Molecular Biology of Demosponge Axial Filaments and Their Roles in Biosilicification

JAMES C. WEAVER AND DANIEL E. MORSE*
Department of Molecular, Cellular, and Developmental Biology, Marine Biotechnology Center, Marine Science Institute, and the Materials Research Laboratory, University of California, Santa Barbara, California 93106

KEY WORDS Tethya aurantium; spicules; silicatein; silicon; biomineralization; biomimetic

ABSTRACT For hundreds of years, the skeletal elements of marine and freshwater sponges have intrigued investigators with a diverse array of remarkably complex morphologies. Early studies of demosponge monaxonal megascleres revealed the presence of a central organic axial filament running their entire length. Until recently, however, the precise function of these axial filaments was largely unknown. The spicules from the temperate Eastern Pacific demosponge, *Tethya aurantia*, comprise ~75% of the dry weight of this species, facilitating the large-scale isolation and purification of the biosilica-associated proteins. Silicateins, the most abundant proteins comprising the axial filaments of these spicules, prove to be members of a well-known superfamily of proteolytic and hydrolytic enzymes and can be easily collected after silica demineralization with hydrofluoric acid. Consistent with these findings, the intact filaments are more than simple, passive templates; in vitro, they actively catalyze and spatially direct the hydrolysis and polycondensation of silicon alkoxides to yield silica at neutral pH and low temperature. Catalytic activity also is exhibited by the monomeric subunits obtained by disaggregation of the protein filaments and those produced from recombinant DNA templates cloned in bacteria. These proteins also can be used to direct the polymerization of organosilicon polymers (silicones) from the corresponding organically functionalized silicon alkoxides. Based on these observations, the silicateins are currently being used as models for the design of biomimetic agents with unique catalytic and structure-directing properties. The presence of axial filaments in a diversity of spicule types and the evolutionary implications of these findings are also discussed. *Microsc. Res. Tech. 62:356–367, 2003.*

INTRODUCTION

Sponges produce an amazing assortment of intricately architectured skeletal elements, the formation of which, in many instances, exceeds modern human engineering capabilities (Hartman, 1981). They are also well known for their ability to synthesize a wide range of complex compounds, many of which have attracted a great deal of attention in recent years for their potential pharmacological activities as antitumor and antifungal chemotherapeutics (Li et al., 1998; Urban et al., 2000). For these reasons, sponges, both freshwater and marine, provide useful research systems for gaining better insight into the mechanisms of biomineral nanofabrication and as potential sources for the large-scale production of medically important natural products. These features, coupled with their overall organizational simplicity, also make them useful research candidates for understanding the fundamental mechanisms of cellular recognition, adhesion, differentiation, and specialization (Gaino and Magnino, 1999; Müller et al., 1999). Although major breakthroughs in recent years have yielded much insight into the biology of the Porifera, the specific requirements for long-term maintenance of sustainable sponge cell cultures for many species have remained enigmatic. In addition, the molecular mechanisms underlying the formation of their mineralized skeletal frameworks, until only recently, have been largely unknown (Cha et al., 1999; Krasko et al., 2000, 2002; Shimizu et al., 1998).

This review will concentrate primarily on current research and future studies of the role of occluded axial filament protein scaffolds and their constituent subunits in directing the synthesis and morphology of siliceous sponge skeletal elements at the macro-, micro-, and nanoscale. In addition, we will discuss recent advances in the field of silicon biotechnology (Morse, 1999, 2001; Shimizu and Morse, 2000), including the design of biomimetic peptides and small molecules based on the catalytic and structure-directing moieties of these proteins for applications in the in vitro synthesis of highly ordered multifunctional silicon-based materials.

