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Isolation and Characterization of Glutamine Synthetase from the Marine Diatom *Skeletonema costatum*[1]

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Two peaks of glutamine synthetase (GS) activity were resolved by anion-exchange chromatography from the marine diatom *Skeletonema costatum* Grev. The second peak of activity accounted for greater than 93% of total enzyme activity, and this isoform was purified over 200-fold. Results from denaturing gel electrophoresis and gel-filtration chromatography suggest that six 70-kD subunits constitute the 400-kD native enzyme. The structure of the diatom GS, therefore, appears more similar to that of a type found in bacteria than to the type common among other eukaryotes. Apparent Michaelis constant values were 0.7 mM for NH_4^+, 5.7 mM for glutamic acid, and 0.5 mM for ATP. Enzyme activity was inhibited by serine, alanine, glycine, phosphinothricin, and methionine sulfoximine. Polyclonal antiserum raised against the purified enzyme localized a single polypeptide on western blots of *S. costatum* cell lysates and recognized the denatured, native enzyme. Western analysis of the two peak fractions derived from anion-exchange chromatography demonstrated that the 70-kD protein was present only in the later-eluting peak of enzyme activity. This form of GS does not appear to be unique to *S. costatum*, since the antiserum recognized a similar-sized protein in cell lysates of other chromophytic algae.

Nitrate and ammonium are the major forms of inorganic N that support primary production in marine ecosystems. Nitrate reductase, which catalyzes the first enzymatic step in nitrate assimilation, has been characterized from several species of marine phytoplankton (Amy and Garrett, 1974; Gao et al., 1993; Ramalho et al., 1995); however, less is known about other enzymes involved with N assimilation in these highly productive, photosynthetic organisms. GS (EC 6.3.1.2) catalyzes the formation of Gln from glutamate and ammonium in the presence of ATP. Many organisms modulate GS activity in response to changes in the cellular C:N ratio or through feedback regulation by amino acids (Orr and Haselkorn, 1982; Mitchell and Magasanik, 1983; Magasanik, 1988). In addition, the assimilation of ammonium into Gln appears to regulate nitrate reductase activity in several species (Reyes and Florencio, 1994a; Sivasankar and Oaks, 1995). Thus, GS is central to both nitrate and ammonium assimilation and is the enzymatic link between C and N metabolism.

Diatoms (Bacillariophyceae) are an ideal taxonomic group in which to examine the biochemical characteristics of GS. From an ecological perspective, diatoms are globally distributed, they are a dominant component of phytoplankton blooms, and they contribute significantly to global C cycles (Treguer et al., 1995). From an evolutionary perspective, morphological studies and molecular analysis of several nuclear- and chloroplast-encoded genes suggest that chromophytes (diatoms and other algae containing chlorophyll *c*) represent an evolutionary lineage that is distinct from that of green algae and vascular plants (Whatley, 1983; Bhattacharya and Medlin, 1995; Reith, 1995). Therefore, studies of the biochemical and genetic characteristics of GS from diatoms will provide not only new insights into our understanding of the regulation of marine primary productivity, but also will provide information concerning the molecular evolution of GS.

Three types of GS have been described based on the molecular size of and the number of subunits in the holoenzyme. Two of the types, GSI and GSIII, are found uniquely in prokaryotic species. GSI is a dodecameric protein with subunits ranging between 44 and 60 kD, and is common among several groups of bacteria (Brown et al., 1994). Recently, GSIII has been described from cyanobacteria and two anaerobic bacteria as a hexameric enzyme composed of 75-kD subunits (Southern et al., 1986, 1987; Goodman and Woods, 1993; Reyes and Florencio, 1994b). In contrast, GSII is found typically in eukaryotes as an octameric enzyme composed of subunits ranging between 35 and 50 kD (Forde and Cullimore, 1989). Several species of soil bacteria also express an octameric form of GS (Darrow and Knotts, 1977; Carlson and Chelm, 1986; Kumada et al., 1993).

