Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 361–365, January 1999
Biochemistry

Silicatein filaments and subunits from a marine sponge direct the polymerization of silica and silicones *in vitro*

JENNIFER N. CHA*†‡, KATSUHIKO SHIMIZU†‡§, YAN ZHOU†‡§, SEAN C. CHRISTIANSEN¶, BRADLEY F. CHMELKA†¶, GALEN D. STUCKY*†‖, AND DANIEL E. MORSE†‡§**

*Department of Chemistry, †Materials Research Laboratory, ‡Marine Biotechnology Center, §Department of Molecular, Cellular, and Developmental Biology, ¶Department of Chemical Engineering, and ‖Department of Materials, University of California, Santa Barbara, CA 93106

Communicated by John A. Carbon, University of California, Santa Barbara, CA, November 3, 1998 (received for review August 21, 1998)

ABSTRACT **Nanoscale control of the polymerization of silicon and oxygen determines the structures and properties of a wide range of siloxane-based materials, including glasses, ceramics, mesoporous molecular sieves and catalysts, elastomers, resins, insulators, optical coatings, and photoluminescent polymers. In contrast to anthropogenic and geological syntheses of these materials that require extremes of temperature, pressure, or pH, living systems produce a remarkable diversity of nanostructured silicates at ambient temperatures and pressures and at near-neutral pH. We show here that the protein filaments and their constituent subunits comprising the axial cores of silica spicules in a marine sponge chemically and spatially direct the polymerization of silica and silicone polymer networks from the corresponding alkoxide substrates *in vitro*, under conditions in which such syntheses otherwise require either an acid or base catalyst. Homology of the principal protein to the well known enzyme cathepsin L points to a possible reaction mechanism that is supported by recent site-directed mutagenesis experiments. The catalytic activity of the "silicatein" (*silica* pro*tein*) molecule suggests new routes to the synthesis of silicon-based materials.**

Silicon, the second most abundant element in the earth's crust, interacts with living systems by mechanisms that have remained poorly understood (1–6). Evidence suggests that silicon is essential for normal growth and biological function in a diversity of plant, animal, and microbial systems (2); silicon compounds have been shown to modulate these activities (1, 2), but the molecular mechanisms of these effects have proved elusive. Studies of marine organisms that produce relatively large masses of silicified structures (3) show the underlying molecular and genetic mechanisms by which living systems process silicon. Hildebrand *et al.* (7) recently characterized the cDNAs coding for a family of silicon transporters in diatoms and showed that the transporters are likely transmembrane proteins. We have found that the marine sponge *Tethya aurantia* produces copious silica spicules (1–2 mm in length and 30 μm in diameter) that constitute 75% of the dry weight of the organism and that each of these spicules contains a central axial filament of protein (1–2 mm in length and 2 μm in diameter) consisting of three very similar subunits that we have named silicateins (for *silica* pro*teins*; ref. 8). Characterization of silicatein α (the subunit comprising nearly 70% of the mass of the filaments) and its cloned cDNA indicated that it is homologous to members of the cathepsin L subfamily of the papain family of proteolytic enzymes (8).

We show here that, at neutral pH, the silicatein filaments and their constituent subunits catalyze the *in vitro* polymerization of silica and silsesquioxanes from tetraethoxysilane and organically modified silicon triethoxides, respectively. These substrates were chosen because of their stability at neutral pH and the similarity of their chemical reactivity to that of the substrates of the proteases. In the absence of the silicateins, polymerization of these precursors usually requires strong acid or base catalysis. These findings, as well as the high structural similarity between the most abundant of the silicatein subunits and a protease that also functions as a general acid–base catalyst at neutral pH, suggest a possible mechanism for the *in vitro* reactions observed.

