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Identification and characterization of a glutamate dehydrogenase in the unicellular cyanobacterium *Synechocystis* PCC 6803

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Glutamate dehydrogenase activity has been detected in permeabilized cells of the unicellular cyanobacterium *Synechocystis* PCC 6803 at similar levels to those of glutamate synthase. The enzyme responsible has been purified by affinity chromatography on 2',5'-ADP-Sepharose and identified as an NADPH-specific glutamate dehydrogenase. The enzyme catalyzes preferentially glutamate formation rather than the reverse reaction, with K_m values for NADPH, 2-oxoglutarate and ammonia of 20 μM, 1.5 mM and 3.7 mM, respectively. It is composed of four identical subunits giving a total molecular mass of 208 kDa for the native protein. Its physiological role is discussed in terms of being an alternative pathway to the glutamine synthetase-glutamate synthase cycle for ammonia assimilation.

Glutamate dehydrogenase; Cyanobacterium; Ammonium assimilation; (*Synechocystis* 6803)

1. INTRODUCTION

Glutamate dehydrogenases (EC 1.4.1.2–4) catalyze the reductive amination of 2-oxoglutarate to glutamate using pyridine nucleotides as electron donors, according to the equation:

$$\text{2-oxoglutarate} + NH_4^+ + \text{NAD(P)H} \rightleftarrows \text{L-glutamate} + H_2O + \text{NAD(P)}^+$$

The reaction is freely reversible, although the rate in each direction varies greatly among enzymes from different sources [1].

The presence of this enzyme in photosynthetic eukaryotes, fungi and heterotrophic bacteria is well documented [1,2], in contrast to the situation in cyanobacteria, where its occurrence has never been conclusively demonstrated. In these microorganisms, the assimilation of ammonia is generally considered to take place through the glutamine synthetase-glutamate synthase pathway [3], with only occasional references to low levels of glutamate dehydrogenase activity assayed in crude preparations [4,5].

Here, we report the presence of an active glutamate dehydrogenase in the unicellular cyanobacterium *Synechocystis* PCC 6803, as well as its purification and main properties.

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2. MATERIALS AND METHODS

2.1. *Growth of cells*

Synechocystis sp. strain PCC 6803 was grown photoautotrophically under continuous fluorescent illumination (25 $W \cdot m^{-2}$, white light) at 35°C on a synthetic medium [6] containing 20 mM potassium nitrate or 15 mM ammonium chloride

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as nitrogen source, in a stream of 1.5% CO_2 in air (v/v).

2.2. *Enzyme assays*

2.2.1. Glutamate dehydrogenase

Glutamate dehydrogenase aminating activity was determined at 30°C in a reaction mixture containing 85 mM Tris-HCl buffer (pH 8.0), 10 mM 2-oxoglutarate, 50 mM NH_4Cl and 0.2 mM NADPH. The reaction was started by addition of ammonium chloride and followed either spectrophotometrically at 340 nm or by glutamate formation. In both cases control experiments were made to correct, respectively, for ammonium-independent oxidation of NADPH or for glutamate present at zero time. Fot the in situ assay, a sample of cells containing about 25 μg chlorophyll was permeabilized in 1 ml reaction mixture, by the addition of 20 μl of 1.25% mixed alkyltrimethylammonium bromide (MTA).

Deaminating activity was followed spectrophotometrically at 340 nm in a reaction mixture containing 80 mM Tris-HCl buffer (pH 9.0), 50 mM L-glutamate and 2 mM $NADP^+$. The reaction was started by the addition of $NADP^+$ and carried out at 30°C.

2.2.2. Glutamine synthetase

Glutamine synthetase biosynthetic activity was determined in situ by following glutamine formation in cells permeabilized as stated above. The assay method was that described in [7] except that 50 mM Hepes-NaOH buffer (pH 7.0) replaced the imidazole buffer. The reaction, carried out in a final volume of 1 ml, was started by the addition of ATP and stopped with 0.6 ml of 1 N HCl after 15 min incubation at 30°C.

2.2.3. Glutamate synthase

Glutamate synthase was determined in situ by following glutamate formation. Cells, containing about 25 μg chlorophyll, were centrifuged and resuspended in 0.9 ml of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.5), 5.5 mM L-glutamine, 5.5 mM 2-oxoglutarate, 5.5 mM aminooxyacetate and 0.28 mM methyl viologen. After adding 20 μl toluene and mixing for 1 min, the reaction was started with 0.8 mg sodium dithionite freshly dissolved in 0.1 ml of 0.12 M $NaHCO_3$. The reaction was carried out for 15 min at 30°C and stopped by the addition of 0.6 ml of 1 N HCl.

2.3. *Analytical methods*

Glutamate and glutamine were determined by the fluorescence of their *o*-phthaldialdehyde (OPA) derivatives measured after separation from other OPA derivatives by reverse-phase HPLC on a μBondapack C18 column [8].