Biochemical and molecular studies have revealed that multiple isoforms of GSII are expressed in the cytosol and chloroplast of several species of vascular plants and green algae, whereas other species express a single form (McNally et al., 1983; Casselton et al., 1986; Forde and Culli-

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Abbreviations: EB, extraction buffer; GS, Gln synthetase; GS_1, isoform eluting early from DEAE column; GS_2, isoform eluting later from DEAE column; MSX, L-Met-D,L-sulfoximine; PPT, phosphinothricin.

more, 1989; Garcia-Fernandez et al., 1994). Analysis of mutants and of transgenic plants have demonstrated that in vascular plants, GS isoforms have unique roles in cellular N metabolism (Wallsgrove et al., 1987; Edwards et al., 1990). The expression of GS isoforms in green algae varies with physiological conditions, although the precise metabolic roles of the isoforms in unicellular algae have not been determined (Florencio and Vega, 1983; Sumar et al., 1984; Garcia-Fernandez et al., 1994).

Even less is known about GS from nongreen algae. Casselton et al. (1986) determined by chromatographic methods that several species of marine algae express multiple GS isoforms and that other species apparently express a single form. To date, however, GS has not been purified from a nongreen algal species, and the subunit size, composition of the holoenzyme, and kinetic parameters are unknown. The objectives of this study were to determine the number of GS isoforms expressed in the marine diatom *Skeletonema costatum*, to purify the most abundant form of GS, and to examine the physical and biochemical properties of the enzyme. We also examined the immunological relatedness of GS among several algal taxa.

MATERIALS AND METHODS

Culture Conditions

Skeletonema costatum Grev. (CCMP 1332) was obtained from the Provosoli-Guillard Culture Center for Marine Phytoplankton at the Bigelow Laboratory for Ocean Sciences (Boothbay Harbor, ME). Stock cultures were maintained axenically in f/2 medium (Guillard, 1975; Cottrell and Suttle, 1993). For protein purification, cells were grown in 20-L containers (Nalgene, Rochester, NY) on f/2 nutrients with either $NaNO_3$ or NH_4Cl as the N source. $NaNO_3$ was added either daily to a final concentration of 60 μM or at the beginning of the incubation to a final concentration of 880 μM. NH_4Cl was added daily to a final concentration of either 60 or 120 μM. The irradiance was 150 μmol quanta m^{-2} s^{-1} (PAR) and was provided by very-high-output and cool-white lights. Cells were grown under a light:dark cycle of 12:12 h at 14:11°C. Cells (approximately 10^5 cells mL^{-1}) were concentrated by filtration using 3MM paper (Whatman) and then pelleted by centrifugation at 2000*g* for 15 min at 15°C. Cell pellets were frozen in liquid N_2 and stored at −80°C until used.

Protein Isolation

Cells (10^8 cells mL^{-1}) were thawed on ice in EB (50 mM imidazole [pH 7.3], 4 mM 2-mercaptoethanol, 1 mM glutathione, 6 mM DTT, 1 mM EDTA, 10 mM $MgSO_4$, 5 mM Glu, 10 mM $MnCl_2$, 10% [v/v] glycerol, 1.0 μg mL^{-1} leupeptin, and 0.005 μM Pefabloc [Boehringer Mannheim]). Cells were disrupted by two passes through a French pressure cell at approximately 12,000 psi, and cellular debris was pelleted by centrifugation at 20,000*g* for 10 min at 4°C. Solid $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 40% saturation, precipitated proteins were removed by centrifugation, and the supernatant was adjusted to 70% $(NH_4)_2SO_4$ saturation (Englard and Seifter, 1990). Precipitated proteins were resuspended in EB to a final concentration of approximately 30 mg protein mL^{-1}.

The protein extract was desalted by gel-filtration chromatography using a 1 × 100 cm column (Sephacryl S-300 HR, Pharmacia) that had been equilibrated with EB at 11°C. The flow rate was 0.3 mL min^{-1}, and 1-mL fractions were collected. For all chromatographic procedures, GS activity was monitored using the transferase assay (described below), and protein concentration was determined colorimetrically with BSA as the standard (Bradford, 1976).

