APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1992, p. 3122–3129
0099-2240/92/093122-08$02.00/0

Vol. 58, No. 9

Glutamine Synthetase and Nitrogen Cycling in Colonies of the Marine Diazotrophic Cyanobacteria *Trichodesmium* spp.†

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Received 1 June 1992/Accepted 1 July 1992

We examined freshly collected samples of the colonial planktonic cyanobacterium *Trichodesmium thiebautii* to determine the pathways of recently fixed N within and among trichomes. High concentrations of glutamate and glutamine were found in colonies. Glutamate and glutamine uptake rates and concentrations in cells were low in the early morning and increased in the late morning to reach maxima near midday; then uptake and concentration again fell to low values. This pattern followed that previously observed for *T. thiebautii* nitrogenase activity. Our results suggest that recently fixed nitrogen is incorporated into glutamine in the N_2-fixing trichomes and may be passed as glutamate to non-N_2-fixing trichomes. The high transport rates and concentrations of glutamate may explain the previously observed absence of appreciable uptake of NH_4^+, NO_3^-, or urea by *Trichodesmium* spp. Immunolocalization, Western blots (immunoblots), and enzymatic assays indicated that glutamine synthetase (GS) was present in all cells during both day and night. GS appeared to be primarily contained in cells of *T. thiebautii* rather than in associated bacteria or cyanobacteria. Double immunolabeling showed that cells with nitrogenase (Fe protein) contained levels of the GS protein that were twofold higher than those in cells with little or no nitrogenase. GS activity and the uptake of glutamine and glutamate dramatically decreased in the presence of the GS inhibitor methionine sulfoximine. Since no glutamate dehydrogenase activity was detected in this species, GS appears to be the primary enzyme responsible for NH_3 incorporation.

Trichodesmium spp., cyanobacteria which occur as colonial aggregates in the oligotrophic open ocean of the tropics and subtropics, are characterized by high rates of N_2 fixation (8, 9). Surprisingly, these organisms have essentially no capacity for the uptake of ammonium, nitrate, or urea at environmental concentrations (9, 14). Thus, it would appear that the colony is almost totally dependent on N_2 fixation for its N requirements.

Recent research with immunogold electron microscopy techniques for localization of nitrogenase in *Trichodesmium thiebautii* colonies revealed that only a fraction of the trichomes in a colony contain nitrogenase. All cells in a diazotrophic trichome appear to contain nitrogenase, and these trichomes are randomly distributed through the colony (4, c.f. reference 20). The N_2-fixing and non-N_2-fixing trichomes are generally considered to be the same species. However, it is also possible that these two trichome types are different species living together. It remains unknown whether each trichome in a colony turns nitrogenase synthesis on and off over time and is essentially self-supporting with regard to N_2 fixation or whether N_2-fixing trichomes supply N to non-N_2-fixing components of the colony.

There are several possible fates for recently fixed NH_4^+. In heterocystous cyanobacteria, fixed N is passed as glutamine from heterocysts to adjacent vegetative cells (27). Heterocysts possess high glutamine synthetase (GS) activity, whereas vegetative cells have high glutamate synthase activity. Moreover, upon differentiation of vegetative cells to heterocysts, GS protein levels increase about twofold (5, 15, 22).

If particular filaments within a *Trichodesmium* colony serve as the source of combined N for other filaments in the colony, N fixed by a N_2-fixing trichome could be released and transferred as ammonium. If this is the case, then N_2-fixing trichomes should have an ammonium incorporation capacity that is less than their ability to fix N_2, thereby permitting release of some of the recently fixed NH_4^+ to the non-N_2-fixing cells. Alternatively, NH_4^+ could be incorporated and then released as amino acids from the N_2-fixing trichomes, which occurs in heterocysts.

To understand whether and how newly fixed N is cycled in the colony, we determined pools and uptake rates of glutamine and glutamate and assessed the activities of possible enzymes associated with primary ammonium incorporation in natural *Trichodesmium* populations. We also determined the specific localization in cells of the ammonium-incorporating enzyme GS and the relationship of GS to nitrogenase.

MATERIALS AND METHODS

General. Research was carried out on two cruises, 9 to 20 April 1990 and 28 January to 17 February 1991, in the northeastern Caribbean Sea and Bahamas on the R/V *Columbus Iselin*. *T. thiebautii* Gomont was collected by towing a 253-μm-mesh, 1-m-diameter net at about a 15-m depth with a ship speed of about 1 knot. The contents of the cod end were diluted with surface seawater, and colonies were then gently picked with plastic inoculation loops and rinsed several times through Whatman GF/F glass-fiber-filtered seawater.

Intracellular glutamine and glutamate. Colonies were

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† Contribution 860 from the Marine Sciences Research Center.

picked (20 for diel 1, 10 for diel 2) from plankton tows by using clean techniques (plastic gloves, acid-washed glassware) and placed in 1.5-ml microcentrifuge tubes containing 1 ml of filtered seawater collected at a 250-m depth. Samples of the same seawater were taken through the extraction process to serve as blanks. The cells were quick frozen in liquid N_2, thawed, sonicated, and then heated at 85°C for 1.5 h. After centrifugation, 100 μl of extract was derivatized with *o*-phthalaldehyde and analyzed by reverse-phase high-performance liquid chromatography with fluorescence detection (18).