MATERIALS AND METHODS

Isolation of Silicatein Filaments and Analysis of Reactions with Silicon Alkoxides. Insoluble silicatein filaments were extracted from the acid- and hypochlorite-cleaned silica spicules of *T. aurantia* by dissolving the silica in buffered HF (1 M HF/5 M NH_4F) as described (8). The HF was removed by dialysis against pure water (Milli-Q, Millapore), and the filaments were collected by filtration. Reactions of the insoluble filaments [either air-dried or suspended (at 0.5 mg/ml) in Tris·HCl buffer (0.6 ml; 25 mM, pH 6.8)] with tetraethoxysilane (TEOS; 1.0 ml/4.5 mmol) were performed with gentle shaking at room temperature for 12 h. The silicatein filaments were added in aqueous Tris buffer for all reactions except the one illustrated in Fig. 1*C* in which the air-dried filaments were reacted with pure TEOS. The reaction also was performed with 1.0 ml (4.1 mmol) of phenyltriethoxysilane (Fig. 1*D*) in place of TEOS. For all samples, the insoluble materials were collected by centrifugation, air-dried, gold sputter-coated, and imaged by scanning electron microscopy with a JEOL JSM 6300F equipped with a cold cathode field-emission source operated at a beam energy of 3.5 kV (Fig. 1).

NMR Analyses. NMR spectra were acquired on a CMX-500 spectrometer (Chemagnetics, Ft. Collins, CO) operating at 11.7 tesla and a ^{29}Si frequency of 99.06 MHz referenced to tetramethylsilane (9). The single-pulse spectrum was acquired for 22 h with an 8.35-μs single pulse and a recycle delay of 300 s, under conditions of magic-angle sample spinning at 3.5 kHz. Crosspolarization magic-angle spinning spectra were acquired for 4 h with a contact time of 4 ms, a pulse width of 6 μs, and a recycle delay of 2 s, while spinning at 6 kHz.

Silicatein Subunits and Analysis of Reactions with Silicon Alkoxides. Silicatein subunits (Table 1, experiment A) were solubilized from the purified filaments (8) by treatment with 10 mM NaOH for 5 min, and the soluble subunits then were dialyzed extensively at 4°C against Tris·HCl buffer (25 mM, pH 6.8). Silicatein α (Table 1, experiment B) was expressed from a recombinant DNA template in *Escherichia coli*, purified, reconstituted and dialyzed as above (Y.Z., K.S., J.N.C., G.D.S., and D.E.M., unpublished results). Denatured proteins were boiled for 15 min. The proteins then were used immediately for

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

Abbreviation: TEOS, tetraethoxysilane.