DEAE Chromatography

Fractions from the gel-filtration column with greater than 0.01 unit of activity were pooled and loaded onto a previously equilibrated, 1 × 15 cm DEAE column (Fast Flow Sepharose, Pharmacia) at 11°C. Proteins were eluted with either a 200-mL linear gradient of KCl (0.0–0.6 M) or a 200-mL two-step linear gradient (0.0–0.22 M followed by 0.22–0.40 M KCl) at a flow rate of 5 mL min^{-1}, and 2-mL fractions were collected.

Analytical Gel-Filtration Chromatography

The M_r of the native form of GS was estimated using gel filtration. The peak fraction (1 mL) from the DEAE column was loaded by gravity onto a 1 × 100 cm column (Sephacryl S-300 HR, Pharmacia). Proteins were eluted with EB at a flow rate of 0.30 mL min^{-1} and collected in 1-mL fractions. The void volume was determined using blue dextran (molecular weight 2,000,000), and total column volume was measured with acetone. The column was calibrated using thyroglobulin (molecular weight 669,000), apoferritin (molecular weight 443,000), β-amylase (molecular weight 200,000), and alcohol dehydrogenase (molecular weight 150,000) as standards. The partition coefficient was calculated as described by Preneta (1989). There was no significant difference between the elution volume of GS_2 purified from NH_4^+-grown cells (n = 4) and that purified from NO_3^--grown cells (n = 3; Student's t test, $t_{df=5}$ = −0.88, P = 0.42), so the apparent molecular weight of GS_2 was estimated from the mean and SD of the seven elution volumes.

Enzyme Activity

GS transferase activity was measured as described by Bressler and Ahmed (1984). One unit of GS transferase activity catalyzed the formation of 1 μmol of product min^{-1} at 25°C. GS biosynthetic activity was used to estimate kinetic parameters (Shapiro and Stadtman, 1970). One unit of biosynthetic activity catalyzed the oxidation of 1 μmol of NADH min^{-1} at 22°C. Peak fractions from the anion-exchange chromatography of NO_3^--grown cells were used to determine apparent Michaelis-Menten parameters for all substrates. The apparent K_m for NH_4^+ also was determined using a cell-free extract from NO_3^--grown cells. The cell-free extract was obtained by sonicating cells, removing cellular debris by centrifugation at 10,000*g* for 10 min at 4°C, and further centrifuging the supernatant at 43,000*g* for 15 min at 4°C. The apparent K_m values, maxi-

mum velocities, and SE values were estimated using the Marquardt-Levenberg curve-fitting algorithm (SigmaPlot, Jandel Scientific, Madera, CA).

Inhibition of GS biosynthetic activity was measured by adding amino acids and PPT to the reaction mixture at final concentrations of 10 mM and 5 μM, respectively. Values reported represent the average of duplicate reactions, which varied by less than 10%. MSX inhibition was measured in duplicate after 15 min using the transferase assay.

PAGE Analysis and Western Blotting

SDS-PAGE, protein visualization, and western blot analysis were conducted using standard techniques (Laemmli, 1970; Dunbar, 1987; Gao et al., 1993). Peak fractions from the gel filtration were analyzed by native gel electrophoresis (Caizzi et al., 1990). GS transferase activity was detected as dark red bands after incubating gels in the assay buffer for 30 min at 37°C. Gels were stained subsequently with Coomassie brilliant blue R-250. The protein band from an adjacent lane, which corresponded with enzyme activity, was excised and analyzed by SDS-PAGE. For western blot analysis, the native gel was incubated in 0.1% (w/v) SDS for 10 min at room temperature before transferring proteins to nitrocellulose.