Uptake of [^{3}H]glutamate and [^{3}H]glutamine. For uptake assays, 10 colonies of *T. thiebautii* were placed in 40 ml of filtered (0.2-μm-pore-size Nuclepore filter) seawater in a clear polycarbonate centrifuge tube and amended with 2 μCi of ^{3}H-labelled glutamine or glutamate (Amersham). The specific activities of glutamine and glutamate stocks were 56 and 47 nCi pmol^{-1}, respectively. Incubations were performed on deck (natural light, ambient seawater temperature) under three layers of neutral density screening (passing 20% ambient sunlight) or in the dark. Individual samples were harvested at 30 and 60 min. Controls included samples filtered at time zero as well as assays run without *T. thiebautii* to correct for nonbiological sorption of the label. Some samples were also amended with methionine sulfoximine (MSX), an inhibitor of GS (1, 2). To terminate incubations, samples were filtered under vacuum (<5 in. [ca. 12.5 mm] of Hg) onto Millipore filters (0.45-μm pore size, 25-mm diameter), rinsed with 1 ml of filtered seawater, placed in plastic scintillation vials containing 5 ml of Ecoscint scintillation cocktail (ICN), and counted on board in a Packard Tri-Carb liquid scintillation counter.

Enzyme activities. Glutamate dehydrogenase (GDH) activity was determined by measuring NADH oxidation (10, 11). Colonies of *T. thiebautii* were placed in 5 ml of 0.1 M Tris-HCl buffer (pH 7.5) with 0.1 ml of 3.0 M NH_4Cl, and the cells were disrupted by sonication. Next, 1.4-ml samples of the cell suspension were centrifuged in a microcentrifuge for 2 min, and the supernatants were placed in 1-cm quartz cuvettes. The reaction was started by adding 0.1 ml of 2-oxoglutarate, and the decrease in A_{340} was read every 10 s for 3 to 4 min. The difference in rate of NADH oxidation in the presence of added NH_4Cl was taken as the GDH activity.

GS activity was measured with the transferase hydroxamate assay (17). For this analysis, 100 *T. thiebautii* colonies were disrupted by sonication and a fraction of the suspension was withdrawn for analysis of protein content (7). The remaining content of each aliquot was further split into fractions for analysis with the transferase assay. Incubations with reagents were for 30 min at 30°C. The effect of MSX was also examined in GS assays.

Immunogold localization and Western blotting (immunoblotting) for GS. Freshly collected *T. thiebautii* colonies were immediately transferred to sterile filtered seawater in microcentrifuge tubes (25 colonies in each). The seawater was carefully removed, and the tubes were placed in liquid N_2 before being freeze-dried. The lyophilized cells were redissolved in sample buffer, boiled, electrophoresed, and immunoblotted (24).

For immunogold labelling, colonies were embedded as described previously (4, 23) with the hydrophilic resin LR White. Ultrathin cross sections from the central areas of the *T. thiebautii* colonies were subjected to anti-GS antiserum (1:100 dilution) and goat anti-rabbit immunoglobulin G conjugated to 10-nm gold spheres (Janssen Pharmaceutica, Oelen, Belgium) (23). The primary antiserum used was raised in rabbits against the GS of *Anabaena* sp. strain PCC 7120 (kindly provided by R. Haselkorn, University of Chicago, Chicago, Ill.). Double labelling in cells for GS and nitrogenase was performed with the same rabbit anti-GS antibody (diluted 1:150) and mouse anti-nitrogenase antibody (diluted 1:50; kindly provided by J. Zehr) with different-sized gold particles (10 and 5 nm, respectively) for each secondary antibody (25, 26). Sections were viewed in a Phillips CM10 transmission electron microscope working at 60 kV. When the primary antiserum was omitted, no label was detected.

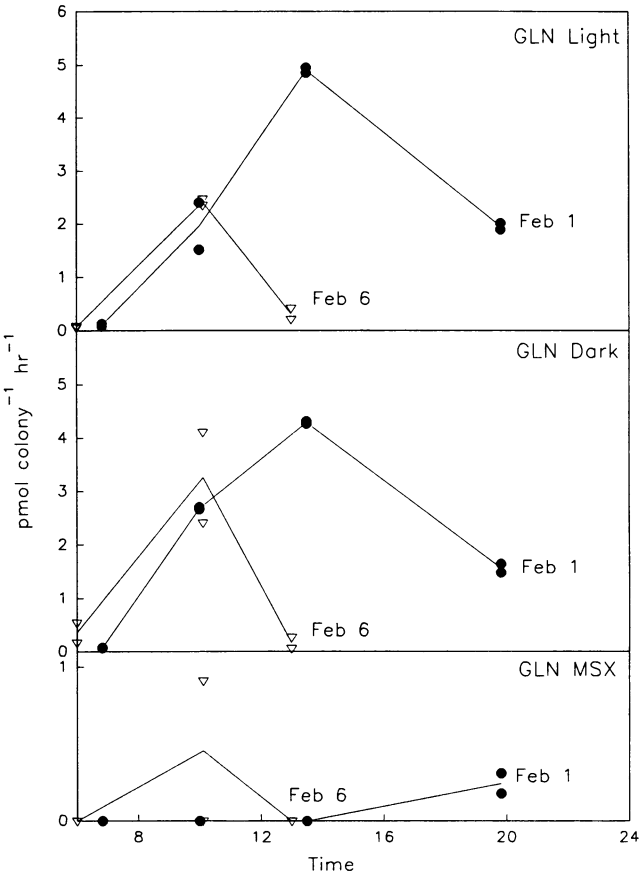

FIG. 1. Rates of glutamine uptake in light (GLN Light) and darkness (GLN Dark) and in the presence of MSX (GLN MSX) in *T. thiebautii* in the Caribbean Sea, February 1989.

