Plant Physiol. (1976) 58, 283-287

An NADP-dependent Glutamate Dehydrogenase in Chloroplasts from the Marine Green Alga *Caulerpa simpliciuscula*[1]

Received for publication April 13, 1976 and in revised form May 24, 1976

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ABSTRACT

NADP-dependent glutamate dehydrogenase was partially purified from extracts of the marine siphonous green alga *Caulerpa simpliciuscula*. The enzyme had an apparent Km NH_4^+ of 0.4 to 0.7 mM and was highly specific for NADPH, α-ketoglutarate, and ammonium ions.

The bulk of the NADP-glutamate dehydrogenase was isolated with the chloroplast fraction in cell-free preparations of this alga and was released from these "chloroplast fractions" as a soluble enzyme on gentle lysis of chloroplast membranes.

The kinetic properties of NAD-dependent glutamate dehydrogenase (EC 1.4.1.2) of higher plants are inconsistent with those required by an enzyme functioning in the synthesis of glutamate from free ammonia (15). In particular, the apparent *Km* values for ammonia are in the range of 5 to 40 mM (2, 11, 21, 25). This should preclude adequate function of the enzyme at the low ambient concentrations of ammonium ions present in many plant tissues. NADP-dependent glutamate dehydrogenase activity has also been described in plant tissues, particularly in chloroplasts (17, 18). No kinetic data on the purified enzyme have been reported to date. However the apparent *Km* for ammonia of impure NAD(P)-dependent glutamate dehydrogenase in chloroplast fractions is 6 mM (17). This is high for an enzyme proposed to function in glutamate synthesis, particularly since 5 mM NH_4^+ partially uncouples phosphorylation from electron transport in chloroplast thylakoids (14).

An alternative pathway from NH_4^+ to glutamate has been found in bacteria (23) and in higher plants (15). This utilizes glutamine synthetase (EC 6.3.1.2) and glutamate synthase (EC 2.6.1.53). Since the *Km* NH_4^+ of glutamine synthetase is 0.02 mM (19), it has been suggested that these enzymes provide an alternative route for glutamate synthesis when ammonium concentrations are low, particularly in chloroplasts and blue-green algae (15, 16). However net synthesis of glutamate from NH_4^+ by this pathway is accompanied by the hydrolysis of one molecule of ATP to ADP and Pi.

An NADP-dependent glutamate dehydrogenase with a sufficiently low *Km* NH_4^+ would provide a more direct and economical route to glutamate, particularly in chloroplasts where NADPH is generated photosynthetically.

This paper describes the properties of an NADP-dependent glutamate dehydrogenase (EC 1.4.1.4) isolated from the siphonous marine alga *Caulerpa simpliciuscula* which has an apparent *Km* NH_4^+ substantially lower than other glutamate dehydrogenases of plants. The enzyme separates in the chloroplast fraction in cell-free preparations of this alga.

[1] This work was supported by the Australian Research Grants Committee.

MATERIALS AND METHODS

Plant Material. *Caulerpa simpliciuscula* plants were gathered at 1 to 3 m depths from the sea at Williamstown, Victoria and kept for up to 2 to 3 weeks in aerated seawater in an aquarium on a 12 hr light/12 hr dark cycle at 16 C.

Extraction of Crude Glutamate Dehydrogenase from *C. simpliciuscula*. Dark green fronds free of visible epiphytic growth were selected, washed in seawater to remove particulate material, and cut into 1 to 2 cm lengths. All subsequent steps were carried out at 0 to 4 C and enzyme preparations were stored at −15 C between stages in the isolation procedure.

Crude extracts were prepared by rolling 500 g of tissue, sandwiched between a double layer of fine nylon mesh, with a wooden roller in1 liter of 0.01 M tris-HCl buffer, pH 8.5, and 1 mM EGTA.[2] The turbid green extract was filtered through glass wool, centrifuged for 10 min at 10,000*g*, and the brown supernatant decanted through Whatman No. 1 filter paper. The detailed procedure for purification of GDH from this crude extract is outlined under "Results."