Contract grant sponsor: the U.S. Army Research Office Multidisciplinary University Research Initiative; Contract grant number: DAAH04-96-1-0443; Contract grant sponsor: the NOAA National Sea Grant Program, U.S. Department of Commerce; Contract grant number: NA36RG0537, Project E/G-2, through the California Sea Grant College System; Contract grant sponsor: the MRSEC Program of the National Science Foundation; Contract grant number: DMR-96-32716 (to the UCSB Materials Research Laboratory); Contract grant sponsor: Department of Energy; Contract grant number: DE-FG03-02ER46006.

*Correspondence to: D.E. Morse, Department of Molecular, Cellular, and Developmental Biology, Marine Biotechnology Center, Marine Science Institute, and the Materials Research Laboratory, University of California, Santa Barbara, CA 93106. E-mail: d_morse@lifesci.ucsb.edu

Received 3 February 2002; accepted in revised form 15 March 2003
DOI 10.1002/jemt.10401
Published online in Wiley InterScience (www.interscience.wiley.com).

AXIAL FILAMENTS AND SPICULE MORPHOLOGY

For many years the intracellular versus extracellular locus of megasclere synthesis in demosponges had been unresolved (Simpson, 1984), although recent work with *Crambe crambe* (Uriz et al., 2000) would seem (at least for this species) to support the latter hypothesis. In this proposed process, the central proteinaceous axial filament (Shore, 1972) is synthesized in its entirety, exocytosed to the mesohyl, and in a microenvironment created between a sclerocyte's pseudopodial plasma membrane and the axial filament, silica deposition takes place (Uriz et al., 2000).

It has long been known that most, if not all, siliceous sponge spicules (including those from both demosponges and hexactinellids) contain organic axial filaments. Although the most frequently observed cross-sectional shape of demosponge axial filaments is hexagonal (Rützler and Smith, 1993), we have observed that it can vary considerably from spicule to spicule, even within the same species, from regular and irregular hexagonal to triangular to nearly circular and is reflected in morphological variations of the surrounding axial channel (Fig. 1). Because of this apparent high degree of intraspecific phenotypic plasticity characteristic of demosponge axial filaments, their cross-sectional morphological characteristics should not be used as a basis for taxonomic characterization or for inferring degrees of evolutionary relatedness between species. Although this may be the case, there are marked differences in axial filament cross-sectional morphologies between hexactinellids and demosponges. Hexactinellid axial filaments are distinctively square in cross section (Reiswig, 1971), while, as discussed above, those from demosponges are hexagonal, or some derivative thereof.

These axial filaments have been proposed to play an essential role in dictating the gross morphological attributes of the spicules that contain them. While previous studies investigating these relationships have predominantly concentrated on analyses of monaxonal megascleres (Garrone, 1969; Simpson and Vaccaro, 1974; Weissenfels and Landschoff, 1977; Wilkinson and Garrone, 1980), there are several specific examples of tetraxonas that beautifully illustrate the roles of these axial filaments in the determination of spicule morphology (Simpson et al., 1985). The dichotriaenes from the temperate Eastern Pacific tetractinellid demosponge *Penares saccharis* clearly illustrate that the genetically predetermined synthesis of the silica precisely follows the contours of the bifurcated axial filaments (Fig. 2). A second dramatic illustration of this can be observed in the rare anomalies of spicule biosynthesis, such as dichotomous defects in strongyloxea from the hadromerid demosponge *Tethya aurantia*. Where the axial filament bifurcates, it precisely guides the deposition of annular siliceous deposits around this 3D organic template (Fig. 3). It should be noted that not all spicule features can be explained based on axial filament morphology. In megascleres, for example, the smallest superficial elaborations in the submicron range typically do not contain axial filament branches guiding their location on the spicule surface (Garrone et al., 1981). Therefore, the synthesis of these features may be determined by the silicalemma that surrounds and deposits the silica during spicule formation. Until recently, however, the detailed composition of the axial filaments and their actual role in spicule biosynthesis was largely unknown (Cha et al., 1999; Shimizu et al., 1998).