Algal species used for immunological comparisons were grown in f/2 medium (Guillard, 1975), and cell-free lysates were obtained by sonication and centrifugation at 20,000*g* for 10 min. Proteins in the supernatant were concentrated by the addition of ice-cold acetone to 80% (v/v), and precipitated proteins were resuspended in EB so that equivalent enzyme activity (10^{-4} units) from each species was analyzed. Algal clones examined were: *Thalassiosira pseudonana* (Bacillariophyceae, CCMP 1335), *Phaeodactylum tricornutum* (Bacillariophyceae, CCMP 1327), *Pelagomonas calceolata* (Pelagophyceae, CCMP 1214), an unclassified pelagophyte that forms "brown tides" (DeYoe et al., 1995), *Coccolithus pelagicus* (Prymnesiophyceae, CCMP 229), and *Dunaliella tertiolecta* (Chlorophyceae, CCMP 1320).

Preparation of Antiserum

Peak fractions from the anion-exchange column were separated by 10% SDS-PAGE, and the 70-kD polypeptide, which correlated with GS activity, was excised from the gels and lyophilized. A total of 250 μg of gel-purified protein was used to immunize one chicken (Cocalico Biologicals, Reamstown, PA). IgG molecules were purified from egg yolk using PEG precipitation (Gassman et al., 1990).

RESULTS AND DISCUSSION

Purification and Biochemical Characterization

Anion-exchange chromatography has been used in a number of studies to separate GS isoforms (Florencio and Vega, 1983; McNally et al., 1983; Sumar et al., 1984; Beudeker and Tabita, 1985; Casselton et al., 1986). Two peaks of GS activity were separated by anion-exchange chromatography when cultures of *S. costatum* were grown with low levels of N (daily additions to 60 μM N; Table I). The isoforms were defined as GS_1 and GS_2 based on their elution from the anion-exchange column. Enzyme activity in the early-eluting peak (GS_1) was always substantially lower than that of the later-eluting peak (GS_2). GS_1 accounted for less than 7% of the total enzyme activity eluted from the anion-exchange column. When cells were grown on higher levels of N (daily additions to 120 μM NH_4Cl or a single addition of 880 μM $NaNO_3$), GS_1 activity was reduced or undetectable (Table I). A similar pattern of GS expression was reported for the green alga *Chlorella*, in which the activity of GS_1 increased in N-starved cells relative to N-replete cells (Sumar et al., 1984). The relative activity of the two isoforms may reflect their cellular expression or indicate that GS_1 is more labile than GS_2 in the early stages of the purification. This issue will be resolved by further characterization of GS_1 in *S. costatum*.

Table I. *Comparison of GS activity from S. costatum grown under different conditions in cell-free extracts and after isoform separation by DEAE chromatography*

GS_1 represents enzyme activity eluting from the DEAE column in the early peak, and GS_2 represents the activity eluting in the later peak. Enzyme activity was measured using the transferase assay. One unit of transferase activity catalyzes the formation of 1 μmol of γ-glutamyl hydroxamate min^{-1} at 25°C.

N Source	Cell-Free Extract Specific Activity	DEAE Chromatography			
		Activity		GS isoform	
		Loaded	Eluted	GS_1	GS_2
	unit mg^{-1}	*unit*	*unit*	%	%
60 μM NH_4^+	0.021	11.1	19.7	1.3	98.7
120 μM NH_4^+	0.011	13.7	29.1	0.1	99.9
60 μM NO_3^-	0.020	4.8	3.0	6.5	93.5
880 μM NO_3^-	0.009	2.9	3.2	3.3	96.7

Specific enzyme activity was highest in cultures grown with the lowest concentrations of N (Table I). Total enzyme activity in NO_3^--grown cells was lower than in NH_4^+-grown cells, although the specific activity in the GS_2 peak fractions was similar in all experiments, ranging from 2.5 to 5.1 units mg^{-1} protein. The consistency in the elution profiles from the DEAE chromatography and the analytical gel filtration indicates that subunit composition of GS_2 is not altered when *S. costatum* is grown with different N sources.