Enzyme Assays. Enzymic activity of GDH, throughout the purification procedure, was assayed in the direction of reductive amination of α-KG. The activity of the extracted GDH was determined at 30 C by the decrease in the absorbance of NADPH at 340 nm in a reaction medium containing, unless otherwise specified, 50 mM glycylglycine buffer, pH 8.5, 2 mM EDTA, 0.87 mM ammonium acetate, 7 mM α-KG, and 0.1 mM NADPH. One unit of enzymic activity (U) converted 1 μmol min^{-1} substrate.

Enzymic activity in intact or partially disrupted particulate fractions was measured by following the production of ^{14}C-glutamate from 5-^{14}C-α-KG. Aliquots (50 μl) of the enzyme fractions were incubated at 30 C in 0.1 ml of 25 mM HEPES-NaOH buffer, pH 8, or 10 mM triethanolamine-HCl buffer, pH 8, and 5 mM EDTA, 0.3 M ammonium acetate, 0.3 mM NADPH, and 0.4 μCi 5-^{14}C-α-KG (2 mM). Products of the reaction were separated by electrophoresis at pH 2.1 as previously described (8) and their radioactivity was determined by liquid scintillation counting.

In experiments where the ammonium concentration was critical, reagents were prepared in, and enzyme preparations were dialyzed against, ammonia-free water prepared by the method of Hird and Marginson (6). In such cases, the ammonium content of reaction media was also analyzed directly by the method of Kirsten *et al.* (12).

Protein concentrations were measured by a modification of the method of Oyama and Eagle (20).

Polyacrylamide Disc Gel Electrophoresis. Polyacrylamide disc gel electrophoresis was performed by the method of Davis (3) except that gels contained no large pore packing region. Pairs of

[2] Abbreviations: EGTA: ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid; GDH: glutamate dehydrogenase; DIECA: diethyl dithiocarbamate; DTT: dithiothreitol; α-KG: α-ketoglutarate.

gels were stained either for protein or for GDH activity in 0.1 M tris-HCl buffer, pH 8.3, 0.1 M glutamate, 0.12 mM NADP, 1 mM nitroblue tetrazolium, and 0.46 mM phenazine methosulfate at 30 C. Dilute samples were concentrated by lyophilization following dialysis against 0.03 M ammonium bicarbonate, pH 8.5.

Preparation of Chloroplast Fraction. The chloroplast fraction was prepared from rhizomes of *C. simpliciuscula* essentially by the method previously described (5) but using an isolation medium of 0.41 M NaCl, 24 mM $MgSO_4$, 22 mM $MgCl_2$, 8 mM KCl, 3 mM $CaCl_2$, 0.1 mM K_2HPO_4, 10 mM HEPES buffer, pH 7.7, 5% (w/v) PVP-40 (mol wt 40,000), 5 mM DIECA, 1 mM DTT, and 0.1% BSA (Cohn Fraction V), unless otherwise specified. After collection of the chloroplasts by centrifugation, the organelles were resuspended in the same medium, excluding DIECA or DTT when it was desired to retain the chloroplasts intact.

Density Gradient Separation of Chloroplast Fraction. Distribution of the chloroplast fraction on a density gradient was carried out by layering 5 ml of the crude suspension onto a discontinuous gradient of 40 to 60% sucrose (5% steps) in 10 mM MES buffer, pH 6.8, 0.1 M EGTA, 1 mM DIECA, and 0.1% (w/v) BSA, and centrifuging at 80,000*g* for 20 min in a Beckman SW25.1 head. The major band of chloroplasts collected at the 50 to 55% sucrose boundary. A similar band formed at approximately 55% sucrose when using a continuous gradient of 20 ml of 40 to 70% sucrose in 10 mM HEPES buffer, pH 7.7, 0.1 M EGTA, and 0.1% (w/v) BSA, topped with a layer of 20% sucrose in the same buffer.

RESULTS

Preparation and Purification of NADP-GDH. Crude extracts of whole *Caulerpa* tissue contained both NAD-GDH and NADP-GDH which could be separated by gel electrophoresis. The enzymic activity in the presence of NADPH was at least 20-fold higher than the activity in the presence of NADH and the rate of nucleotide reduction was entirely dependent on the presence of α-KG and added ammonium. Analysis of the crude soluble extract obtained by sonication of the isolated chloroplast fraction (Table I) also showed that the major GDH present was NADP-specific. Neither glutamate synthase nor alanine dehydrogenase activities were detected in the crude extracts (Table I).