**To whom reprint requests should be addressed. e-mail: d_morse@lifesci.lscf.ucsb.edu.

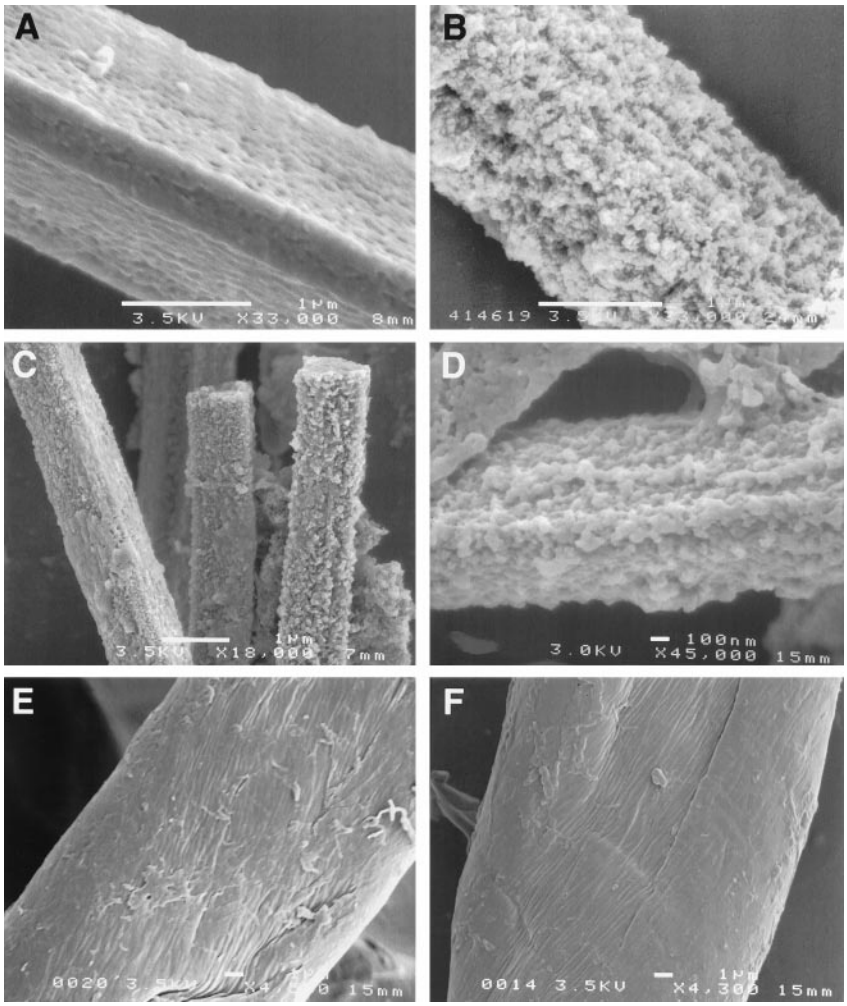

FIG. 1. Scanning electron micrographs of the products of the reaction between silicon alkoxides and silicatein or cellulose filaments. (*A*) Silicatein filaments before the reaction. (*B*) Silicatein filaments after a 12-h reaction with TEOS (1.0 ml; 4.5 mmol) plus Tris·HCl buffer. (*C*) Air-dried silicatein filaments incubated with TEOS as in *B*, but with no additional water. (*D*) Silicatein filaments after an 8-h reaction with phenyltriethoxysilane (1.0 ml; 4.1 mmol) plus Tris·HCl buffer. (*E*) Cellulose fiber. (*F*) Cellulose fiber after a 12-h reaction with TEOS as in *B*.

an assay in which TEOS (1 ml; 4.5 mmol) was added to 0.6 ml protein (0.26 or 0.5 mg/ml in Tris buffer, as specified). The mixtures were thoroughly resuspended by pipetting, and the reactions were allowed to continue for 15–60 min at 20°C. The samples then were centrifuged to collect the silica products; the pellets were washed a minimum of three times with ethanol to remove unreacted TEOS, collected by centrifugation, and then either hydrolyzed with 1 M NaOH for 10 min or suspended only with water to quantify residual adsorbed TEOS. The samples then were diluted, and the released silicic acid was quantified with a modification of the colorimetric molybdate assay (10) with the reagent blank of Brzezinski and Nelson (11), yielding a detection limit of 50 nM $Si(OH)_4$.

Table 1. Silicatein subunits catalyze polymerization of silica

Protein	Polymerized Si, nmol
A. Silicatein subunits	
Native	214.0 ± 2.0
Denatured	24.5 ± 2.0
BSA	42.1 ± 0.7
Papain	22.9 ± 1.0
Trypsin	16.2 ± 2.6
(None)	10.2 ± 1.3
B. Recombinant silicatein α	
Native	140.0 ± 6.2
Denatured	8.8 ± 1.9
(None)	6.7 ± 2.1

Proteins in 0.6 ml of Tris·HCl buffer (25 mM, ph 6.8) were incubated with 1 ml (4.5 nmol) of TEOS, and the polymerized silica was quantitated after centrifugation and hydrolysis as described in *Materials and Methods*. For experiment A, proteins at 0.3 mg; reaction for 15 min. For experiment B, proteins at 0.06 mg; reaction for 60 min.

RESULTS

The silicatein filaments can be dissociated to their constituent subunits: α, β, and γ (8). These subunits accelerate the *in vitro* polymerization of silica $(SiO_2)_n$ from the monomeric TEOS at neutral pH (Table 1, experiment A). Electron microscopy confirms the formation of a dendritic silica precipitate (not shown). Little polymerization is seen in the absence of these proteins; normally, under these conditions, polymerization of silica from TEOS requires either an acid or base catalyst (12). The activity of the silicatein subunits is abolished by thermal denaturation, indicating a dependence on the native three-dimensional conformation of the subunit proteins. Denaturation with the detergent SDS also abolishes activity (results not shown). Specificity of the observed effect is indicated by the finding that the condensation of TEOS under these conditions is significantly slower when trypsin, papain, or BSA is substituted for the silicatein.

Silicatein α comprises $\approx$70% of the mass of the silicatein filaments in *T. aurantia* (8). This subunit, when expressed in bacteria from a recombinant DNA template and subsequently purified and reconstituted, proves to be sufficient to accelerate the polymerization of silica from TEOS at neutral pH (Table 1, experiment B). In this case also, thermal denaturation abolishes reactivity with the silicon alkoxide. These findings are significant, because the complete amino acid sequence of the α subunit has a high similarity to members of a well characterized enzyme superfamily (8).