GS_2 was purified over 200-fold compared with the crude extract. Results from a representative purification are summarized in Fig. 1A and Table II. SDS-PAGE revealed that a 70-kD polypeptide was present in both NH_4^+- and NO_3^--grown cells (Fig. 1B). Although other, less-abundant proteins were present in the fraction, their abundance pattern was not correlated with GS_2 enzyme activity (data not shown). Further characterization of GS_2 by native PAGE revealed a single band of enzyme activity (Fig. 2A), and subsequent denaturing PAGE of the active enzyme revealed a 70-kD polypeptide (Fig. 2B).

The M_r of the native enzyme, as determined by analytical gel filtration, was 400,000 (± 44,000 SD; data not shown) and is consistent with an enzyme constituted of six 70-kD

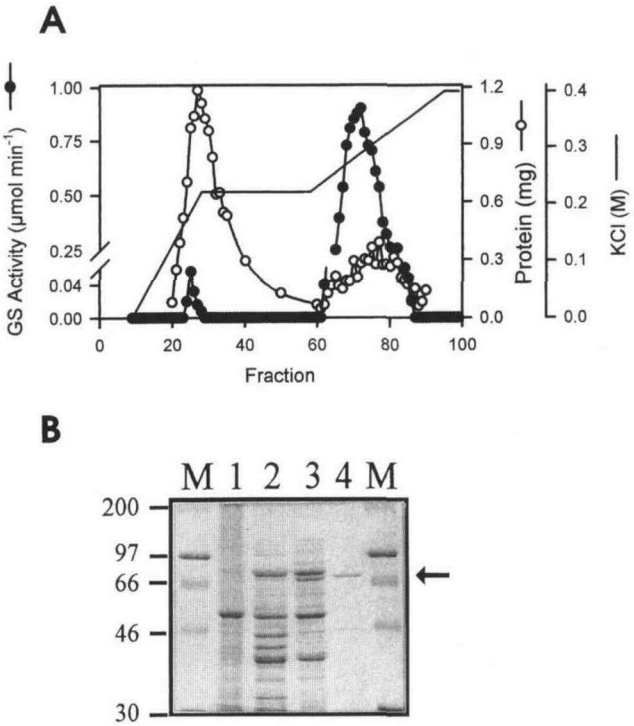

Figure 1. Purification of GS_2 from *S. costatum* cells grown on ammonium. A, Typical elution profile of GS transferase activity and protein from a DEAE-Sepharose column. GS activity in each fraction (●), protein yield in each fraction (○), and KCl (—) in each fraction. B, SDS-PAGE analysis of GS purification. Lane 1, Crude extract; lane 2, resuspended 40 to 70% saturated $(NH_4)_2SO_4$ pellet; lane 3, Sephacryl S-300 HR column peak fraction; lane 4, DEAE-Sepharose column peak fraction. Lanes 1 and 2, 10^{-3} units of GS transferase activity; lanes 3 and 4, 10^{-2} units of GS transferase activity. GS is marked with an arrow. M, Molecular mass markers in kD. Similar protein profiles were obtained with NO_3^--grown *S. costatum*.

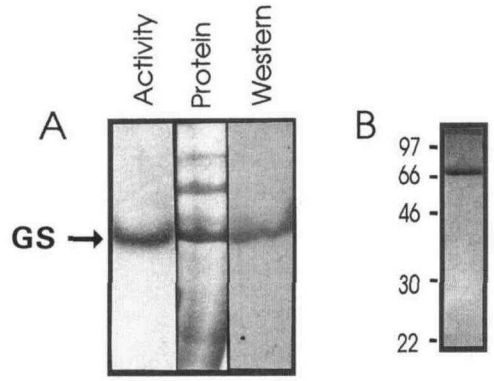

Figure 2. Detection of GS_2 by native gel electrophoresis and immunoblotting. Peak fractions from chromatography of NO_3^--grown *S. costatum* cells were analyzed by native gel electrophoresis. A, Activity lane, Native gel stained for GS transferase activity; Protein lane, native gel stained with Coomassie blue to detect total protein; and Western lane, after electrophoresis the native protein was treated with 0.1% SDS, transferred to nitrocellulose, and challenged with *S. costatum* anti-GS_2 serum. The anti-GS_2 serum identifies a single immunoreactive protein corresponding to the GS activity band. GS is marked with an arrow. B, Silver-stained SDS-PAGE of active band reveals a 70-kD protein.