The NADP-GDH was partially purified from extracts of whole tissue by the following procedure. Crude extract (1035 ml) was concentrated 20-fold by ultrafiltration through a Diaflo UM20E membrane of retentivity 10,000 to 20,000 mol wt and the concentrate was brought to 1% (w/v) with streptomycin sulfate. After centrifugation at 10,000*g* for 10 min, the clear brown supernatant was dialyzed against 3 liters of 0.05 M tris-HCl and 1 mM EGTA buffer, pH 8.5, for 12 hr. It was then applied to a DEAE-cellulose column (30 × 4 cm^2) which had been previously equilibrated with the same buffer. The column was eluted at 2.5 ml min^{-1} firstly with 700 ml of 0.05 M tris-HCl and 1 mM EGTA buffer, pH 8.5, and then with a linear concentration gradient of 0 to 0.2 M NaCl in 650 ml of the same buffer. Enzymic activity eluted at approximately 0.1 M NaCl.

The eluate from the DEAE-cellulose column (84 ml) was further purified by $(NH_4)_2SO_4$ precipitation. Saturated $(NH_4)_2SO_4$ solution (pH 6.8) was used to bring the solution successively to 30, 45, and 60% saturation, then solid $(NH_4)_2SO_4$ (99.8 gl^{-1}) was finally added to give 75% saturation. Each precipitate was collected by centrifugation at 10,000*g* for 10 min and redissolved in 3 ml of 0.2 M tris-HCl and 1 mM EGTA buffer, pH 8.5. Enzyme precipitating between 45% and 75% saturation was combined, mixed with an equal volume of 0.2 M tris-HCl and 1 mM EGTA buffer, pH 8.5, in 40% (w/v) sucrose, and layered onto a Bio-Gel A-5m column (60 cm × 4 cm^2) of fractionation range 10,000 to 5,000,000 mol wt. Proteins were eluted with 0.2 M tris-HCl and 1 mM EGTA buffer, pH 8.5, at 10 ml hr^{-1} (Fig. 1). Pooled fractions 101 to 108 (14.5 ml) were used to determine the kinetic and electrophoretic properties of the GDH.

Overall, this procedure achieved a 78-fold increase in GDH activity mg^{-1} protein and 8% recovery of the total enzymic activity (Table II). Measurements of the A_{260}/A_{280} ratio also indicated a significant removal of nucleic acids by the streptomycin sulfate treatment. There was also a substantial removal of color from the preparation at several stages in the procedure.

The enzyme was relatively stable in the crude extracts losing only 25% of its activity at −20 C over 4 months. Negligible loss of activity occurred at 4 C over 24 hr. However, the more purified enzyme was far less stable, and during chromatography on Bio-Gel A-5m there was substantial inactivation of the enzyme during the 24-hr period. Two peaks of NADP-GDH activity were eluted from the Bio-Gel A-5m column (Fig. 1). The smaller mol wt component contained the bulk of the activity recovered but nevertheless contained only 29% of the enzymic activity applied to the column.

Polyacrylamide gel electrophoresis was performed on the crude extract and the ammonium sulfate-precipitated material. The distribution of proteins on these gels after staining with Amido Schwarz indicated that although there had been concentration of the band of protein corresponding to the NADP-GDH activity, there was still much inactive protein present. The further purification of this material on Bio-Gel A-5m reduced the concentration of protein eluting with the enzymic activity to the point where it was not detectable on gels.

Characterization of Partially Purified GDH. The most interesting feature of the enzyme is its capacity for reductive amination when ammonium ions are present at the low concentration of 1 μmol g^{-1} fresh weight present in *C. simpliciuscula in vivo*. The apparent *Km* values were 3.8 mM for glutamate and 6.7 mM

Table I. *Glutamate Dehydrogenase Activity in Caulerpa Chloroplast Extracts*

The complete reaction mixture contained 50 mM glycylglycine, pH 8.5, 2 mM EDTA, 0.87 mM ammonium acetate, 7 mM α-KG, 0.1 mM NADPH or NADH, and enzyme extract in 1.15 ml. Glutamine was 0.87 mM and pyruvate 7 mM.

