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Intracellular Localization of Glutamine Synthetase in a Nitrogen-fixing Cyanobacterium *Anabaena cylindrical*

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Abstract: The major route of ammonia assimilation is the reaction which is catalyzed by glutamine synthetase to give ammonia. Cell-free extracts and purified thylakoid membranes using differential centrifugation and density gradient techniques were assayed for the percentage activity of the enzyme. Glutamine synthetase was detected in all cell-free extracts. Seventy six percent of the enzyme activity was found associated with the thylakoid membranes. Using antiserum raised to the thylakoids, 78.5% inhibition of the enzyme activity was obtained.

Key words: Anabaena cylindrical, glutamine synthetase activity, thylakoid membrane, ammonia, chlorophyll concentration, sucrose density gradient

INTRODUCTION

Glutamine synthetase (GS) is a key enzyme in the assimilation of ammonia by nitrogen fixing cyanobacteria (Muro-Pastor *et al*., 2005; Sampaio *et al*., 1979; Stewart, 1980). The enzyme was also described for its cardinal importance in biosynthetic metabolism (Shapiro and Shtadtman, 1970) and as a key element of nitrogen assimilation in enteric bacteria (Tyler, 1978; Flores and Herrero, 2005). In *Rhodospirillum rubrum*, the enzyme was reported to be associated with or bound to the chromatophore membranes (Yoch *et al*., 1983). However, Romero *et al*. (1988), using two species of Rhodospirillaceae, demonstrated a cytosolic location for the enzyme. Bergman *et al*. (1985) showed an equal distribution of GS in the cytoplasm of *Anabaena cylindrica*.

In this study, localization of GS in the thylkoid membranes of *A. cylindrica* was investigated.

MATERIALS AND METHODS

Organism and growth conditions: *Anabaena cylindrica*, strain was obtained from the Culture Center of Algae and Protozoa, Cambridge, UK. The organism was grown xenically in BG-11 medium (Stanier *et al*., 1971) in 250 mL conical flasks. Cultures were incubated under constant illumination of 60 μmol $m^{-2} s^{-1}$.

Preparation of thylakoid membranes: A 5 L culture was grown in 10 L aspirator equipped with a magnetic stirrer and sparged with sterile air. Cells were harvested by centrifugation at 5000 x g for 20 min. The cell pellet was resuspended in 50 mL of Tricine buffer, pH 7.5, containing 10 mM NaCl. Cells were then disrupted by four 15 sec periods of ultrasonication punctuated with 15 sec rest periods in an ice bath.

The resulting material was immediately diluted with an equal volume of Tricine buffer containing 400 mM sucrose. All subsequent centrifugation were performed at 4°C. Unbroken cells were removed by centrifugation at 2500 x g for 30 min. The resulting supernatant was centrifuged at 35000 x g for 30 min to sediment the thylakoid membranes. These membranes was gently washed and suspended in Tricine buffer containing 400 mM sucrose.

Density gradient centrifugation: Aliquots (2 mL)of the washed thylakoids and of the 2500 x g cell-free supernatant were each layered onto a 20 mL 10 to 60% (w/w) linear gradient of sucrose in Tricine buffer in a polycarbonate tubes. After centrifugation at 80000 x g for 4 h, a 1.0 mL fraction was collected from the bottom of each tube.

Glutamine synthetase assay: The biosynthetic activity of the enzyme was followed according to the method of

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Sampaio *et al.* (1979). The glutamine synthetase (GS) activity was measured as μmole of NADH oxidized\mg of protein\h.

Preparation of antiserum: Thylakoid membranes of *A. cylindrica* were emulsified with an equal volume of Freunds complete adjuvant and 1.0 mL of this mixture (containing 1.9 mg of thylakoid protein)was injected subcutaneously into the hind foot of a rabbit. After 16 days, a booster dose containing 2.5 mg protein but without adjuvant was injected subcutaneously into the neck. Blood was collected 2 weeks later and serum was prepared and stored at -20°C.

Electron transport reactions: Ferricyanide-Hill reaction, Mehler-photosystem I reaction and NADP photo-reduction were carried out using cell-free extracts and thylakoid membranes as described by Sallal *et al.* (1987).

Chlorophyll and protein determinations: Chlorophyll a was measured according to Kirk (1967) and protein was measured following the method of Lowry *et al.* (1951), using crystalline BSA as a standard.

RESULTS AND DISCUSSION

Glutamine synthetase, biosynthetic activity was assayed in all of the differential centrifugation fractions of cell extracts of *A. cylindrica* as presented in Table 1. The enzyme activity retained in washed pellet produced after

Table 1: Distribution of glutamine synthetase activity in fractions of *A. cylindrica*

Fraction	Enzyme activity Sp. Activity a	Activity b
2500x g for 15 min supernatant	21.12	100.00
2500x g for 30 min pellet	2.01	3.00
2500x g for 30 min supernatant	22.00	87.20
35000x g for 30 min pellet	24.50	80.00
35000x g for 30 min supernatant	1.26	6.00
Washing of 35000x g pellet		
Pellet	28.45	76.00
Supernatant	2.05	1.50

a, specific activity: u moles NADH oxidized/mg protein/h, b, percentage activity originally present in 2500x g for 15 min supernatant