RESULTS

Uptake of glutamine and glutamate. Rates of uptake of both glutamine and glutamate changed over the course of the day (Fig. 1 and 2). In the early morning (ca. 0600 to 0630 h), before nitrogenase activity was present (8), uptake of both amino acids in the light was relatively low: ambient rates averaged 0.10 and 0.07 pmol colony^{-1} h^{-1} for glutamine (Fig. 1) and 1.8 and 5.3 pmol colony^{-1} h^{-1} for glutamate (Fig. 2) on the sample dates of 1 and 6 February, respectively. Later in the morning, when nitrogenase activity is typically high (8), uptake rates increased to about 2.5 to 5 pmol of glutamine colony^{-1} h^{-1} and about 25 pmol of glutamate colony^{-1} h^{-1}. Rates of uptake of both amino acids in *T. thiebautii* collected after midday or late in the afternoon

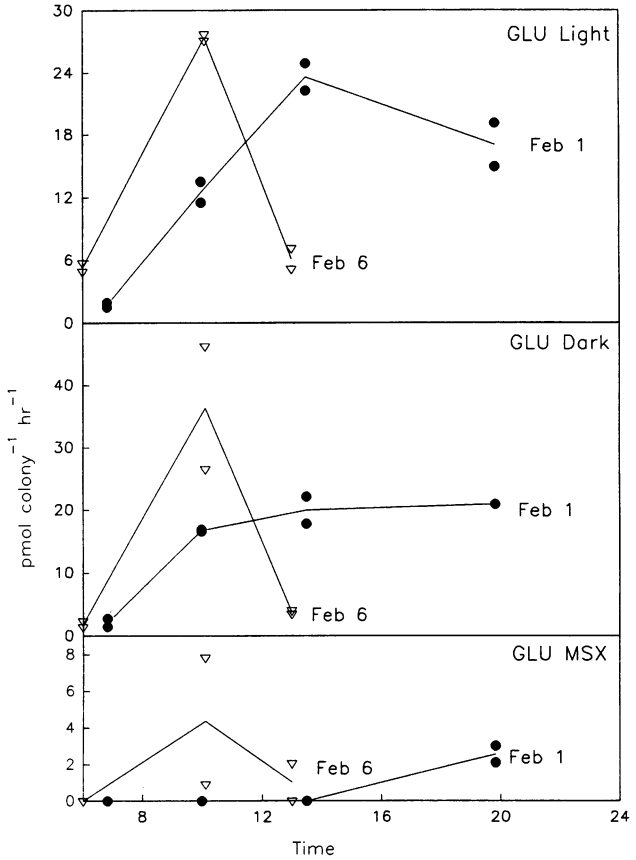

FIG. 2. Rates of glutamate uptake in light (GLU Light) and darkness (GLU Dark) and in the presence of MSX (GLU MSX) in *T. thiebautii* in the Caribbean Sea, February 1989.

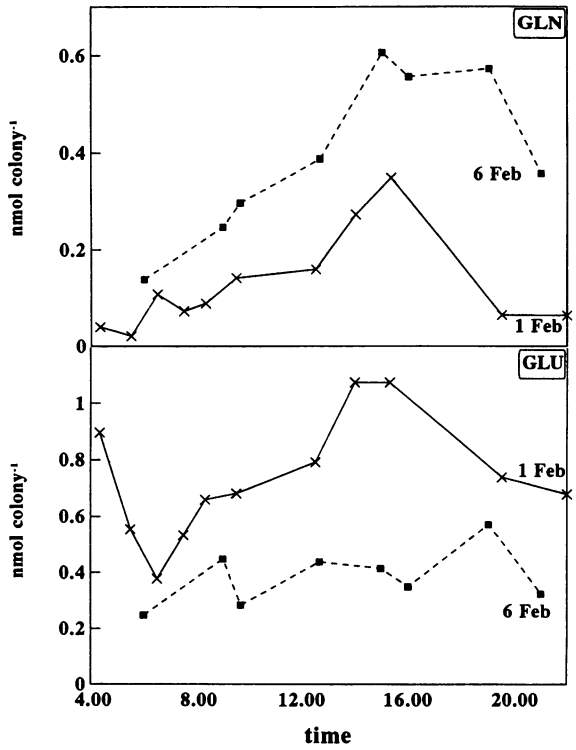

FIG. 3. Concentration of glutamine (GLN) and glutamate (GLU) in colonies of *T. thiebautii* through a diel cycle in the Caribbean Sea, February 1989.

decreased in relation to those of the midmorning samples. Rates in samples incubated in the dark were similar to those in samples incubated in the light (Fig. 1 and 2).

The maximum uptake rates differed on the two dates, although the overall pattern was similar (Fig. 1 and 2). On 6 February, the maximum occurred at 1000 h with a marked drop-off to an almost negligible rate at 1300 h. In contrast, the maximum on 1 February occurred at 1330 h and then decreased to less than half that value at 2000 h. With the limited number of samples taken, it is possible that the true maximum was missed on each date.