Assay Medium		Rate of Oxidation of Reduced Nucleotide mU mg^{-1} protein
NADPH:	complete medium	90
	$-NH_4^+$	20
	-α-KG	15
	$-NH_4^+$ + glutamine	18
	-α-KG + pyruvate	15
NADH :	complete medium	10
	-α-KG + pyruvate	8

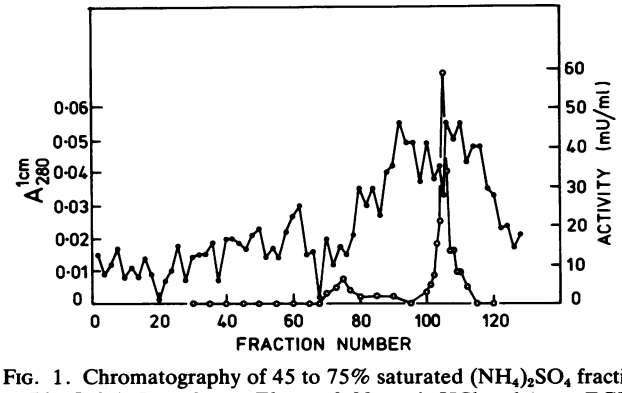

FIG. 1. Chromatography of 45 to 75% saturated $(NH_4)_2SO_4$ fraction on a Bio-Gel A-5m column. Eluant: 0.02 M tris-HCl and 1 mM EGTA buffer, pH 8.5; fraction volume; 2.05 ml. Fractions 101 to 108 were pooled. O: enzymic activity, ●: A_{280}^{1cm}.

Table II. *Purification of Glutamate Dehydrogenase*

Values of protein concentration and enzymic activity were the average of at least two determinations. The degree of purification was defined as the ratio of the specific activities (units of enzymic activity mg^{-1} of protein) before and after stages in the procedure. Recoveries of enzymic activity were corrected for the actual proportion of the initial tissue extract used in each purification step.

Enzyme Preparation	Volume (ml)	Total Protein (mg)	Total Activity (mU)	Specific Activity (mU mg^{-1})	Overall Purification (fold)	Overall Recovery (%)
Crude extract	1035	290	12,900	44.7	1	100
Extract concentrated on Diaflo UM20E	54.5	169	9,530	56.3	1.3	75
Dialyzed streptomycin sulphate supernatant	55.5	111	9,090	81.7	1.8	72
Pooled fractions after DEAE-cellulose chromatography	84	21.8	6,350	292	6.6	51
45-75% $(NH_4)_2SO_4$	6.1	5.1	3,480	680	15	28
Pooled fractions after Biogel A-5m chromatography	14.5	0.094	327	3,480	78	8

for α-KG. V_{max} for reductive amination of α-ketoglutarate was 4.1 times V_{max} for the reverse reaction, and in each of these properties the *Caulerpa* enzyme is similar to a range of other GDH's. However, the apparent *Km* for ammonium of 0.67 mM is at least an order of magnitude lower than those of NAD-GDH's from higher plant tissues.

The substrate specificities of the purified GDH are shown in Table III. The enzyme was highly specific for NADPH and α-KG after purification. Neither pyruvate nor oxaloacetate acted as amino group acceptors. In fact we found no evidence in *Caulerpa* extracts of the alanine dehydrogenase activity described in unicellular algae (10, 13). The small degree of activity with α-ketovalerate is similar to that shown by the bovine liver enzyme (4).

Although ammonium ion is by far the preferred amino group donor, there was a small reaction rate in the presence of 0.2 M glutamine but not 0.2 M asparagine. Similar activity with glutamine has been shown for the GDH from *Neurospora crassa* and the bovine liver enzyme (1). The levels of glutaminase or of glutamate synthase determined by radioisotope analyses in these *Caulerpa* extracts were insufficient to account for the activity observed with glutamine in Table III.

Localization of GDH. Three independent pieces of evidence were obtained which suggest that a major part of the NADP-GDH in *Caulerpa* is located within the chloroplast.

Firstly, the enzyme was present in isolated chloroplasts but could only be detected upon lysis of the chloroplasts. Chloroplasts were isolated from rhizomes of *Caulerpa* and incubated in a medium in which they had previously been shown to retain the ability to fix CO_2 and to evolve O_2. Under such conditions the chloroplasts were also impermeable to exogenous 3-P-glycerate and potassium ferricyanide (5). The rate of formation of ^{14}C-glutamate from ^{14}C-α-KG in such a chloroplast preparation was unaffected by the addition of ammonium and reduced nucleotides. In intact chloroplasts, therefore, there was no detectable GDH activity, only glutamate formation apparently due to aminotransferase activity. By contrast, GDH activity was detected when the same chloroplast preparation was resuspended in 0.01 M HEPES buffer, pH 7.7, and sonicated three times for 30 sec. Such treatment also makes these chloroplasts permeable to potassium ferricyanide (5). Hence GDH in the chloroplast preparation was inaccessible to exogenous NADPH in intact chloroplasts.