subunits. The subunit size and predicted hexameric structure of the *S. costatum* GS_2 are more similar to GSIII found in several bacteria than to GSII found typically in eukaryotes (Southern et al., 1986, 1987; Forde, et al., 1989; Goodman and Woods, 1993; Reyes and Florencio, 1994b; see Rasulov et al., 1977 for an exception). Although the partition coefficient more accurately reflects the Stokes radius of a protein, gel filtration is commonly used to estimate the mol wt when similar types of protein are examined, and an accuracy of ±10% suffices (Nozaki et al., 1976). The determination of the precise quaternary structure of the diatom GS_2 will require additional analysis.

Apparent Michaelis-Menten kinetic parameters were estimated for the three substrates using the DEAE-purified GS_2 (Table III). The kinetic analysis for each substrate was described by a rectangular hyperbola. Apparent K_m values for NH_4^+, measured with either the cell-free extract (0.16 mM ± 0.07 SE) or the 200-fold, purified sample (0.70 mM ± 0.12 SE), were greater than the K_m values previously measured in cell-free extracts of *S. costatum* or the centric diatom *Thalissiosira pseudonana* (Table III). The values we obtained, however, are within the range of those reported for other species of green algae, vascular plants, and bacteria (see Table III). In contrast, the apparent K_m values for Glu (5.71 ± 0.43 SE) are similar among all studies of *S. costatum*. The apparent K_m for ATP (0.53 ± 0.04 SE) is within the range reported for GS from other organisms.

GS_2 biosynthetic activity was inhibited by the addition of Ala, Ser, or Gly (Table IV). Ala, Ser, and Gly inhibit the activities of GSI, GSII, and GSIII (Southern et al., 1987; Forde and Cullimore, 1989) and interact at a common site in bacterial GS (Orr and Haselkorn, 1981). Therefore, de-

Table II. *Summary of GS_2 purification from NH_4^+-grown S. costatum*

NH_4Cl was added daily to a final concentration of 60 μM. Proteins were eluted from the DEAE-Sepharose column using the two-step gradient described in "Materials and Methods." Enzyme activity was measured using the GS transferase assay. One unit of transferase activity catalyzes the formation of 1 μmol of γ-glutamyl hydroxamate min^{-1} at 25°C.

Step	Total Activity	Total Protein	Specific Activity	Purification	Activity Yield
	unit	*mg*	*unit mg^{-1}*	*-fold*	*%*
Crude extract	6.73	454.0	0.01	1	100
70% $(NH_4)_2SO_4$	2.57	56.5	0.05	3	38
Sephacryl S-300 HR	8.45	11.6	1.30[a]	88	126
DEAE Sepharose (GS_2)	8.07	2.9	4.27[a]	288	120

[a] Specific activity was calculated for the peak fraction.

Table III. *Apparent K_m values (mM) for GS from different sources*

Species	NH_4^+	Glu	ATP	Ref.
S. costatum[a]	0.702	5.7	0.53	This study
S. costatum[b]	0.008	6.8	NR	Bressler and Ahmed, 1984
S. costatum[a,b]	0.029	29.0	NR	Falkowski and Rivkin, 1976
Thalissiosira pseudonana[b]	0.002	7.3	0.19	Bressler and Ahmed, 1984
Chlamydomonas reinhardtii[a] (cytosol)	0.083	2.5	0.32	Florencio and Vega, 1983
Chlamydomonas reinhardtii[a] (chloroplast)	0.244	10.0	0.29	Florencio and Vega, 1983
Oryza sativa[c] (cytosol)	1.000	2.0	1.00	Hirel and Gadal, 1980
Oryza sativa[c] (chloroplast)	1.000	20.0	2.00	Hirel and Gadal, 1980
Saccharomyces cerevisiae[c]	NR[d]	6.3	1.30	Mitchell and Magasanik, 1983
Anabaena 7120[a]	<0.020	2.1	0.32	Orr and Haselkorn, 1981

[a] GS activity measured as the ADP-dependent oxidation of NADH. [b] GS activity measured as the glutamate-dependent formation of phosphate. [c] GS activity measured as the formation of γ-glutamyl hydroxamate from glutamate and hydroxylamine. [d] NR, Not reported.

spite the structural differences among the GS holoenzymes, the forms share several catalytic and regulatory properties (Forde and Cullimore, 1989).