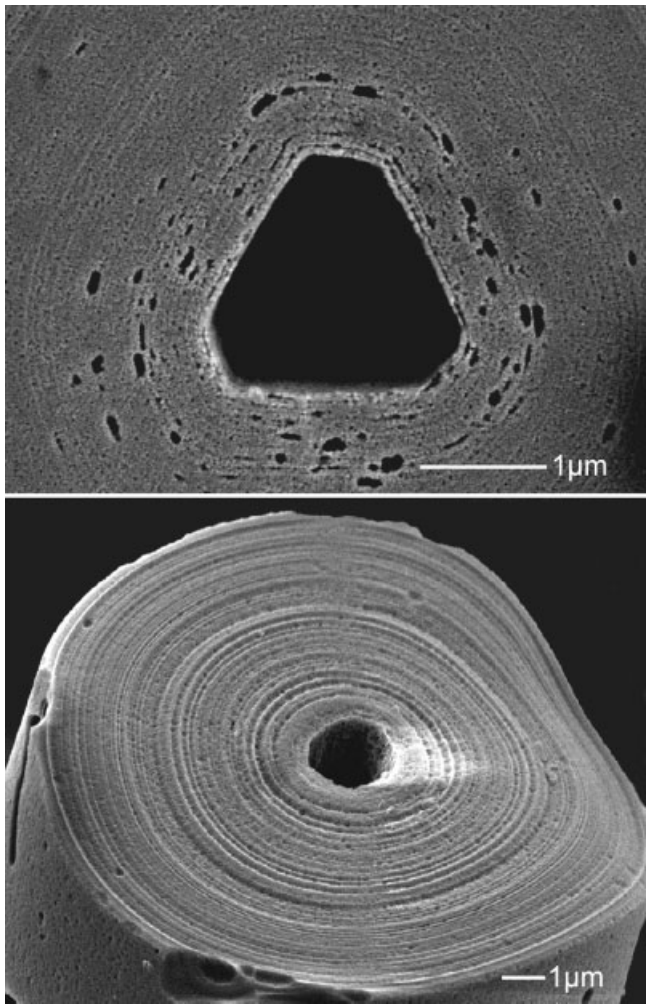

Fig. 1. Scanning electron micrographs of cross-sections through strongyloxea from the temperate Eastern Pacific demosponge, *Tethya aurantia*, revealing the diversity in axial channel morphologies. See also Fig. 3. Cross-sections include regular and irregular hexagonal, triangular, and nearly circular. The annular siliceous deposits surrounding the preexisting axial filaments are clearly visible.

SILICATEIN BIOCHEMISTRY AND SILICON BIOTECHNOLOGY

The temperate Eastern Pacific demosponge *Tethya aurantia* provides an excellent model system for studying the detailed molecular mechanisms of biosilicification. The supporting skeleton of this species is dominated by siliceous spicules (strongyloxea) measuring ~2 mm in length and 30μm in diameter (Fig. 4) which comprise ~75% of the dry weight of this organism (Cha et al., 1999; Shimizu et al., 1998). The large quantities of biosilica available make this species a uniquely tractable model system for investigating spicule formation.