Chlorophyll was determined as in [9]. Protein was determined as in [10], except for the elution profile in fig.1, determined by the method of Bradford [11] using BSA as standard.

2.4. *Electrophoresis*

Polyacrylamide gel electrophoresis was carried out in 5% acrylamide gels, in glass tubes, according to [12]. SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborn [13] in 10% acrylamide gels. The gels were stained with Coomassie brilliant blue and scanned at 600 nm.

2.5. *Gel filtration*

The molecular mass of the native protein was determined by gel filtration through a Sephacryl S-300 column (1.6 × 30 cm) using the following standards: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), glyceraldehyde-3-phosphate dehydrogenase (120 kDa) and bovine serum albumin (67 kDa).

3. RESULTS AND DISCUSSION

The enzymes of the glutamate synthase cycle, namely glutamine synthetase and glutamate synthase, are found at high levels in *Synechocystis* cells using nitrate as nitrogen source (table 1). However, in cells grown in ammonia-containing medium, glutamine synthetase activity is only about 10% of the control value in nitrate (table 1). The same levels are obtained by adding ammonium, even at low concentrations, to cells growing in nitrate [14].

Since this 10-fold decrease in glutamine synthetase activity did not affect the growth rate of the microorganism, we investigated the presence of an alternative route for ammonia incorporation into carbon skeletons. As shown in table 1, in situ assays of glutamate dehydrogenase showed the

Table 1

Effect of the nitrogen source on ammonia-assimilating enzymes

Enzyme activity	Nitrogen source	
	Nitrate (mU/mg protein)	Ammonium (mU/mg protein)
Glutamine synthetase	72.4 (100%)	6.4 (8.8%)
Glutamate synthase	15.4 (100%)	13.0 (84.4%)
Glutamate dehydrogenase	7.6 (100%)	10.0 (131.6%)

Enzyme activities were determined in situ in cells cultivated in the presence of nitrate (20 mM) or ammonium (15 mM), as described in section 2

presence of this enzyme activity at levels similar to those of glutamate synthase, and high enough to sustain the exponential growth of the microorganism. Furthermore, glutamate dehydrogenase activity is 30% higher in ammonia-grown cells than it is in nitrate.

In order to avoid interference from other enzymes which could be present in the crude extract, such as alanine dehydrogenase and transaminases, we proceeded to purify it. Cell-free extracts were obtained by sonic disruption of ammonia-grown cells followed by centrifugation for 30 min at 36000 × *g*. The supernatant was adsorbed onto a DE-52 DEAE-cellulose column (2.5 × 25 cm) equilibrated with 50 mM Hepes-NaOH buffer, pH 7.0 (hereafter referred to as standard buffer). After washing the bed with 4 column volumes of standard buffer, the enzyme was eluted with a 0.05–0.5 M KCl gradient (400 ml) in standard buffer. Fractions with high glutamate dehydrogenase activity, eluting at 0.2–0.25 M KCl, were combined and applied to a 2′,5′-ADP-Sepharose column (1 × 12 cm) equilibrated with standard buffer supplemented with 0.2 M KCl. After washing out weakly adsorbed proteins with 4 column volumes of 0.5 M KCl in standard buffer, the enzyme was eluted by the inclusion of 2 mM $NADP^+$ in the washing buffer. The elution profile

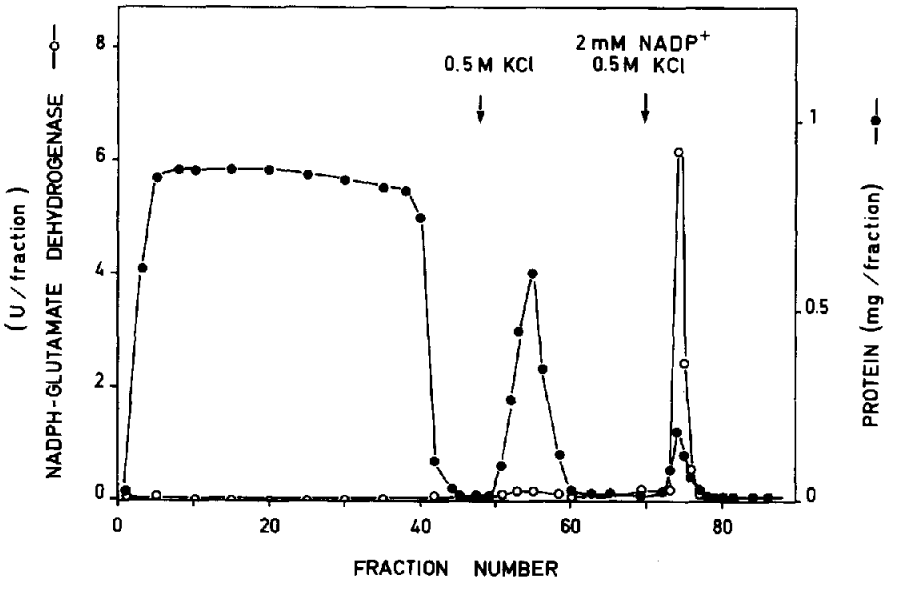

Fig.1. Affinity chromatography of *Synechocystis* glutamate dehydrogenase on 2′,5′-ADP-Sepharose. The volume of the fractions was 5 ml (fractions 1–50) and 3 ml thereafter. For additional details see text.