Secondly, chloroplasts retained NADP-GDH activity during fractionation on sucrose gradients. Crude "chloroplast fraction" contains a significant amount of contaminating starch granules and cell debris (5). The material banding at 55% sucrose after 30 min at 80,000*g* on sucrose gradients was freed of a major proportion of visible material. Measurement of glutamate formation from ^{14}C-2-oxoglutarate was carried out on similar samples before and after fractionation on the gradient. Both fractions were deliberately lysed in 0.01 M HEPES buffer, pH 7.7, before assay (Table IV). The chloroplasts retrieved from the gradient retained a higher GDH activity/unit Chl despite the removal of the major portion of cell debris.

Thirdly, the distribution of succinate dehydrogenase and NADP-GDH activities among the subcellular particulate fractions is markedly different. The total activity of GDH separating

Table III. *Alternative Substrates for Glutamate Dehydrogenase*

The reaction mixture contained 50 mM glycyglycine, pH 8.5, 2 mM EDTA, 10 mM α-KG, 8.7 mM NH_4Cl, 0.1 mM NADPH, and Bio-Gel A-5m eluate unless otherwise indicated.

		Rate of Utilization of Substrate mU ml^{-1} enzyme extract	
A.	Reduced Nucleotide	- α-KG + NH_4^+	+ α-KG + NH_4^+
	NADPH	0	12.5
	NADH	0	0.6
B.	α-Keto Acid (10 mM)	- NH_4^+ + α-Ketoacid	+ NH_4^+ + α-Ketoacid
	None	0	0
	α-KG	6.5	54.2
	α-Ketovalerate	0	3.3
	Pyruvate	0	0
	Oxaloacetate	10.3	10.3
C.	Amino Group Donor	- α-KG	+ α-KG
	None	0	2.8
	NH_4Cl	0	43.0
	Alanine (20 mM)	0	2.7
	Glycine (20 mM)	0	2.7
	Asparagine (20 mM)	0	2.8
	Glutamine (20 mM)	0	7.5

Table IV. *Synthesis of ^{14}C-Glutamate in Isolated Chloroplasts*

Chloroplasts were prepared and subjected to gradient centrifugation as described under "Materials and Methods." Material at the 50 to 55% sucrose boundary was collected off the gradient. Both chloroplast fractions before and after the gradient were suspended in 0.01 M HEPES buffer, pH 7.7, and 0.1 mM EGTA prior to addition of ^{14}C-2-oxoglutarate. Other conditions as under "Materials and Methods."

Additions to Medium		Glutamate Formation Chloroplasts before gradient	Chloroplasts after gradient
Nucleotide	NH_4^+	mU mg^{-1} Chl	
None	-	1.8	2.8
	+	1.8	2.0
NADH	-	2.8	2.2
	+	2.7	1.7
NADPH	-	2.5	4.0
	+	5.2	8.5*

* Maximum activity recovered from chloroplasts from a continuous gradient 35 mU mg^{-1} Chl.

Table V. *Distribution of Glutamate Dehydrogenase in Particulate Fractions*

Chloroplasts were prepared as described under "Materials and Methods" and collected by centrifugation at 750*g* for 1 min. Material sedimenting in the subsequent centrifugation at 750*g* for 5 min and 10,000*g* for 20 min was also collected. Pellets were resuspended in 0.01 M HEPES and 1 mM EGTA, pH 7.7, sonicated three times for 30 sec, centrifuged at 10,000*g* for 10 min, and the supernatants assayed for GDH as in Table I. Chl was measured after extracting the final pellets into 90% acetone.

