme has been obtained by following activity changes in enzyme extracts devoid of stabilising ligands (Cullimore, unpublished results). The half-life of the methyl viologen activity of 4.6 hr was virtually identical to that found with

Table 2. Some hydrodynamic and kinetic properties of ferredoxin-dependent and NADH-dependent glutamate synthase

Properties	Ferredoxin enzyme	NADH enzyme
Stokes' radius (nm)	4.85	5.63
Sedimentation coefficient ($s_{20,w}$)	8.4	10.5
Molecular weight	165000	240000
App. K_m for glutamine (μM)	190	900
App. K_m for 2-oxoglutarate (μM)	170	7
App. K_m for ferredoxin (μM)	0.01	—
App. K_m for NADH (μM)	—	13
pH optimum	7.5	7.5

The kinetic values shown here were determined using the fixed substrate concentrations described in the Experimental.

Table 3. NH_3 assimilation and activities of ferredoxin-dependent and NADH-dependent glutamate synthase in *Chlamydomonas* grown on NH_3 under different conditions

	NH_3 assimilation		Glutamate synthase	
Growth condition	Light	Dark	Ferredoxin	NADH
	(pkat/mg dry wt)			
Phototrophic	75	8	453	776
Mixotrophic	82	33	146	314
N-starved	270	170	1030	1810

The enzyme activities shown are corrected for full substrate saturation.

NADH, whereas the half-life of the ferredoxin enzyme was *ca* 21 hr. In view of these observations and the similarity between the algal and plant enzymes it will be of interest to critically re-examine whether the NADH enzyme is entirely absent from the leaves of higher plants. This is of particular importance in deciding whether the chloroplastic ferredoxin enzyme is exclusively concerned with NH_3 assimilation in this organ.

An important feature of the nitrogen metabolism of *Chlamydomonas* is that it can, under some conditions, assimilate NH_3 in the dark almost as rapidly as in the light [8] and see also Table 3) and it is possible that the large differences in dark assimilation observed in cells cultivated under different carbon and nitrogen regimes could be specifically attributable to differences in the level of the NADH-dependent glutamate synthase. Activity measurements made in cells exhibiting very different rates of dark assimilation (Table 3) do not support this simple explanation. Although the amounts of the two enzymes did vary, the ratio of their activities remained unchanged. It would appear therefore that under these varied conditions no preferential synthesis of the NADH enzyme occurred, but rather that a mechanism operated to ensure a strictly balanced synthesis of the two forms of the enzyme. Significantly, under all these conditions the activity of either enzyme alone was sufficient to sustain the measured rate of dark assimilation.

It is now apparent that higher plants [9], as well as certain bacteria [10], possess dual systems of NH_3 assimilation and experiments using mutants have provided some insight of the advantages of this pattern of organization. Recent work with mutants of *Arabidopsis* lacking the ferredoxin glutamate synthase [11] have shown that although these plants grow quite normally in air supplemented with CO_2, it is a lethal mutation if the plant is subjected to photorespiratory stress. Apparently then, although the pyridine nucleotide enzyme localized in the root can assimilate sufficient nitrogen to sustain normal growth (it is now suggested that this enzyme rather than glutamate dehydrogenase has this role—C. R. Somerville and W. L. Ogren, personal communication), the ferredoxin-specific enzyme must function almost exclusively to reassimilate the NH_3 released by photorespiration. Since it is equally well documented that under normal conditions the chloroplastic enzyme contributes to the primary assimilation of NH_3 by the leaf [11], it is evident that this enzyme plays two distinct roles in photosynthetic cells and that a change of function is dictated by the prevailing environmental conditions. Elegant studies on bacterial mutants defective in either glutamate synthase or glutamate dehydrogenase have emphasized the real energy saving achieved by a cell in switching from the glutamate synthase cycle (a very efficient NH_3 scavaging pathway but requiring an additional mole of ATP for every mole of NH_3 assimilated) to the more economical dehydrogenase pathway following an increase in NH_3 availability [10]. Presumably these considerations also apply to *Chlamydomonas*. Clearly a very substantial saving in energy expenditure must be achieved if the carbon reserves of the alga are mobilized in the dark to generate NADH rather than reduced ferredoxin for the assimilation of NH_3. Equally in this instance it would seem to make sense to forgo the saving of energy and nutrients achieved in repression of the synthesis of the two synthase enzymes in order to accomplish an immediate and freely reversible switch to the ferredoxin pathway when the organism is illuminated. Certainly the coordinate behaviour of the enzymes described here is consistent with this view; the actual contributions of the two enzymes in nitrogen assimilation under different physiological conditions is presently being investigated.