Table 2

Purification of glutamate dehydrogenase from *Synechocystis*

Purification step	Volume (ml)	Activity (U)	Protein (mg)	Spec. act. (U/mg protein)	Purification (fold)	Yield (%)
Crude extract	350	8.45	998	0.0085	1	100
DE-52	200	9.6	68	0.141	16.6	114[a]
2',5'-ADP-Sepharose	6	8.55	0.3	28.5	3353	101

[a] See text. Starting material: 74 g fresh wt

of the enzyme is presented in fig.1. As shown, the enzyme remains firmly bound to the gel even at high ionic strength, allowing extensive washing of contaminating proteins and, as a consequence, a 200-fold purification in a single step.

The results from a typical purification are summarized in table 2. The specific activity of the preparation was increased more than 3000-fold, up to 28 U/mg protein (aminating, NADPH). It is interesting to note that the ion-exchange step increased total activity, probably due to the removal of interfering substance(s).

The purified enzyme ran as a single band under non-denaturing electrophoresis and was stable for at least 2 months when stored at 0–4°C in standard buffer.

Gel-filtration data give a molecular mass for the native enzyme of 208 ± 5 kDa. Electrophoresis in the presence of SDS revealed a single protein band, migrating at a position which corresponds to a subunit molecular mass of 46 kDa. According to these values, the enzyme appears to be composed of four identical subunits.

The pure enzyme was able to catalyze the reaction in either direction, although at different rates. The requirements of the reaction in the aminating direction (glutamate formation) are presented in table 3. It can be seen that no reaction took place in the absence of NADPH, 2-oxoglutarate or ammonium, or when the enzyme was boiled. The stoichiometry found between NADPH oxidized and glutamate formed was 1:1, as expected for a reaction catalyzed by glutamate dehydrogenase. NADH could substitute for NADPH as electron donor for the reaction, although at a much lower rate (NADPH/NADH activity ratio 35–40). The requirements of the reaction using NADH as reductant were the same as for NADPH (not shown).

In the deaminating direction (2-oxoglutarate formation) NAD^+ was unable to sustain any significant activity. In contrast, $NADP^+$-dependent formation of 2-oxoglutarate from glutamate occurs readily in the presence of the enzyme. The reaction does not proceed in the absence of glutamate, $NADP^+$ or enzyme.

Since Ca^{2+} is considered to stimulate glutamate dehydrogenase [1], we have investigated the effect of different concentrations of calcium chloride on both aminating and deaminating activities. Glutamate formation is stimulated by only 25% at 20 mM calcium chloride, however, deaminating

Table 3

Characterization of glutamate formation by *Synechocystis* glutamate dehydrogenase

System	NADPH oxidized (nmol)	Glutamate formed (nmol)
Complete	32.0	31.2
– NADPH	–	0.6
– 2-oxoglutarate	0.8	0.0
– ammonium	1.3	0.0
Complete (enzyme boiled)	1.3	0.2

The assay was carried out for 15 min as described in section 2

activity increased by 108% at the same concentration. NAD^+ supported deaminating activity was undetectable even at calcium chloride concentrations as high as 0.1 M.

Under our assay conditions, the ratio between aminating and deaminating activities using NADP(H), ranges from 8.2 (no calcium present) to 4.9 (20 mM calcium), therefore suggesting that the enzyme has an assimilatory rather than catabolic role, as is usually the case with NADPH-dependent glutamate dehydrogenases [2].

The optimal pH for the aminating activity is between 7.5 and 8.0. At this pH, the K_m values for NADPH, 2-oxoglutarate and ammonium are 20 μM, 1.5 mM and 3.7 mM, respectively.

This last value, although two orders of magnitude higher than that of glutamine synthetase, is lower than in most organisms [1,2] and will allow the operation of glutamate dehydrogenase in cells growing at high ammonium concentration, where it is not worthwhile to spend ATP to obtain the extra affinity of glutamine synthetase for ammonia.

On the other hand, ammonia, having an unprotonated diffusible species, will equilibrate across the membranes following the pH gradients and, therefore, will accumulate in low pH compartments such as thylakoids. In this respect, the topological location of the enzyme is of the utmost importance and is presently being investigated.

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