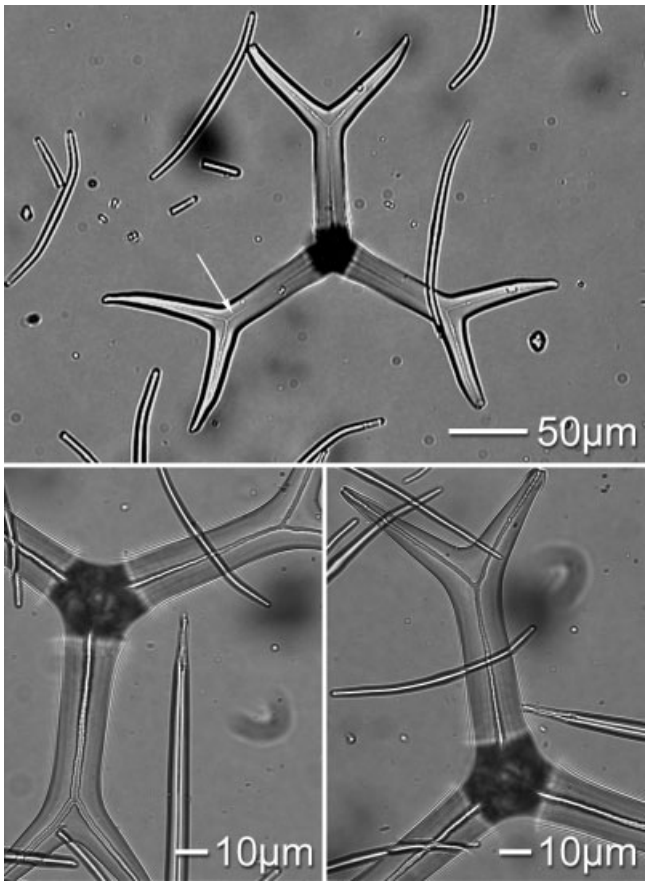

Fig. 2. Optical micrographs revealing locations of axial filaments in dichotriaenes from the temperate Eastern Pacific demosponge *Penares saccharis*. The upper image illustrates an unaltered spicule, the arrow indicating the axial filament. Similar spicules in the lower images have been treated with an alkaline etchant (NaOH), dissolving the distal portions of the megascleres and solubilizing the axial filaments. The remaining axial channel clearly illustrates the previous location of the axial filament.

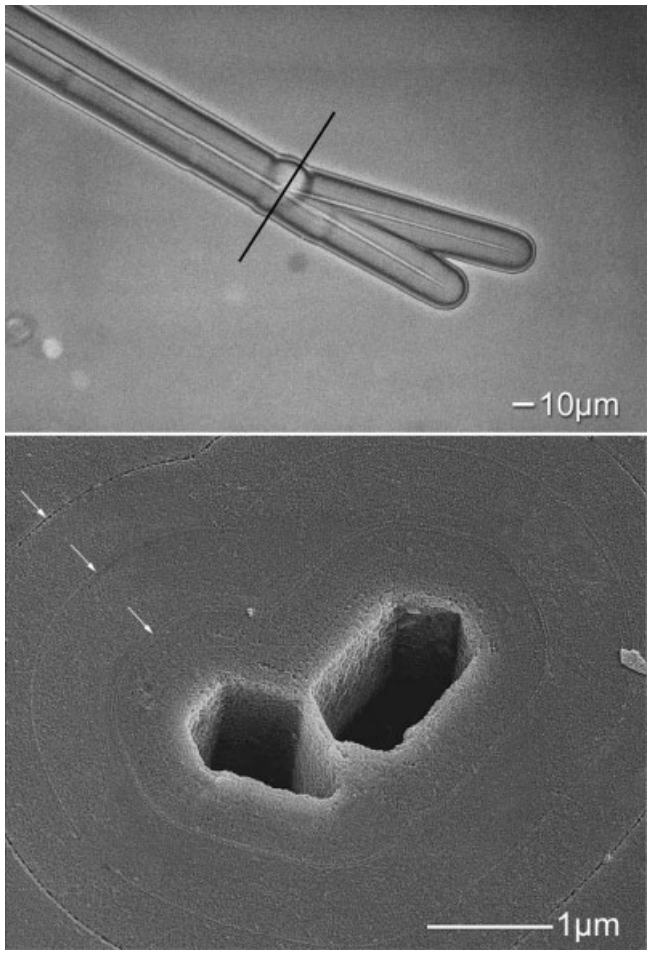

Fig. 3. Microscopic analyses of dichotomous defects in *Tethya aurantia* strongyloxea. The upper image illustrates the dramatic effect that axial filament anomalies can manifest in a mature megasclere. The black line indicates the location of sectioning for the lower SEM through a similar spicule. The lower SEM not only illustrates a rare instance of filament bifurcation (reflected in the morphology of the axial channel), but also clearly illustrates the essential role of the axial filaments in dictating the microscale arrangement of the deposited silica; the arrows indicate the successive annular deposits.