The changes in mass uptake through the day were not due to changes in the external substrate concentrations. Ambient concentrations of glutamate and glutamine did not show any systematic variation in the sample bottles during an experiment or through the day. On 1 February, the average glutamine and glutamate concentrations were 10.5 (standard deviation [SD] = 2.1) and 107.8 (SD = 27.9) nmol, respectively; and on 6 February, the averages were 8.6 (SD = 2.6) and 150 (SD = 21.6) nmol, respectively.

Internal concentrations of free glutamine and glutamate were determined in parallel with uptake studies (Fig. 3). For glutamine, a diel fluctuation was evident on both dates. On 1 February, glutamine concentrations increased from less than 0.1 nmol colony^{-1} in the early morning to about 0.4 nmol colony^{-1} at 1600 h, falling off thereafter. A similar trend but with higher overall concentrations was noted on 6 February. A similar diel pattern for glutamate was noted on 1 February, but diel variation was minimal on 6 February. Concentrations of glutamate exceeded those of glutamine by about fivefold on 1 February, in contrast to the results of 6 February, when the concentration difference between these two pools was less pronounced. Subsequent work indicates that the values of 1 February, including the diel trend in glutamate (7a), are more representative.

An increase in the content of glutamine through the morning to midday in colonies of *Trichodesmium erythraeum* was also observed (Fig. 4). The glutamate content was relatively low in midmorning, about 0.25 nmol colony^{-1}, but it increased to over 1.0 nmol colony^{-1} by late afternoon.

GS activity. Freshly collected *T. thiebautii* colonies had high GS activity. However, there were no apparent diel changes in GS activity during the two cycles studied (Table 1). On 12 and 13 April, the GS activities ranged from 1.1 to 1.8 nmol of glutamyl hydroxamate formed (μg of protein)$^{-1}$ min^{-1}. These differences appear to be simple random variation, since the minimum and maximum rates both occurred in late evening on successive days (Table 1). On 15 and 16 April, the GS activities were similar, ranging from 1.3 to 1.7 nmol of glutamyl hydroxamate formed (μg of protein)$^{-1}$ min^{-1}, again showing little difference during the day.

GDH activity. Twenty-five assays of GDH activity were performed in parallel with GS measurements (data not shown). No NADH-dependent GDH activity was detected in crude extracts of freshly collected *T. thiebautii* colonies collected during the day or night.

Effects of MSX. Exposure of *T. thiebautii* colonies to MSX resulted in a marked reduction in GS activity. Pretreatment of *T. thiebautii* in vivo with 100 μmol of MSX for 15 min

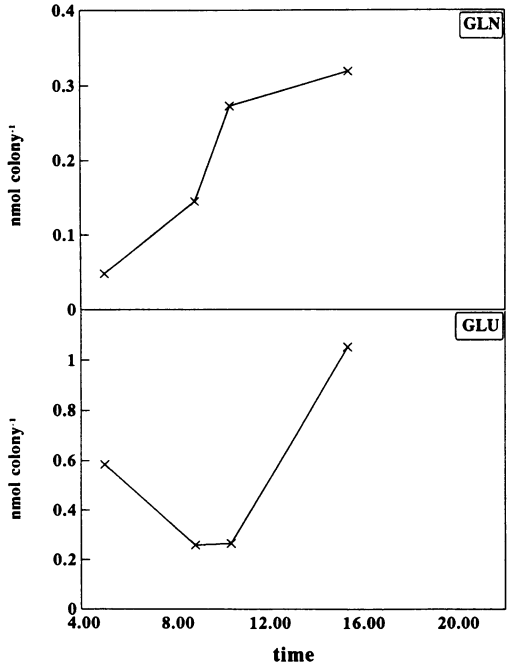

FIG. 4. Concentration of glutamine (GLN) and glutamate (GLU) in colonies of *T. erythraeum* from early morning to midafternoon through a diel cycle in the Caribbean Sea, February 1989. Samples were incubated in natural sunlight with screening to remove 60% ambient light in an incubator cooled with surface-flowing seawater.

reduced the GS activity to 59% of the control value (control mean, 1.30 nmol of GS activity [μg of protein]$^{-1}$ min^{-1}; s^2 = 0.005; n = 4), whereas exposure of sonicated samples in vitro to 1 and 10 mmol of MSX reduced the GS activities to 37% (s^2 = 0.097) and 15% (s^2 = 0.001), respectively, of the control value. The addition of MSX also resulted in a dramatic and almost complete inhibition of uptake of both amino acids (Fig. 1 and 2).

GS protein levels. Immunoblotting with an anti-*Anabaena* sp. strain PCC 7120 GS antiserum revealed one polypeptide of about 50 kDa in *T. thiebautii* colonies. Approximately 12.5 μg of protein was loaded for each lane. The concentration of GS, as evidenced by Western blotting, appeared to be similar from the early morning (0655 h) to late in the day (1800 h) (Fig. 5). The GS content of the 2400-h sample appeared lower than those collected earlier, but GS was clearly present. To locate the GS assay activities detected within the colonies, GS immunogold labelling was used.