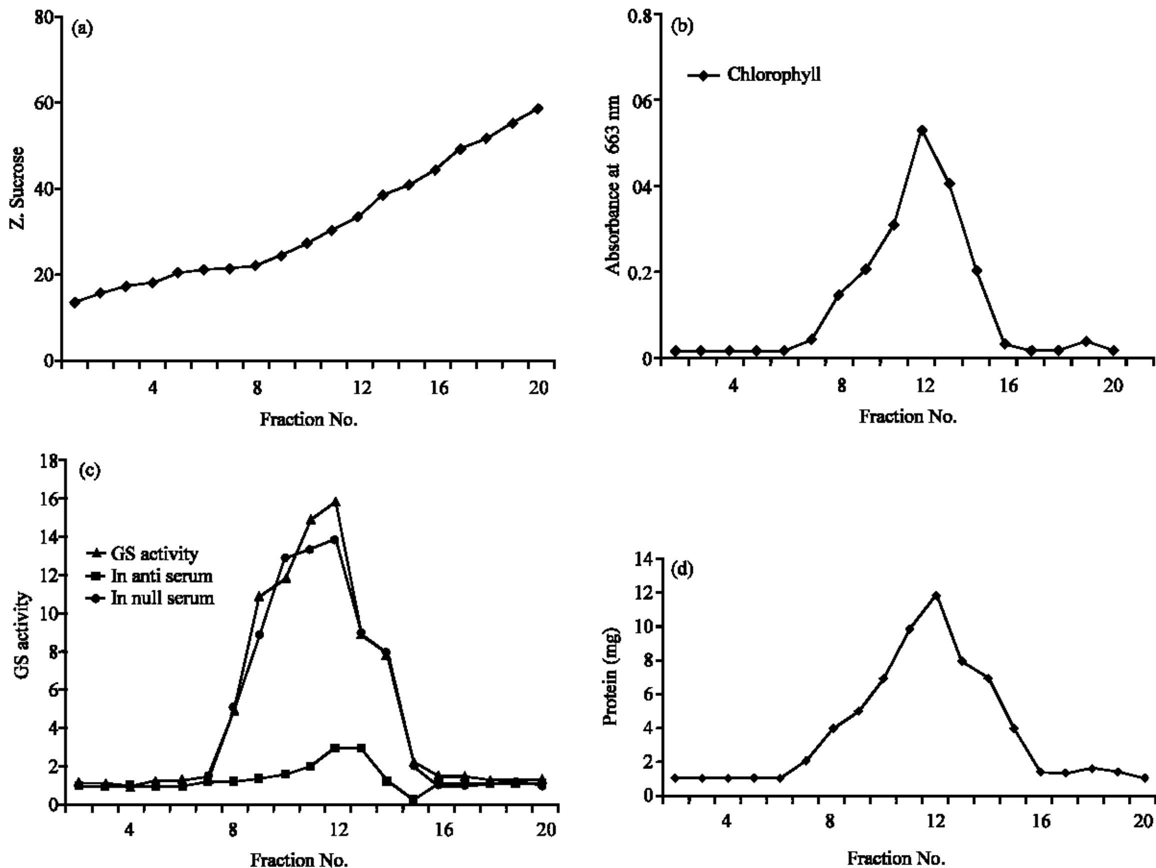

Fig. 1: Distribution of *A. cylindrica* chlorophyll and glutamine synthetase activity after sucrose density gradient centrifugation. (a) represents sucrose concentration; (b) represents chlorophyll concentration; (c) represents: (▲) GS activity, (■) GS activity+ antiserum and (◆) GS activity +null serum and (d) represents protein concentration

Table 2: Photosynthetic reactions of the thylakoid membrane fraction of *A. cylindrica*

Reaction	Activity
Photosystem II a	26
Photosystem I b	395
NADP- photoreduction c	35

a, specific activity: umol ferricyanide reduced/mg chlorophyll/h, b, specific activity:umol O2 uptake/mg chlorophyll/h, c, specific activity: umol NADP reduced/mg chlorophyll/h

35000 x g for 30 min was 76% of the original activity, while the enzyme activity in the supernatant was 6% (Table 1). The procedure for the isolation of the chlorophyll-containing pellet was essentially that described for thylakoid isolation (Codd and Sallal, 1978). Figure 1a-c show the sucrose density gradient of the thylakoid membranes, where the highest enzyme activity correlated with the maximal chlorophyll a concentration.

The 35000 x g pellet also catalyzed the photoreduction of ferricyanide from water in the photosystem II-Hill reaction and the photosystem I-Mehler reaction using DCPIP/ascorbate couple as electrons donor and methylviologen as electron acceptor.

This pellet also catalyzed the photoreduction of NADP via photosystem II and I as presented in Table 2. Further data in support of this concept were obtained using an antiserum prepared against *A. cylindrica* thylakoids. As shown in Fig. 1b and c the antiserum caused 78.5% inhibition of the enzyme activity found in the sucrose density gradient fraction, containing the highest chlorophyll concentration. In *Anabaena cylindrica*, GS was found to be distributed throughout the cytoplasmic region using an immunocytochemical method for localization (Bergman *et al*., 1985). In this immunological technique, the specific antiserum reacts with both active and inactive enzyme, in addition, this technique the antiserum reacts only with exposed molecules of the enzyme (antigen), since the antiserum cannot penetrate the membrane structure to react with the embedded parts of the antigen. As shown in Table 1, GS activity was associated with the thylakoid membranes of *A. cylindrica*. The results obtained from the sucrose density gradient and the inhibition of the antiserum raised to the thylakoid membranes to GS activity, support in a more quantitative measures the presence of the major enzyme activity in the thylakoids.

The present results support other studies on the involvement of thylakoids in respiration and photosynthesis (Grodzinski and Colman, 1976; Sallal and Nimer, 1988, 1990).

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