An additional advantage of this species is that unlike many other demosponges, *T. aurantia* lacks extensive intraspecific morphological variability, making in situ species identification nonproblematic. These attributes, coupled with its ease of culture under laboratory conditions, make *T. aurantia* a useful research subject.

The axial filaments occluded within the strongyloxea can easily be collected after demineralization of the surrounding silica with hydrofluoric acid. The isolated filaments (Fig. 5) measure ~2 μm in diameter and 2 mm in length and comprise ~0.5% of the spicule weight (Cha et al., 1999; Shimizu et al., 1998). These filaments consist primarily of three closely related protein subunits, named silicateins (for ***silica*** pro***teins***) α, β, and γ (Shimizu et al., 1998).

These axial filaments appear to have a previously unanticipated role in spicule formation. In addition to their role as templates or guides for silica deposition, a catalytic role is suggested by the discovery that the filaments can catalyze the hydrolysis and polycondensation of various silicon alkoxides, such as tetraethylorthosilicate (TEOS) and the methyl- and phenyl-triethoxysilanes, to yield silica and the corresponding silsesquioxanes (silicones) in vitro (Cha et al., 1999; Shimizu et al., 1998). In marked contrast to industrial conditions, this protein-catalyzed siloxane polycondensation occurs at ambient temperature and pressure and neutral pH.

While it is unlikely that simple silicon alkoxides are the in vivo precursors of biogenic silica polymerization, they may be related to conjugates of silicic acid through association with intracellular ionophores, or the formation of covalent complexes via condensation with alcohols, polyols, sugars, or catechols (Bhattacharyya and Volcani, 1983; Evans et al., 1990; Harrison, 1996; Harrison and Loton, 1995; Kinrade et al., 1999a,b; Perry and Yun, 1992) that may be formed prior to silica deposition and recently suggested to be involved in diatom biosilicification (Kinrade et al., 2002). In dia-