The intact silicatein filaments also are active, promoting the condensation of silicon alkoxides and organically modified silicon alkoxides to form the corresponding polymerized silica or silsesquioxanes $(RSiO_{3/2})_n$ (silicones in which *R* represents an organic side chain) at neutral pH (Figs. 1 and 2). The macroscopic filaments serve as scaffolds to organize the deposition of the resulting silica and silsesquioxanes (Fig. 1). Organization of the resulting silica is seen more clearly when the condensation of TEOS is performed without adding water (except for the water of protein hydration); the absence of added water restricts the dendritic growth of the silica by limiting hydrolysis of the precursor to create a silica substructure that follows the longitudinal axis of the protein filament (Fig. 1*C*). In the absence of the filaments, no polymerization of TEOS was observed at neutral pH during the course of the experiments. This result is consistent with the known requirement for acid or base catalysis (12). The activity of the silicatein filaments is abolished by thermal denaturation, indicating a dependence on the native conformation of the constituent proteins. Neither silk (not shown) nor cellulose (Fig. 1 *E* and *F*) fibers exhibit any activity with TEOS under the same conditions, indicating that polymeric fibers with high surface densities of hydroxyl groups are not sufficient to accelerate or organize silica polymerization from TEOS at neutral pH. The acceleration of polymerization and structure-directing activities of the silicatein filaments also are evident with organically substituted triethoxysilane precursors with the general structure R—Si$\equiv$$(OEt)_3$, where *R* represents phenyl, methyl, etc. When phenyltriethoxysilane is provided as substrate, a polymerized product is formed on the protein filaments (Fig. 1*D*). However, under the same conditions (pH 6.8, 24 h) in the absence of the protein filaments, little or no condensation of the precursor was observed. Similar results were observed with methyltriethoxysilane as well.

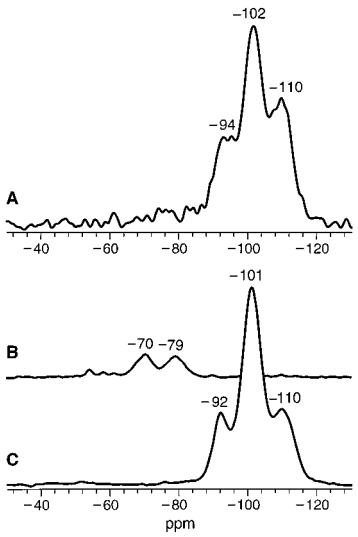

FIG. 2. ^{29}Si magic-angle spinning NMR spectra of silica and silsesquioxane products on silicatein filaments were acquired. Samples were prepared as described for Fig. 1 *B* and *D*. (*A*) A single-pulse ^{29}Si magic-angle spinning spectrum of the reaction product of silicatein filaments and TEOS. (*B* and *C*) Crosspolarization magic-angle spinning spectra of the reaction products of silicatein filaments and phenyltriethoxysilane (*B*) and TEOS (*C*).

Solid-state ^{29}Si NMR was used to analyze the extent of polymerization of the siloxanes on the protein filaments. Analysis of the products formed from TEOS (Fig. 2 *A* and *C*) showed three inhomogeneously broadened peaks corresponding to Q^2 (≈ -92 ppm), Q^3 (≈ -101 ppm), and Q^4 (-110 ppm) siloxane species, indicative of a disordered, incompletely polymerized silica network characteristic of the silica found in biological materials. In contrast to these results, crosspolarization ^{29}Si NMR analysis of the product formed from the phenyltriethoxysilane precursor showed no Q^4 species (Fig. 2*B*). This result is consistent with the silsesquioxane structure of the polymerized product, as would be predicted from polymerization of the precursor that contains only three functional groups available for the formation of siloxane linkages. The phenylsilsesquioxane exhibits a T^3 resonance at -79 ppm (shifted downfield by 20 ppm because of the phenyl substituent; ref. 9), a T^2 signal at -70 ppm, and weak downfield T^1 resonances. The ^{29}Si crosspolarization magic-angle spinning spectrum for the product formed by using phenyltriethoxysilane (Fig. 2*B*) displays much weaker intensity compared with the ^{29}Si crosspolarization magic-angle spinning spectrum for the product formed by using TEOS (Fig. 2*C*). This weaker intensity is caused by the reduced degree of functionalization of the silsesquioxane in the final product.