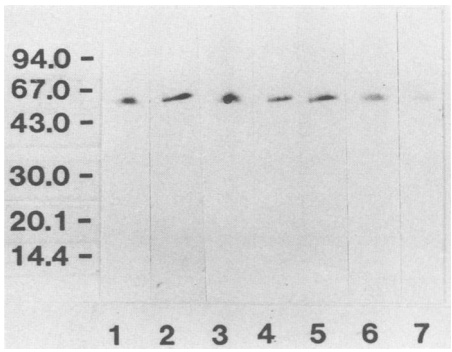

FIG. 5. Immunoblot of GS in *T. thiebautii* colonies. Molecular masses (kilodaltons) are shown to the left. Lanes: 1, 0655 h; 2, 0733 h; 3, 0915 h; 4, 1200 h; 5, 1500 h; 6, 1800 h; 7, 2400 h.

GS was present in all *T. thiebautii* trichomes examined with in situ immunocytochemistry-transmission electron microscopy (Fig. 6 to 8). The enzyme was present throughout the cell in regions of medium electron transparency that represent the cytoplasm. GS was present during the day and night (Fig. 6 and 7), although the label density at night was slightly reduced. Cells collected during the day averaged 34 (SD = 1.6) gold spheres μm^{-2}, and those at night averaged 30 (SD = 1.7) gold spheres μm^{-2}. Double-immunolabeling experiments were performed to examine whether there was any difference between GS levels in nitrogenase-containing cells and GS levels in cells with little or no nitrogenase (Fig. 8). Cells collected at 1200 h, with a high level of nitrogenase, had an average of 52 (SD = 2) spheres that localized GS μm^{-2}, whereas adjacent cells (trichomes) with low nitrogenase labels had a mean of 24 (SD = 1.5) GS-localizing spheres μm^{-2}, i.e., only 46% of the GS of the nitrogenase-containing trichomes. The GS protein label was seen in the 0.5-μm-wide filamentous cyanobacterium in the LPP group that is associated with *Trichodesmium* spp. (23). However, the biomass of the 0.5-μm cyanobacterium was 1,000-fold less than that of *Trichodesmium* spp., and it would be expected that the small cyanobacteria would have only a minor contribution to the total GS activity of a colony.

DISCUSSION

We report for the first time on the occurrence and activity of GS protein in freshly collected colonies of the marine cyanobacterium *T. thiebautii* during the day and night. On the basis of our results, including the effect of MSX on glutamate and glutamine uptake and the absence of GDH

TABLE 1. GS activity in *T. thiebautii* colonies on 12, 13, 15, and 16 April 1990[a]

Date (April)	Time (h)	GS activity (nmol μg^{-1} min^{-1})	Date (April)	Time (h)	GS activity (nmol μg^{-1} min^{-1})
12	1200	1.32 (0.04)	15	1615	1.52 (0.01)
12	2000	1.15 (0.01)	15	1900	1.67 (0.02)
12	2300	1.11 (0.01)	15	2330	1.41 (0.02)
13	0230	1.34 (0.01)	16	0230	1.46 (0.02)
13	0530	1.57 (0.02)	16	0515	1.51 (0.01)
13	0830	1.39 (0.01)	16	0800	1.29 (0.01)
13	1500	1.44 (0.01)	16	1200	1.35 (0.01)
13	2000	1.84 (0.01)			

[a] Shown are means and variances (within parentheses) of eight replicate measurements at each time point. Rates are nanomoles of glutamyl hydroxamate formed per microgram of protein per minute.

