

NOTE

GLUTAMINE SYNTHETASE IN MARINE ALGAE: NEW SURPRISES FROM AN OLD ENZYME<sup>1</sup>

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**Glutamine synthetase (GS), which catalyzes the formation of glutamine from ammonium and glutamate in the presence of ATP, is encoded by three distinct gene families: GSI, GSII, and GSIII. Genes encoding GSI are found in the Bacteria and Archaea, whereas GSII genes are found in eukaryotes and a few species of Bacteria. Members of the third family, GSIII, have been described from a limited number of bacteria; however, recent biochemical and molecular data suggest that this type of enzyme is broadly distributed among the algae. Peptide fragments obtained from GS purified from the marine diatom *Skeletonema costatum* (Greville) Cleve are 77% identical to a partial sequence of GSIII from *Chaetoceros compressum* Lauder, which permits the unambiguous assignment of the biochemically characterized enzyme to the GSIII gene family. The N-terminal sequence was 43% identical to the GSIII-like enzyme purified from the haptophyte *Emiliania huxleyi* (Lohm.) Hay et Miller and several residues were conserved among bacterial and eukaryotic GSIII enzymes. The presence of genes encoding GSIII in diatoms and haptophytes indicates that this enzyme family is more broadly distributed in eukaryotes than previously suspected.**

**Key index words:** diatoms; evolution; glutamine synthetase; nitrogen metabolism; *Skeletonema costatum*

**Abbreviations:** GS, glutamine synthetase

Glutamine synthetase (GS), an enzyme essential for ammonium assimilation and glutamine biosynthesis, is found in all organisms and has been the focus of numerous biochemical, molecular, and evolutionary studies. Three distinct forms of GS have been described (GSI, GSII, and GSIII), which differ both in primary and tertiary structure. Genes encoding GSI

have been analyzed from species of Archaea and Bacteria and were thought to be restricted to prokaryotes until Mathis et al. (2000) described the presence of GSI-type enzymes in vascular plants. GSII, although considered typical for eukaryotes, has been described from both eukaryotes and soil bacteria (Brown et al. 1994, reviewed in Brown and Doolittle 1997). Until recently, genes encoding the third form, GSIII, had been described from only a few prokaryotes; however, biochemical and molecular studies of GS in marine algae show that GSIII is more broadly distributed than previously suspected.

Biochemical studies of GS isoenzymes in the diatom *Skeletonema costatum* (Robertson and Alberte 1996) and the haptophyte *Emiliania huxleyi* (Maurin and Le Gal 1997) identified enzymes that were structurally similar to GSIII. The holoenzymes were comprised of six subunits that ranged between 60 and 78 kDa and were in striking contrast to the octameric GSII holoenzymes typical of eukaryotes. Recently, Kinoshita et al. (1998) identified a cDNA sequence from the diatom *Chaetoceros compressum* that is homologous to GSIII from bacteria. Here we show that two peptide fragments obtained from the *S. costatum* GSIII-type enzyme are 77% identical to the predicted amino acid sequence of the cloned *Chaetoceros* GSIII. The similarity allows for the unambiguous assignment of the biochemically purified diatom enzyme, and by extension, the haptophyte GS enzymes, to the GSIII family of enzymes.

Peptide fragments of GSIII were obtained by protease digestion (Rosenfeld et al. 1992) of GS purified from *S. costatum* as described by Robertson and Alberte (1996). Anion exchange fractions containing GSIII were resolved by denaturing gel electrophoresis, and proteins were visualized using CBB (20% methanol, 0.5% acetic acid, 0.2% Coomassie Brilliant Blue) and destaining in 30% methanol. Proteins were excised and washed twice with 150  $\mu$ L of 50% acetonitrile in 200 mM ammonium carbonate at 37° C for 20 min. Gel slices were cut into 1-mm<sup>2</sup> pieces and dried using vacuum centrifugation (Savant Instruments, Holbrook, NY).