DISCUSSION

Nanoscale control of polymerization of silicon and oxygen determines the structures and properties of a wide range of siloxane-based materials now in use or under development for advanced applications (13–18). The possibility that the molecular mechanisms governing nanofabrication of the remarkable diversity of silicified structures produced in living organisms (1–2, 19–21) might offer new insights for synthesis motivated the present study.

The discovery that the proteins of the axial filament of the sponge spicule are closely related to members of a well known enzyme family raised the interesting possibility that these proteins might be capable of catalysis (8) in addition to their long-suspected function as template-like organizers of polysiloxane nucleation (6). The results reported here confirm that possibility for a reaction studied *in vitro*. Comparison of the silicatein α and cathepsin L sequences (8) shows that the six cysteine residues that form intramolecular disulfides in cathepsin L are conserved fully in the silicatein, suggesting that the three-dimensional structures of the two proteins are quite similar. Of the three residues of the ''catalytic triad'' of the cathepsin active site, two, His and Asn, are conserved also in silicatein α, but the third active-site residue in cathepsin, Cys, is replaced in the silicatein by Ser[26], preventing this protein from being an effective protease (8). At this position, the structure of silicatein α resembles that of the other major class of proteases, the serine proteases, typified by trypsin and chymotrypsin. Both the condensation of silicon alkoxides promoted by the silicateins and the cleavage of peptides catalyzed by the proteases must proceed through an obligatory hydrolysis reaction, and both are known to be accelerated by general acid–base catalysis, suggesting that the mechanism of action of silicatein α in this process may be related fundamentally to that of its homologous enzyme counterparts. Recent site-directed mutagenesis results (22) confirm the requirement for the specific Ser[26] and His[165] residues of silicatein α for catalysis of the siloxane polymerization described here. These results suggest that the mechanism of silicatein-mediated catalysis of siloxane polymerization from the alkoxide substrates may be closely parallel to that of the well characterized Ser/His and Cys/His active-site proteases (23). As illustrated in Fig. 3, such a mechanism may help to explain the observed

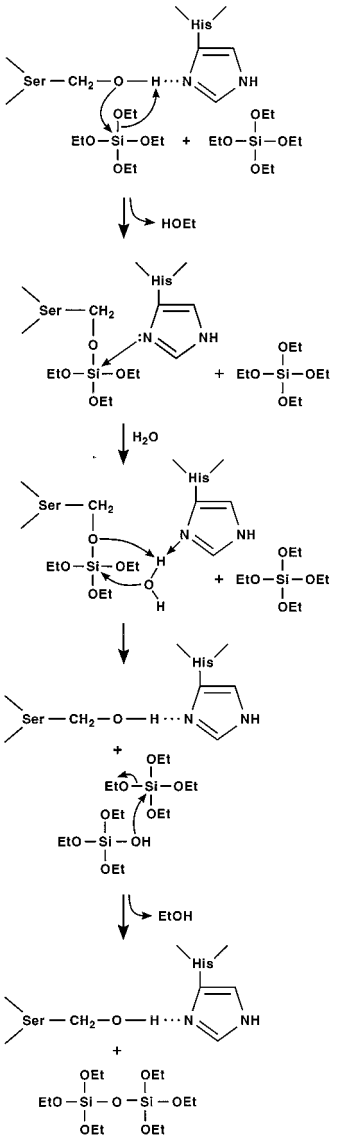

FIG. 3. Proposed reaction mechanism of silicon ethoxide condensation catalyzed by silicatein α, based on the well characterized mechanism of catalysis by the Ser/His and Cys/His active-site proteases (22). R = phenyl- or methyl- for the silicon triethoxide substrates, and $R = CH_3CH_2$—O— (= EtO—) for TEOS. Hydrogen-bonding between the imidazole nitrogen of the conserved histidine and the hydroxyl of the active-site serine is proposed to increase the nucleophilicity of the serine oxygen, potentiating its attack on the silicon atom of the substrate. Nucleophilic attack on the silicon displaces ethanol, forming a covalent protein—O—Si intermediate (potentially stabilized as the pentavalent silicon adduct via donor bond formation with the imidazole nitrogen). The addition of water completes hydrolysis of the first alkoxide bond. Condensation initiated by nucleophilic attack of the released Si—O^- on the silicon of the second substrate molecule then forms the disiloxane product.