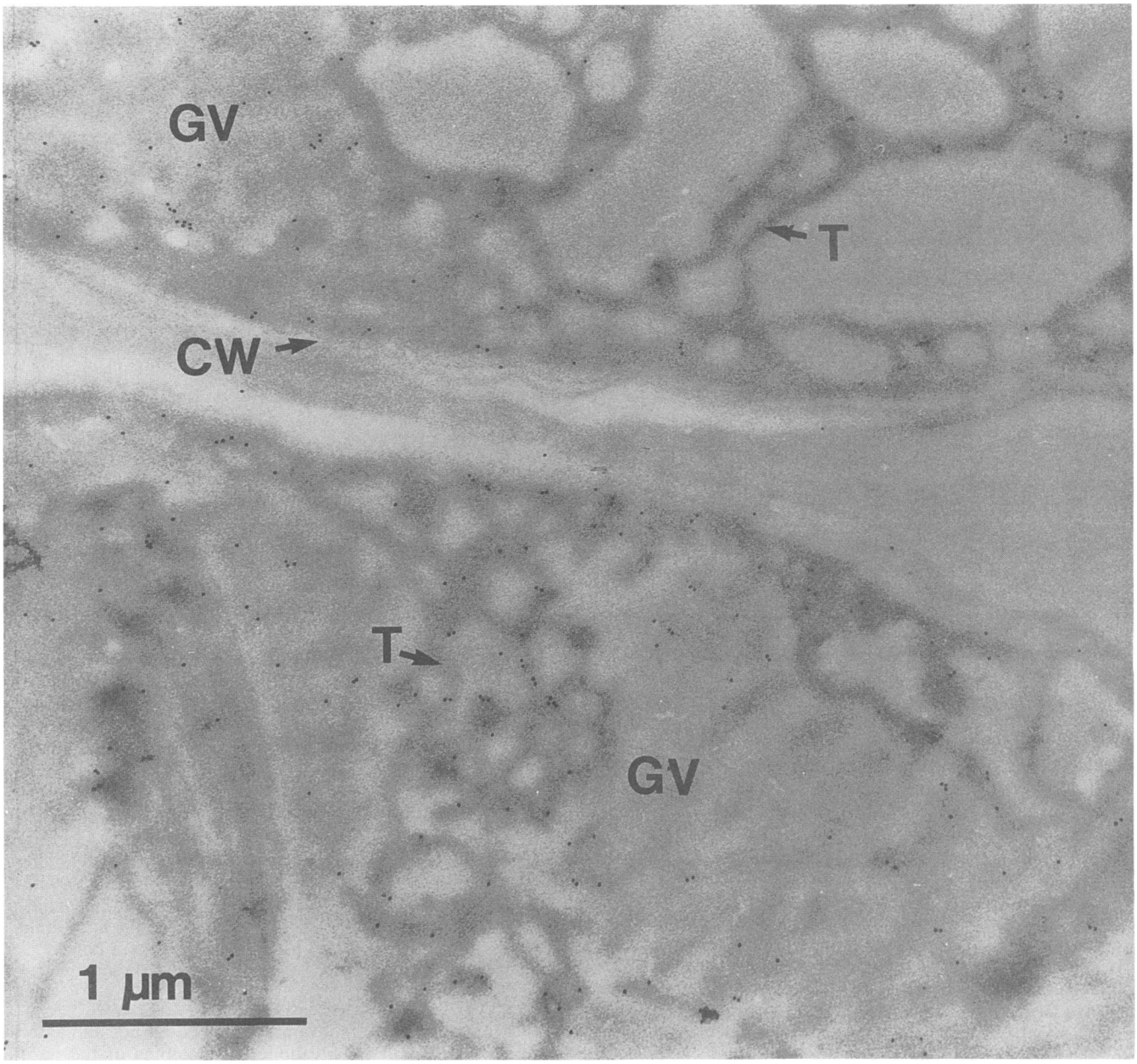

FIG. 6. Immunogold localization of GS in a *T. thiebautii* colony collected during the day. Note the distribution of the enzymes in cytoplasmic areas of the cells. CW, cell wall; GV, gas vesicle; T, thylakoid.

activities, we conclude that GS is the primary enzyme responsible for incorporation of ammonium in this organism.

In contrast to our observation that the nitrogenase protein occurred only in a subset of the trichomes of a colony (5), GS was found in cytoplasmic areas of all *T. thiebautii* cells examined. GS levels were about twofold higher in cells containing nitrogenase, suggesting coregulation of GS and nitrogenase induction in *Trichodesmium* spp. like the coregulation that appears to occur in *Plectonema boryanum* PCC 73110 (21). In heterocystous cyanobacteria, GS protein levels are substantially higher in heterocysts than in vegetative cells (5, 17, 22), indicating a heterocystlike character of the nitrogenase-containing *Trichodesmium* trichomes. However, we have previously demonstrated that, unlike heterocysts, nitrogenase-containing trichomes of *T. thiebautii* do not have a thickening of the cell wall and contain photosynthetic proteins such as phycoerythrin and phycocyanin as well as ribulose 1,5-bisphosphate carboxylase:oxygenase in similar quantities in cells with or without nitrogenase (24).

The uptake of glutamine and glutamate was time specific; the maxima occurred either in the midmorning or at midday, coinciding with the maxima in nitrogenase activity. More recent and detailed analysis of *T. thiebautii* colonies (7a) indicates that glutamate composes about 44% of the intracolonial free amino acid pool whereas glutamine composes less than 5%. The very high intracolonial concentration of glutamate, coupled with observations that GS was present in higher quantities in N_2-fixing cells and that the overall