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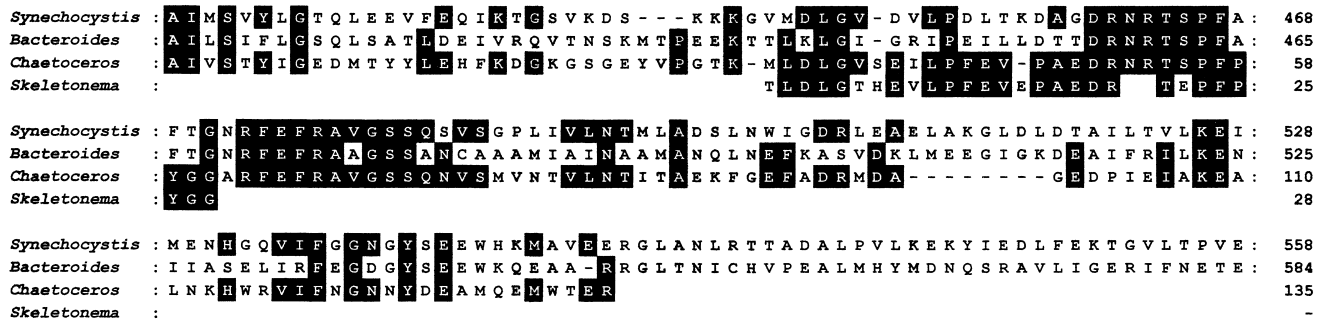


FIG. 1. Sequence alignment of GSIII predicted amino acid sequences from bacteria and diatoms. Shaded residues are identical to the *Chaetoceros* sequence. Alignment was obtained using the PileUp program of the Wisconsin Package Version 9.0 (Genetics Computer Group, Madison, WI). Gaps are indicated as dashes. Numbers indicate position relative to the first amino acid of the sequence. GenBank accession numbers are *Synechocystis* X76719, *Bacteroides* M28252, and *Chaetoceros* AB016770.

The gel slices were partially rehydrated with 10  $\mu$ L of 200 mM ammonium carbonate containing 2  $\mu$ L of trypsin (200  $\mu$ g $\cdot$ mL<sup>-1</sup>; Promega Corp., Madison, WI). After absorption of the protease solution, gel pieces were covered with 200 mM ammonium bicarbonate and incubated at 37° C overnight. The supernatant was collected after centrifugation, transferred to a clean tube, and digested peptides were recovered from the gel slices using two 20-min extractions with 100  $\mu$ L of a solution containing 60% acetonitrile and 0.1% trifluoroacetic acid at 30° C with shaking. Extracts were combined, concentrated by vacuum centrifugation, and stored at -20° C until analyzed.

For N-terminal sequencing, anion exchange fractions containing the GSIII holoenzymes were desalted and concentrated using a Centricon 50 following the manufacturer's instructions (Amicon, Bedford, MA). Proteins were resolved by denaturing PAGE using a Tris:tricine buffer (50 mM Tris, 40 mM tricine, 0.1% SDS, pH 7.9); the upper reservoir buffer contained 0.1 mM thioglycolic acid (LeGendre and Matsudaira 1989). Gels were run for 30 min at 120 V before sample loading and at 120 V for 1.2 h after sample application. Proteins were transferred to polyvinylidene difluoride membranes (transfer buffer: 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid, 10% methanol) at 100 V for 2 h and were visualized by staining (50% methanol, 0.2% Coomassie Brilliant Blue) and destaining (50% methanol, 10% acetic acid). The region of the membrane containing GSIII was excised and stored until sequenced. Sequencing was done at

the UCLA School of Medicine's Protein Microsequencing Facility.

Amino acid sequences from four peptide fragments were obtained and ranged between 8 and 20 residues in length. Two fragments aligned with the partial GSIII sequence from *Chaetoceros* in a region predicted to form part of the active site; the sequences were 77% identical (Fig. 1). Trypsin cleaves peptides on the carboxyl side of arginine and lysine residues (Stryer 1995); the amino acid sequence from *Skeletonema* GSIII was consistent with cleavage following the carboxyl arginine of the first fragment. In addition, the cleavage pattern suggested that the next arginine, conserved among all GS sequences, was also conserved in *Skeletonema*. The sequence similarity between GSIII and either GSI or GSII is extremely low (less than 20% identity; Reyes and Florencio 1994) and none of the peptide fragments sequenced in this study could be aligned with either GSI or GSII sequences.