GS biosynthetic and transferase activities were inhibited by the addition of PPT and MSX, respectively. Inhibition by PPT followed an exponential decay pattern similar to that of MSX inhibition of GS from *Anabaena* (Orr and Haselkorn, 1981). Maximal inhibition occurred within 7 min, with less than 1% of enzyme activity remaining. GS transferase activity was maximal at 47°C, with activity decreasing above this temperature. The ratio of the rate of enzyme activity at temperatures differing by 10°C was 1.4 in the cell-free extract (slope = 0.014, $r^2 = 0.92$).

Immunological Characterization

Polyclonal antiserum was raised against the gel-purified, 70-kD polypeptide, and immunoreactivity was observed both in chicken serum and in eggs after 73 d. The antiserum recognized a unique, 70-kD polypeptide in western blots of partially purified GS_2 and *S. costatum* cell lysates (Figs. 3 and 4A). There was no cross-reactivity observed with the preimmune serum when similar dilutions and development times were used. Lower dilutions or longer development times, however, revealed an immunoreactive protein of approximately 48 kD (Fig. 4B).

Native gel electrophoresis of partially purified GS revealed a single band of enzyme activity. Western analysis of the native gel revealed an immunoreactive polypeptide that co-localized with the GS activity-stained band (Fig. 2A) and confirmed that the antiserum raised against the 70-kD polypeptide recognized GS from *S. costatum*. When the band from a lane adjacent to the GS activity-stained band was analyzed by denaturing PAGE and by subsequent western blotting, the diatom GS_2 antiserum recognized the resultant 70-kD protein (data not shown). The antiserum neither inhibited nor precipitated enzyme activity, suggesting that the serum recognized epitopes on either the interfaces of the subunits or internal to the native structure.

The immunological relatedness of samples from the two chromatographically separated peaks of enzyme activity was examined by western blot analysis. Samples containing equivalent GS activity (5×10^{-3} units) from the two peak fractions were analyzed. The diatom anti-GS_2 serum detected a 70-kD protein in the sample from the second

Table IV. *Effect of amino acids and inhibitors on GS_2 activity from S. costatum*

Enzyme activity was measured using aliquots of DEAE-purified GS_2. Amino acids, PPT, and MSX were added to the reaction mix at a final concentration of 10 mM, 5 μM, and 10 μM, respectively. The effects of amino acids and PPT were measured using the biosynthetic assay. Controls had 31 units of activity. One unit of biosynthetic activity catalyzed the oxidation of 1 μmol of NADH min^{-1} at 22°C. The effect of MSX was measured using the transferase assay.

Addition	GS Activity
	%
None	100
Gln	104
Ser	77
Ala	68
Gly	65
PPT	<1
MSX	5

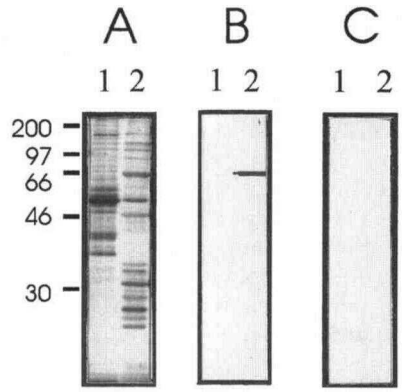

Figure 3. Demonstration of immunological variation between the two GS isoforms separated by anion exchange. A, Silver-stained denaturing PAGE of partially purified proteins from DEAE-chromatography; B, western blot with GS_2 antiserum; C, western blot with preimmune serum. Lanes 1, Protein from early-eluting peak fraction; lanes 2, protein from later-eluting peak fraction. Lanes were loaded with equal enzyme activity (approximately 5×10^{-3} units).