acceleration of silicon alkoxide condensation promoted by silicatein α and the silicatein filaments *in vitro*, because the rate-limiting step in this condensation is the initial hydrolysis of the alkoxide required to generate the reactive Si—O species and because the rate of spontaneous hydrolysis is lowest at neutral pH (12). It is possible that the activity exhibited by the filaments and subunits isolated from the sponge may reflect some contribution from an as yet undetected component from the purification. However, we have shown (*i*) that the purified and reconstituted silicatein α subunit produced in bacteria from a cloned cDNA template is active aone (Table 1); (*ii*) that this activity is abolished by prior heating of the α protein; and (*iii*) that this activity is reduced significantly by site-directed mutagenesis replacing either of two specific amino acid side chains (22). These results indicate that the α subunit alone is sufficient to accelerate the polycondensation of silicon tetraethoxide to form silica at neutral pH. The observation that the activities of the mixture of subunits obtained from the filaments—and of the intact filaments themselves—are also abolished by prior heating supports the idea that some or all of the activity was contributed by the silicatein proteins themselves.

Because the axial protein filament is occluded wholly within the silica spicule, it had long been suspected of participating in the control of biosilicification, although the mechanism by which it might do so remains unclear (24). Hecky *et al.* (4) postulated that the hydroxyl-rich proteins of the silicified diatom wall might condense with silicic acid monomers, thus serving as scaffolds to organize the growth of the silica. Thermodynamic calculations have been presented in support of that suggestion (25). Such a mechanism may also contribute to the results reported here. Although the lack of activity of the hydroxyl-rich cellulose and silk polymers indicates that the simple density of hydroxyls alone is not sufficient for polymerization of the silicon alkoxides, the conformation of such groups in the silicatein molecule may be important for this activity. Indeed, several runs of contiguous hydroxyls were noted in silicatein α (8), with the suggestion that these might be important in orienting the siloxane groups of either the substrate or product. Other researchers have suggested that various organic conjugates of silicic acid might serve as the proximate substrates for polymerization *in vivo*. Silicon catecholates have been used by Perry *et al.* in extensive studies of silica polymerization promoted by sugars and polysaccharides from silicified plants (26–28), and Mann and his colleagues recently showed that bacterial filaments can direct the deposition of a colloidal silica gel that, after calcination, yielded a macroporous filamentous material (29). Identification of the biological substrate for silicification in the sponge is needed to assess the possible physiological significance of the catalytic activity that we have observed with the silicon alkoxides *in vitro*. Knowledge of the biological substrate will help determine whether this activity can contribute to the biological mechanism of silica synthesis and nanofabrication under physiological conditions or whether it is simply a vestigial reflection of the evolutionary history of the silicatein molecule (30). Independent of this question, the synthesis of polymeric networks of phenyl- and methyl-silsesquioxanes by the silicatein filaments at neutral pH *in vitro* suggests that this mechanism may be harnessed for the development of environmentally benign routes to the synthesis of patterned silicon-based materials.

We thank M. Brzezinski, G. Zank, N. Auner, and an anonymous reviewer for helpful suggestions. This work was supported by grants from the U.S. Office of Naval Research, the U.S. Army Research Office Multidisciplinary University Research Initiative Program, the National Oceanic and Atmospheric Administration National Sea Grant College Program of the U.S. Department of Commerce, the California Sea Grant College System, the Materials Research Sciences and Engineering Centers Program of the National Science Foundation, the U.S. National Science Foundation, the Packard Foundation, and a donation from the Dow-Corning Corporation. B.F.C. is a Camille and Henry Dreyfus Teacher-Scholar and an Alfred P. Sloan Research Fellow.