Finally it is possible that other advantages accrue from the alga possessing two spatially separate glutamate synthase cycles. They could function to provide sites for the synthesis of glutamate and amino acids in the cytosol as well as the chloroplast and could also ensure under appropriate conditions a spatial separation of the photorespiratory nitrogen cycle from the assimilation of exogenous NH_3.

EXPERIMENTAL

Culture. Chlamydomonas reinhardii (Dangeard) CCAP 11/32a was grown in wide-bottomed conical flasks as in ref. [8] except that phototrophic cultures were aerated (30 l./hr), and mixotrophic cultures were supplemented with NaOAc (24 mM). NH_4Cl (5 mM) was used as a sole source of nitrogen.

Assay of glutamate synthase. Crude extracts were obtained by sonication (Dawe Soniprobe; 100 W; 2 periods of 30 sec at 4°) in 50 mM K-Pi buffer pH 7.5 including 0.5 mM Na_2EDTA, 0.1% mercaptoethanol and 300 mM sucrose which was purged of O_2 by bubbling N_2 through it. Cell debris was removed by centrifugation and the clear supernatant desalted by passing through a column of Sephadex G-25. Samples of cells for permeabilization were obtained as in ref. [2] and rendered permeable either by freezing in liquid N_2 for 15 sec or by the addition of nystatin (450 units/assay) for the determination of ferredoxin or NADH activity respectively. The enzymes were assayed radiochemically by determining the formation of Glu from Gln following their separation by ion-exchange chromatography. The reaction mixture contained in 1.5 ml: 50 μmol imidazole acetate, pH 7.5; 6 μmol 2-oxoglutarate; 1.5 μmol Gln containing 7.4 kBq (0.2 μCi) and either 0.9 μmol NADH or 0.1 mg ferredoxin and 0.2 ml of a freshly prepared soln of 100 mg Na dithionite and 100 mg $NaHCO_3$ in 6 ml. Methyl viologen (0.8 μmol) and azaserine (0.75 μmol) were included where indicated. The assay, at 30°, was initiated by addition of reductant and terminated by vigorous aeration at 0°. One ml was applied to a 0.5 × 2 cm column of Dowex AG 1-X8 (acetate form) and followed by 3 ml 50 mM HOAc. Glu was eluted with 4 ml 0.3 M KNO_3 and its ^{14}C content determined. Using this method an assay may be completed within 20 min.

Analytical methods. The methods used for determining NH_3 assimilation by the alga based on ^{15}N incorporation or increase in total N have been described [12]. In the expt involving N-starved cells, an NH_3 grown culture was transferred to a complete medium lacking NH_3 for 16 hr. NH_3 uptake was then measured as described in ref. [13], by following its depletion from the medium after the addition of 0.25 mM NH_3. It has been shown that these methods give identical results in *Chlamydomonas* [1].

Full details of the methods used for determining the sedimentation coefficients and Stokes' radii of the enzymes are described in ref. [14] except that care was taken to minimize exposure to O_2 by using degassed buffers throughout. 1.5 l. NH_3 grown cells in the exponential phase of growth were harvested by centrifugation at 4° and extracts were prepared as described above. The enzymes were partially purified by $(NH_4)_2SO_4$ precipitation (30–60% saturation) and desalted into the same buffer on Sephadex G-25. For gel filtration 0.6 ml out of a total of 2 ml was applied to a 1.1 × 47 cm column of Sephacryl S-200

equilibrated in the same buffer and the column was run at a rate of 0.11 ml/min and *ca* 0.75 ml fractions were collected in preweighed tubes. The enzyme activities were assayed in 0.1 ml samples. Extracts in which sedimentation coefficients were determined were desalted into the buffer containing 2.5% sucrose, and 0.2 ml was applied to the gradient. Both determinations could be completed in 20 hr and a quantitative recovery of the ferredoxin enzyme and 60% recovery of the NADH enzyme was obtained.

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