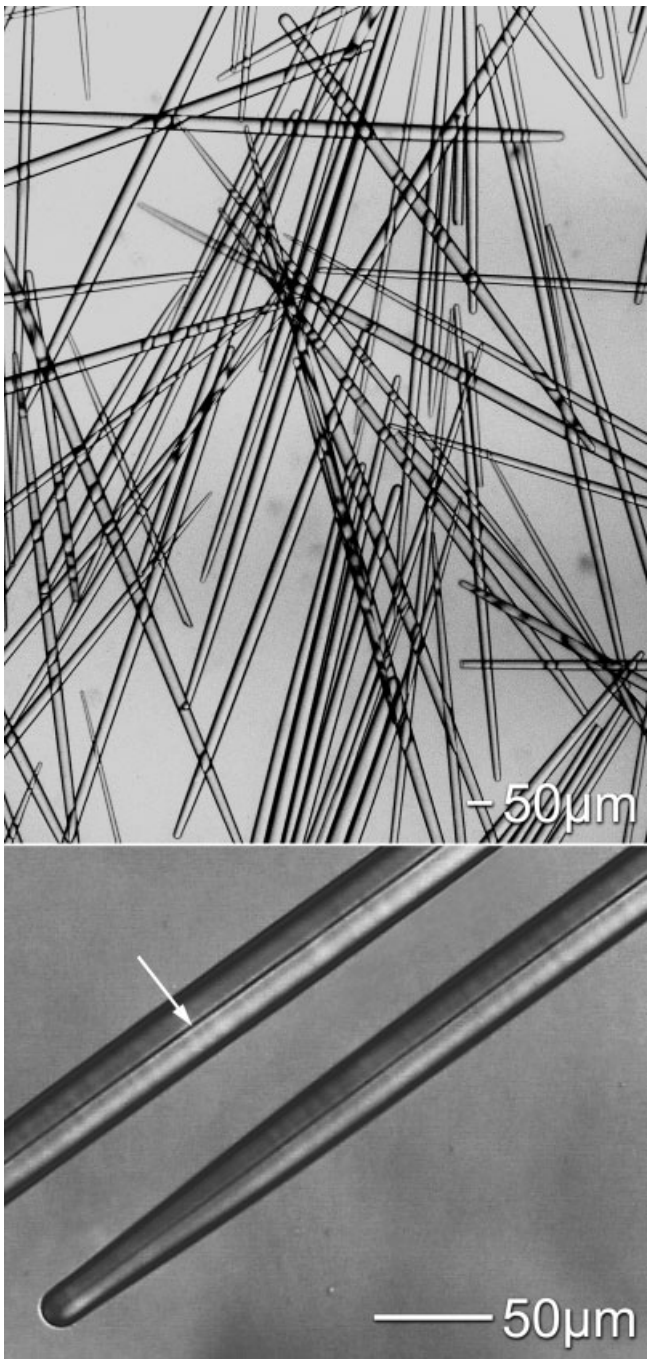

Fig. 4. Optical micrographs of strongyloxea from *Tethya aurantia* measuring ~30 μm in diameter and 2 mm in length. The lower image reveals the axial filaments (indicated by the arrow).

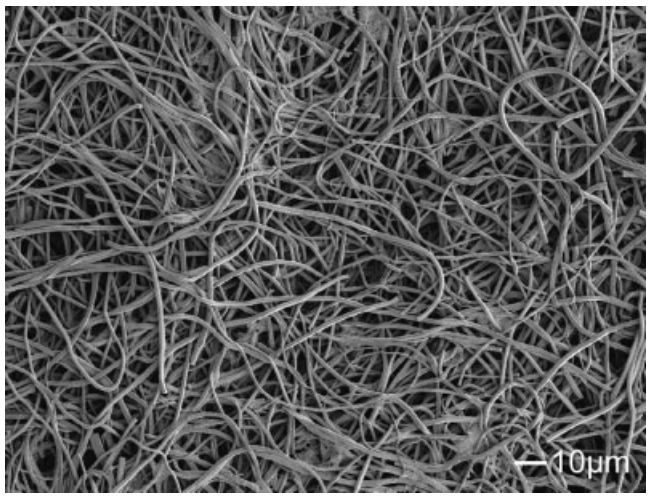

Fig. 5. Scanning electron micrograph of axial filaments collected from *Tethya aurantia* strongyloxea after demineralization with HF. Each filament measures ~2 μm in diameter and 2 mm in length.

toms, silicic acid is accumulated in intracellular pools at much higher concentrations than that at which it would otherwise normally spontaneously precipitate, suggesting some form of sequestration or chemical conjugation (Chisholm et al., 1978; Sullivan, 1986). If silicic acid is conjugated with organic moieties after transport and concentration in the sclerocytes during spicule formation in demosponges, the catalytic mechanism observed in vitro may be essential for the in vivo induction of silica condensation. Although there is currently no evidence to support the silicatein-mediated silicon alkoxide hydrolysis mechanism in vivo, further analyses of the early stages of spicule formation in demosponges can be expected to shed new insight into the biological role these proteins play in the mineralization process.

A second activity of the axial filaments is templating; in vitro, they guide the deposition of the polycondensed product along the entire filament length (Cha et al., 1999). The silica produced in vitro by this process is nanoparticulate (mean diameter ~70 nm), deposited on the filament in a coating several particles thick. Neither silk fibroin nor cellulose fibers, both of which possess high densities of surface hydroxyls (as does the axial filament), promote the condensation of silicon alkoxides at neutral pH, demonstrating that more than the simple presence of hydroxyl-rich domains are responsible for the observed activity (Cha et al., 1999).