Fraction	Chl	Glutamate Dehydrogenase	
	mg	mU	mU mg^{-1} protein
750g, 1 min	1.05	295	75
750g, 5 min	0.35	170	-
10,000g, 20 min	0.06	11	-
Supernatant	-	166	6

with the chloroplast fraction and other particulate fractions was measured during the isolation of intact chloroplasts from the crude cell contents (Table V). The bulk of particulate GDH precipitated with the major chloroplast fraction which was collected by an initial 750*g* centrifugation. By contrast, the "mitochondrial fraction" (10,000*g*, 20 min) contained very little GDH activity. The distribution of succinate dehydrogenase activity in fractions prepared the same way has been reported previously (5). In contrast to the distribution of GDH, succinate dehydrogenase activity in the mitochondrial fraction was four times greater than in the initial 750*g* chloroplast fraction. The NADP-GDH activity measured in the chloroplast fraction therefore could not have been caused by adhering mitochondria.

Finally, the specific activity of GDH mg^{-1} protein was also higher in the chloroplast fraction (Table V) than in the supernatant. This too was expected if the main source of NADP-GDH was the chloroplast.

Characterization of Chloroplast-GDH Extract. In all the major properties tested, the crude enzyme extracted from chloroplasts matched closely with that purified from whole tissue: *Km* NH_4^+, 0.4 mM; pH optimum, 7.5 to 8.5; rate glutamine to rate NH_4^+, less than 0.05; and rate NADPH to rate NADH, 9. The enzymic activity from both sources also had the same electrophoretic mobility on polyacrylamide gels.

DISCUSSION

Comparison of the properties of previously characterized GDH's with the enzyme purified from *Caulerpa simpliciuscula* shows two significant differences. Firstly, the enzyme is NADP-specific and has an apparent *Km* for ammonium ions of 0.4 to 0.7 mM. Secondly it is largely if not completely chloroplastic.

The low apparent *Km* for ammonium of the *Caulerpa* enzyme sets it apart not only from the NAD-specific GDH's of higher plants but also from the NAD- and NADP-specific GDH's of both *Neurospora* and yeasts (7, 22). The only exception appears to be an NAD-specific GDH in peas where the exceedingly low apparent *Km* values for ammonium and α-KG of 42 and 2.9 μM, respectively, have been reported (24).

A variety of GDH activities have been described previously in chloroplasts isolated from higher plants. *Vicia faba* contained an NAD(P)-GDH activity which remained tightly bound to the particulate fraction when either intact chloroplasts or naked chloroplast-lamellae were prepared (18). Although the levels of enzyme present in these preparations were difficult to assess because of the assay procedures used, the chloroplast enzyme had both NADP- and NAD-dependent activity and was firmly bound to the membranous material. By contrast the enzyme we have found in *Caulerpa* was readily released as a soluble enzyme from the chloroplast fraction with such gentle treatments as suspension in low osmotic medium. It was also 9-fold more active with NADPH than NADH in crude chloroplast extracts and 75-fold more active with NADPH than NADH when purified. It therefore also differs from the GDH activity associated with a chloroplast-containing fraction from pea leaves, described by Tsenova, which was six times more active with NADH than NADPH (24).

Even in most extracts of whole *Caulerpa* plants, GDH activity was predominantly NADP-specific, although a separate NAD-GDH was identified. Both NADH- and NADPH-dependent GDH activities have also been detected in crude extracts of other algae, the relative amounts varying with species (9, 10). It should be noted however that growth in high ammonium, low nitrate media caused a large *de novo* synthesis of NADPH-dependent GDH in *Chlorella*, leading to a similar predominance of NADP-GDH in that alga (13).

The marked specificity of the purified enzyme we have studied for NADPH, its location in the chloroplast, and its low apparent *Km* for ammonium all make it eminently suitable for reductive amination in the chloroplast. In addition, chloroplast fraction extracts were found to contain up to 10 μmol hr^{-1} mg^{-1} Chl of NADP-GDH activity when assayed in the presence of 0.6 mM α-KG. Therefore there was more than enough GDH activity to account for the rate of $^{14}CO_2$ accumulation of 5 to 10 μmol hr^{-1} mg^{-1} Chl into amino acids by chloroplasts from *Caulerpa* (5), even though the concentration of α-ketoglutarate used in the assays was well below that required for saturation.

Hence, the NADP-GDH has both suitable kinetic properties and also the capacity for reductive amination at low ammonium concentrations in the chloroplast of *Caulerpa* independent of the dual enzyme system proposed elsewhere (15).

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