Previous biochemical work established that *S. costatum* and *E. huxleyi* express isoenzymes that are more similar in subunit size and composition to GSIII than to either GSI or GSII (Robertson and Alberte 1996, Maurin and Le Gal 1997). The amino acid sequence of two peptide fragments presented here confirmed the identity of the biochemically purified GS from *S. costatum* as a member of the GSIII family (Fig. 1). Full-length GSIII sequences have been described from only a few species of bacteria, including cyanobacteria (*Synechocystis* [Reyes and Florencio 1994], *Pseudanabaena* [Crespo et al. 1998]), *Bacteroides fragilis* (Hill et

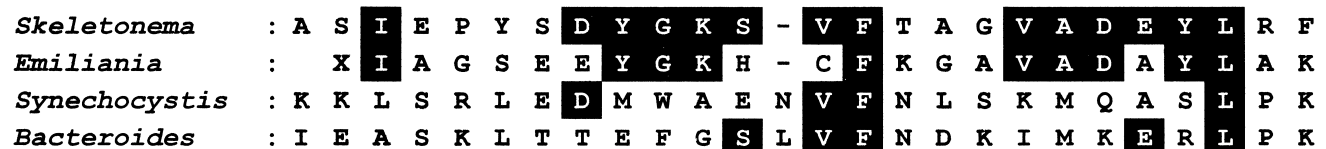


FIG. 2. Sequence alignment of N-terminal amino acid residues. Shaded residues are identical to the *Skeletonema costatum* sequence. Symbols as in Figure 1. GenBank accession numbers are *Synechocystis* X76719 and *Bacteroides* M28252. Sequence for *Emiliana huxleyi* is from Maurin and LeGal (1997). The first amino acid from the N-terminal peptide sequences is given for *Skeletonema* and *Emiliana*; the *Synechocystis* sequence begins at amino acid residue 21; the *Bacteroides* sequence begins with the second residue of the full-length protein.

al. 1989), and *Butyrivibrio fibrisolvens* (Goodman and Woods 1993). Partial GSIII sequences have been reported from the diatoms *Thalassiosira pseudonana* and *Chaetoceros debilis* (AF251002 and AF251003, respectively; deposited by M. C. Schaefer and F. P. Wilkerson), but the partial sequences did not overlap with sequenced peptides from the purified *S. costatum* GSIII.

N-terminal sequences obtained from *S. costatum* and *E. huxleyi* GSIII enzymes were 43% identical (Fig. 2). Although the similarity with bacterial GSIII sequences was low (15%–20%), several residues were conserved among eukaryotic and prokaryotic GSIII sequences. The lack of an N-terminal methionine in either of the algal sequences may be an artifact in peptide sequencing or may indicate the proteins are posttranslationally processed. Currently, it is unknown whether the GSIII enzymes are located in the cytoplasm, chloroplast, or mitochondria. If the proteins are located in either cellular organelle, the lack of a methionine residue may be due to cleavage of a transit sequence.

Most photosynthetic eukaryotes express multiple GS isoenzymes that are compartmentalized in either the chloroplast or the cytoplasm. In vascular plants, the well-characterized GS isoenzymes are members of the GSII gene family and are thought to have arisen by recent gene duplication (Coruzzi et al. 1989). Marine algae also express multiple isoenzymes. However, in contrast to vascular plants and green algae, *S. costatum* expresses both GSII- and GSIII-type enzymes (Robertson et al. 1999). It is unclear whether a similar pattern will be observed in other chromophytes.

Although GS has been used to examine the evolutionary relationships between organisms in different domains, the relationship among GSI, GSII, and GSIII remains unresolved. The diversity of organisms that contain genes from different families (e.g. GSI and GSII or GSI and GSIII genes in bacteria; GSII and GSIII genes in diatoms; GSI and GSII in vascular plants) supports the hypothesis that the genes have their origin in the last common ancestor of prokaryotes and eukaryotes. The alternative hypothesis would be one of rampant horizontal gene transfers.

The presence of GSIII-type enzymes in diatoms and haptophytes suggests that the distribution of this enzyme family may be broader than previously anticipated. It will be interesting to unravel the evolutionary relationships among the members of the GSIII gene family. For example, are the sequences from photosynthetic eukaryotes more closely related to cyanobacterial sequences, suggesting gene transfer from the ancestral chloroplast? Or, was the GSIII gene in the ancestral nuclear genome and therefore broadly distributed among protist lineages? The diatoms are particularly interesting in this regard, because they are members of a larger assemblage, the

Stramenopiles, that contains both photosynthetic and nonphotosynthetic eukaryotes (Leipe et al. 1996). Clearly, investigation of the gene family in a broad number of eukaryotic taxa will provide insight into the early evolution of cellular metabolism.

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