1. Volcani, B. E. (1981) in *Silicon and Siliceous Structures in Biological Systems*, eds. Simpson, T. L. & Volcani, B. E. (Springer, New York), pp. 157–201.
2. Voronkov, M. G. (1997) in *Biochemistry of Silicon and Related Problems*, eds. Benz, G. & Lindqvist, I. (Plenum, New York), pp. 395–434.
3. DeLaRocha, C. L., Brzezinski, M. A. & DeNiro, M. J. (1997) *Geochim. Cosmochim. Acta* **61,** 5051–5056.
4. Hecky, R. E., Mopper, K., Kilham, P. & Degens, E. T. (1973) *Mar. Biol. (Berlin)* **19,** 323–331.

5. Swift, D. M. & Wheeler, A. P. (1992) *Phycology* **28,** 209–213.
6. Schwab, D. W. & Shore, R. E. (1971) *Nature (London)* **232,** 501–502.
7. Hildebrand, M., Volcani, B. E., Gassman, W. & Schroeder, J. I. (1997) *Nature (London)* **385,** 688–689.
8. Shimizu, K., Cha, J., Stucky, G. D. & Morse, D. E. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 6234–6238.
9. Smaihi, M., Jermoumi, T. & Marignan, J. (1995) *Chem. Mater.* **7,** 2293–2299.
10. Strickland, J. D. H. & Parsons, T. R. (1972) *A Practical Handbook of Seawater Analysis* (Fisheries Research Board of Canada, Ottawa), 2nd Ed.
11. Brzezinski, M. A. & Nelson, D. M. (1986) *Mar. Chem.* **19,** 139–151.
12. Iler, R. K. (1979) in *The Chemistry of Silica: Solubility, Polymerization, Colloid and Surface Properties, and Biochemistry,* ed. Iler, R. K. (Wiley, New York), pp. 98–99.
13. Zhao, D. Y., Yang, P. D., Huo, Q. S., Chmelka, B. F. & Stucky, G. D. (1998) *Curr. Opin. Solid State and Mater. Sci.* **3,** 111–121.
14. Tolbert, S. H., Firouzi, A., Stucky, G. D. & Chmelka, B. F. (1997) *Science* **278,** 264–268.
15. Zhao, D., Feng, J., Huo, Q., Melosh, N., Frederickson, G. H., Chmelka, B. F. & Stucky, G. D. (1998) *Science* **279,** 548–552.
16. Green, W. H., Le, K. P., Grey, J., Au, T. T. & Sailor, M. J. (1997) *Science* **276,** 1826–1828.
17. McGrath, K. M., Dabbs, D. M., Yao, N., Aksay, I. A. & Gruner, S. M. (1997) *Science* **277,** 552–556.
18. Mann, S. & Ozin, G. (1996) *Nature (London)* **382,** 313–318.
19. Schwab, D. W. & Shore, R. E. (1971) *Biol. Bull.* **140,** 125–136.
20. Levi, C., Barton, J. L., Guillemet, C., Le Bras, E. & Jehuede, P. J. (1989) *Mater. Sci. Lett.* **8,** 337–339.
21. Cattaneo-Vietti, R., Bavestrello, G., Cerrano, C., Sa, M., Benatti, U., Giovine, M. & Gaino, E. (1996) *Nature (London)* **383,** 397–398.
22. Zhou, Y., Shimizu, K., Cha, J. N., Stucky, G. D. & Morse, D. E. (1999) *Angew. Chem.,* in press.
23. Lehninger, A., Nelson, D. & Cox, M., eds. (1993) *Principles of Biochemistry* (Worth, New York), pp. 223–227.
24. Shore, R. E. (1972) *Biol. Bull.* **143,** 689–698.
25. Lobel, K. D., West, J. K. & Hench, L. L. (1996) *Mar. Biol. (Berlin)* **126,** 353–360.
26. Harrison, C. C. (1996) *Phytochemistry* **41,** 37–42.
27. Harrison, C. C. & Loton, N. J. (1995) *J. Chem. Soc. Faraday Trans.* **91,** 4287–4297.
28. Perry, C. C. & Yun, L. J. (1992) *J. Chem. Soc. Faraday Trans.* **88,** 2915–2921.
29. Davis, S. A., Burkett, S. L., Mendelson, N. H. & Mann, S. (1997) *Nature (London)* **385,** 420–423.
30. Morse, D. E. (1999) in *Organosilicon Chemistry IV: From Molecules to Materials,* eds. Auner, N. & Weis, J. (Wiley, New York), in press.