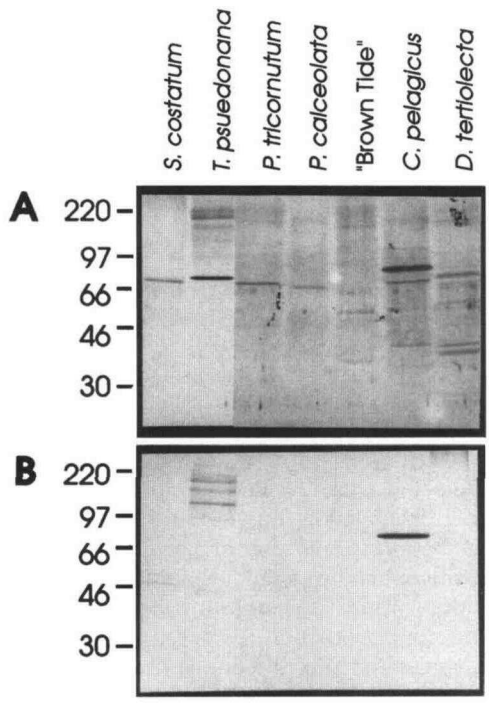

Figure 4. Comparison of immunological relatedness of GS_2 among species of marine phytoplankton. Cell-free extracts were prepared as described in "Materials and Methods." Each lane was loaded with equal GS transferase activity (approximately 10^{-4} units). Lanes are labeled with species names. Taxonomic groupings are described in "Materials and Methods." A, Western blot using GS_2 antiserum (1:5000 dilution). Lanes labeled *S. costatum* and *T. pseudonana* were developed for 2 min. The remaining lanes were developed for 15 min. B, Western blot using preimmune serum (1:5000 dilution). The entire blot was developed for 15 min.

peak of enzyme activity, but there were no immunoreactive proteins in the sample from the first peak (Fig. 3). Thus, the antiserum is specific for GS_2, and the immunological results support our hypothesis that the two peaks of enzyme activity represent distinct GS isoforms.

The *S. costatum* GS_2 antiserum recognized similar-sized proteins in cell lysates of other species of algae (Fig. 4). An immunoreactive polypeptide was detected in cell lysates from the centric diatoms *S. costatum* and *T. pseudonana*, the pennate diatom *P. tricornatum*, and the pelagophyte *P. calceolata*. The preimmune serum recognized a protein in the coccolithophorid *C. pelagicus*, confounding the interpretation of the pattern observed with the immune serum. There were several immunoreactive polypeptides in the sample from the chlorophyte *Dunaliella tertiolecta*. The intensity of the immunoreactive signal was greatest in lysates of the centric diatoms, and the signal in other chromophyte species required longer development times. In our study GS_2 activity accounted for more than 93% of the total GS activity in *S. costatum*, whereas in *P. tricornatum* GS_1 accounted for the majority of the activity (Casselton et al., 1986). Therefore, the observed difference in immunorecognition of GS among algal taxa could be due to differences in antigenicity or in the relative expression of the two GS isoforms. Additional studies are required to verify that the immunoreactive polypeptides observed in other algal species are GS.

CONCLUSIONS

The hexameric structure of *S. costatum* GS_2, composed of 70-kD subunits, is more similar to GSIII, which is found in bacteria, than to GSII, which is found in most eukaryotes (Rasulov et al., 1977; Southern et al., 1986, 1987; Forde and Cullimore, 1989; Goodman and Woods, 1993; Reyes et al., 1994b). Although structurally divergent, several catalytic and regulatory mechanisms are conserved among all types of GS. Our immunological data suggest that the 70-kD form of GS may not be unique to *S. costatum*, since antiserum raised against the diatom GS_2 recognized a polypeptide of similar size in other chromophytic taxa. The molecular analysis of this novel form of GS in chromophytic algae should provide new insights into the evolution of genes encoding GS, as well as into the molecular evolution of these algae.

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