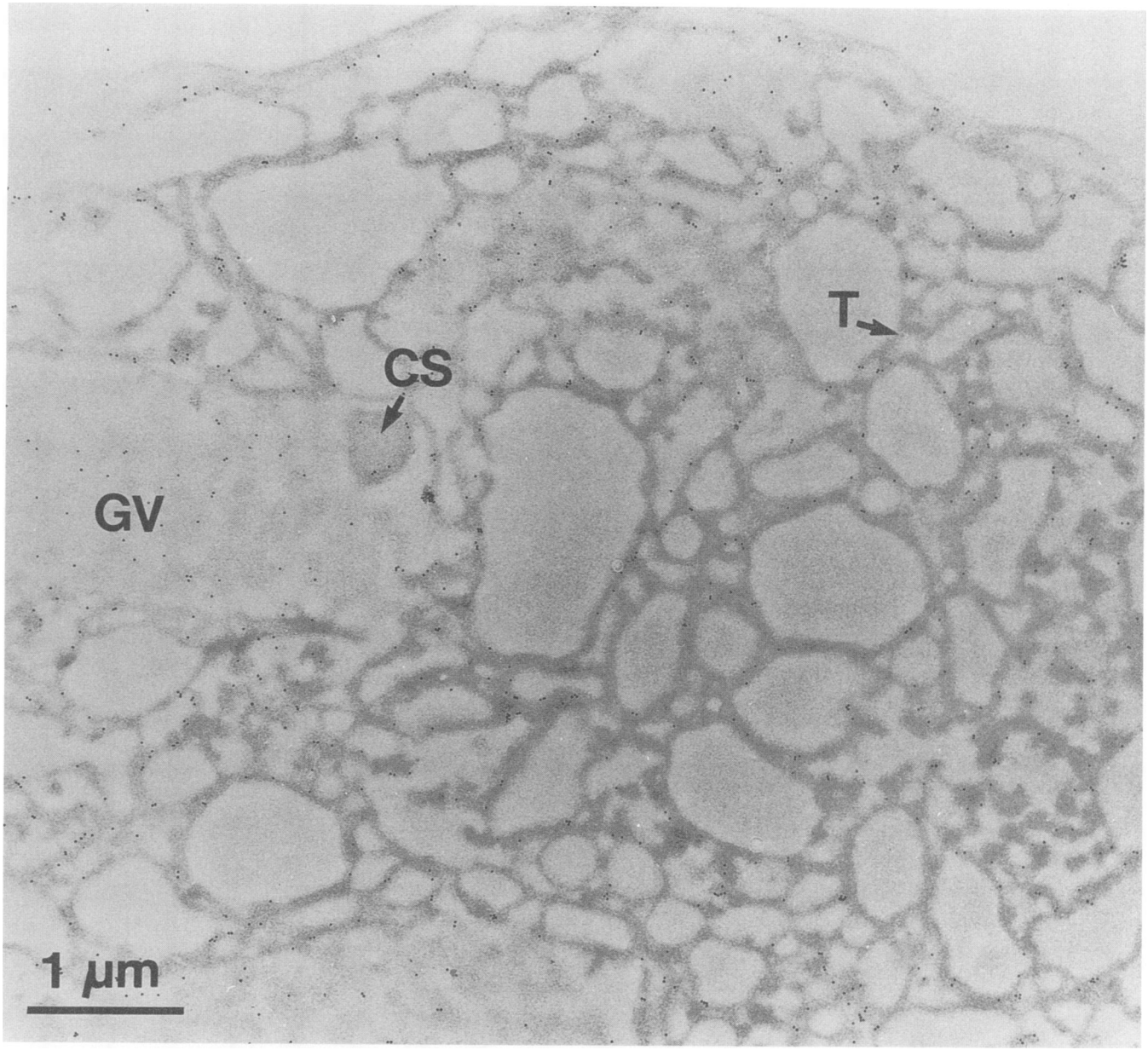

FIG. 7. Immunogold localization of GS in a *T. thiebautii* colony collected at night. GV, gas vesicle; T, thylakoid; CS, carboxysome.

colonial glutamate uptake rate greatly exceeded that for glutamine (Fig. 1 and 2), suggests that, if there is a net exchange of N between N_2-fixing and non-N_2-fixing trichomes, glutamate may be the major N transfer molecule. We did not examine glutamate synthase in our studies; if glutamate is indeed the major N-transfer molecule, glutamate synthase might also be expected to occur with elevated levels and activities in N_2-fixing trichomes. This would be in contrast to the situation in heterocystous cyanobacteria (5, 15, 22).

GS activity per colony and immunogold localization indicate a relatively constant level of the enzyme on a diel basis. The large diel changes in the uptake rates of glutamine and glutamate must be explained in light of these findings. Intracolonial glutamine and glutamate increase in concentration during the day, since they are synthesized after NH_3 is formed by nitrogenase. Increases in the flux of glutamate from N_2-fixing cells would increase the concentration of glutamate in the interfilamental spaces and, in turn, possibly stimulate mass transport and assimilation of glutamate by non-N_2-fixing trichomes and/or to the associated microflora. Indeed, the minimal variation in ambient (external) concentrations of glutamate and glutamine during incubations (Table 1), despite internal pool size increases, may reflect highly efficient internal cycling. If an exogenously supplied tracer does undergo substantial isotope dilution in the internal spaces of the colony, uptake rates (based on external concentrations) would be underestimated. In this regard, it would be instructive to examine the concentration dependence of glutamate uptake by individual filaments to determine whether K_Ss are more reflective of ambient environmental (nanomolar) or internal (micromolar) concentrations.

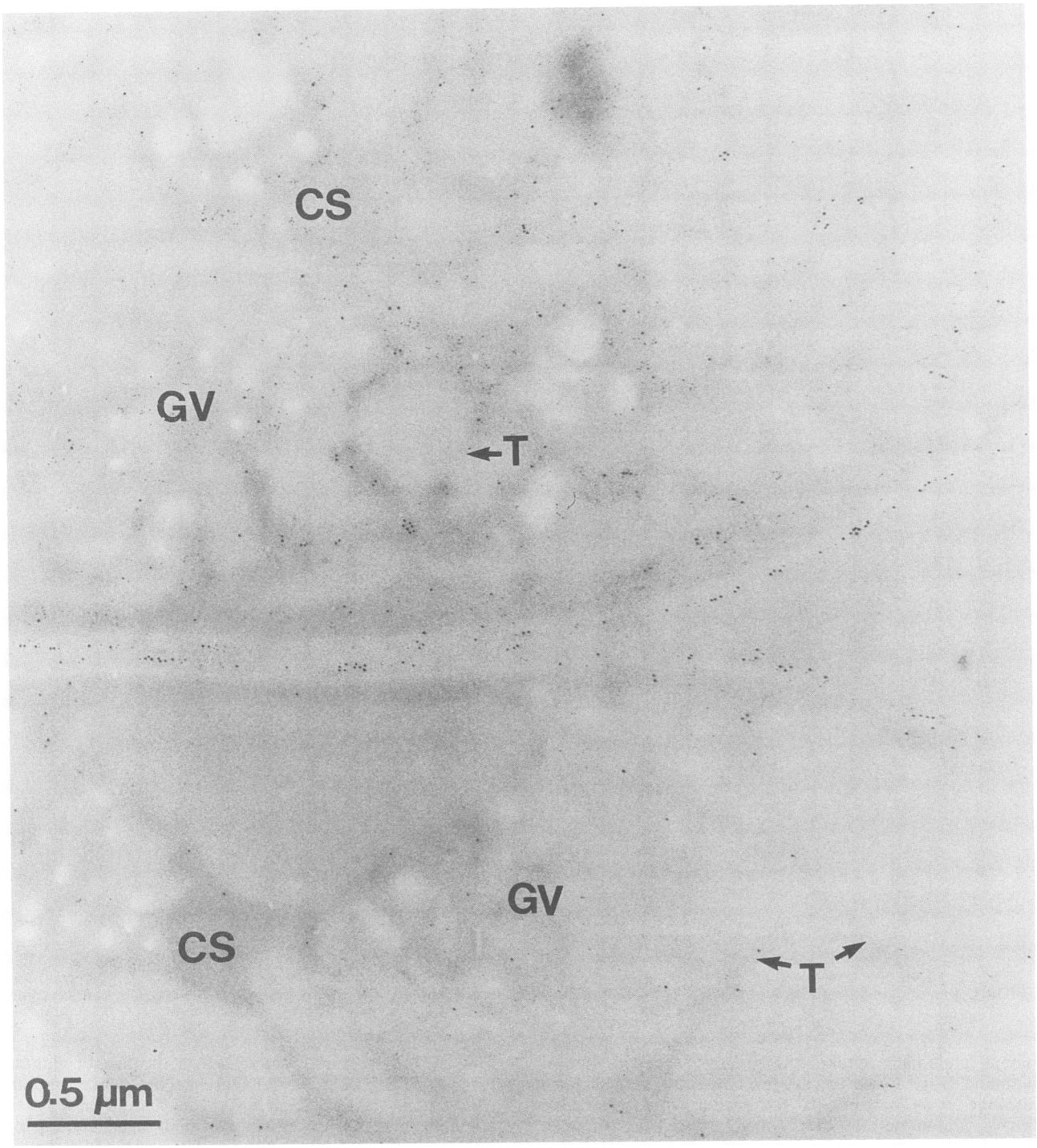

FIG. 8. Immunogold localization of GS (10-nm gold spheres) and nitrogenase (5-nm gold spheres) in a *T. thiebautii* colony. Abbreviations are defined in the legends to Fig. 6 and 7. Note that the cell on top contains high concentrations of both nitrogenase and GS and the cell on the bottom has low concentrations of both proteins.

The uptake by *Trichodesmium* spp. of NH_4^+, NO_3^-, and urea at ambient oceanic concentrations is virtually undetectable (8, 14, 19). This is paradoxical because these N compounds (in particular, NH_4^+) have been considered to be preferred over energetically more costly (e.g., N_2 or NO_3^-) or other more complex N compounds (3). If the intracolonial concentrations of amino acids are high, this may help to partially explain the enigmatic lack of uptake capacity for these three commonly used N sources. In the diatom *Phaeodactylum tricornutum*, arginine severely inhibits the uptake of NH_4^+ (13). Other amino acids inhibit the uptake of NO_3^- in diatoms (6) and of urea in chlorophytes (16). A similar

mechanism may explain the non-use of NH_4^+ in *Trichodesmium* spp.

Since colonies have virtually no ability to take up NH_4^+, NO_3^-, and urea, it is also unlikely that they could be major N transfer molecules within the colony. Furthermore, the twofold-higher level of GS in N_2-fixing cells reinforces the proposition that ammonium is an unlikely transfer solute.

The substantial diel changes in glutamine and glutamate uptake and content in colonies suggest that *Trichodesmium* spp. may go through major changes in N sufficiency through the day. The variations in amino acid pools may play a direct role in the daily cycle of nitrogenase synthesis (4, 8). These changes and observed changes in ultrastructure and protein synthesis (4, 8) indicate that this organism is highly dynamic with regard to its diel cycle.

In summary, GS appears to be the major enzyme responsible for the assimilation of fixed N in *T. thiebautii*. The higher concentrations of GS in N_2-fixing cells suggest that synthesis of GS protein may be coregulated with nitrogenase. Nitrogen fixed in N_2-fixing trichomes may be transferred, possibly as glutamate, to non-N_2-fixing trichomes and associated organisms. The high concentrations of glutamate in colonies may explain the previously observed inhibition of uptake of NH_4^+, NO_3^-, and urea in *Trichodesmium* spp.

ACKNOWLEDGMENTS

We thank Judith O'Neil, Michael Doall, Maryann Maertz, and Mara Diaz for help with assays while at sea and the captain and crew of the R.V. *Columbus Iselin* for all their efforts.

This research was supported by National Science Foundation grants OCE 90-15606 and OCE 88-16584 to E.J.C. and OCE 90-12199 to D.G.C., by the Bank of Sweden Tercentenery Foundation and Teyggers Foundation grant to B.B., and by a grant from the Government of Pakistan to P.J.A.S.

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