Additional support for the suggestion that this mechanism of axial-filament-induced silica polycondensation may actually occur in vivo is provided by recent studies of the annular substructure of *Tethya aurantia* strongyloxea. Etching of intact spicules with hydrofluoric acid (Cha, 2001), and recent AFM analyses of spicule cross-sections, reveal that the deposited silica is nanoparticulate, with a particle diameter of ~50–80 nm (Weaver et al., submitted) forming alternating layers that are approximately monoparticulate in thickness, with characteristic etchant reactivity (Fig. 6) [Nanoparticles are the kinetically most favored form of silica deposited from solution (Iler, 1979) and this motif is also seen in the biosilica walls of diatoms (cf. below)]. Bütschli (1901) first observed this annular patterning of silica deposition in demosponge spicules, and Schwab and Shore (1971) proposed that the differential sensitivity to etching resulted from variations in water content, the more hydrated (and less condensed) zones corresponding to regions that etch more easily and are

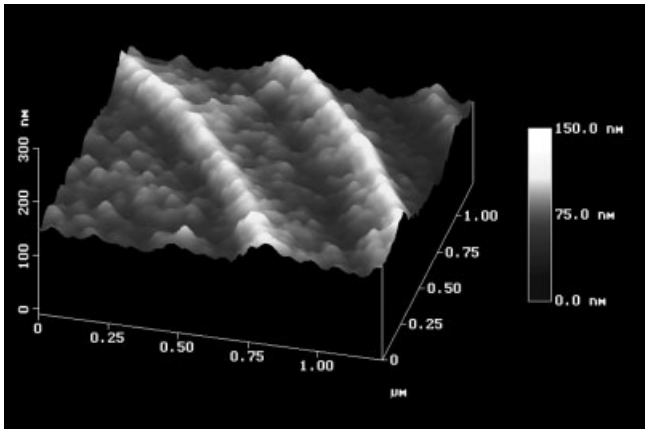

Fig. 6. Atomic force micrograph of a *Tethya aurantia* strongyloxea cross-section, illustrating the nanoparticulate nature of the deposited silica. Each particle measures ~50–80 nm in diameter.

less refractile. The annular deposits of silica nanoparticles are morphologically consistent with the product formed catalytically on the surfaces of purified axial filaments in vitro (Cha et al., 1999), and those obtained from the synthesis catalyzed by the silicatein monomers made from recombinant DNA templates cloned in bacteria (Fig. 7).

Small-angle X-ray diffraction analysis of the protein filaments reveals a distinct ultrastructural periodicity, suggesting that the macroscopic filaments are constructed from a highly regular, repeating subassembly of its protein subunits (Cha et al., 1999). These results are consistent with previous transmission electron microscopy studies that first revealed the apparent paracrystallinity of demosponge axial filaments (Garrone et al., 1981). While the templating activity of the silicateins is lost upon filament disaggregation and solubilization, the catalytic activity is retained by the constituent monomers. Heat denaturation of both the intact axial filaments and their constituent subunits abolishes their catalytic activity, suggesting that the native conformation of the proteins is required for their catalysis of silica polymerization (Cha et al., 1999; Zhou et al., 1999).

These subunits are present in the approximate ratio of α:β:γ = 12:6:1 (Shimizu et al., 1998), although the detailed distribution of the different proteins within individual filaments is not yet known. Electrophoretic characterization of silicateins α, β, and γ reveals that they are very similar in terms of both molecular weight and isoelectric point. The amino acid sequences of both silicatein α and β have been determined from the sequences of the cloned cDNAs, in conjunction with partial sequence analyses of the purified proteins initially isolated from the strongyloxea (Shimizu et al., 1998; Zhou, Cao, and Morse, unpubl.). Native silicatein α and β purified from the axial filaments, and those produced from recombinant DNA cloned in bacteria, yield functional, catalytically active proteins, indicating that the silicateins are in fact the constituents of the axial filaments responsible for their observed activity (Cha et al., 1999; Zhou et al., 1999).

Sequence analysis of silicateins α and β revealed the surprising fact that they are closely related to members

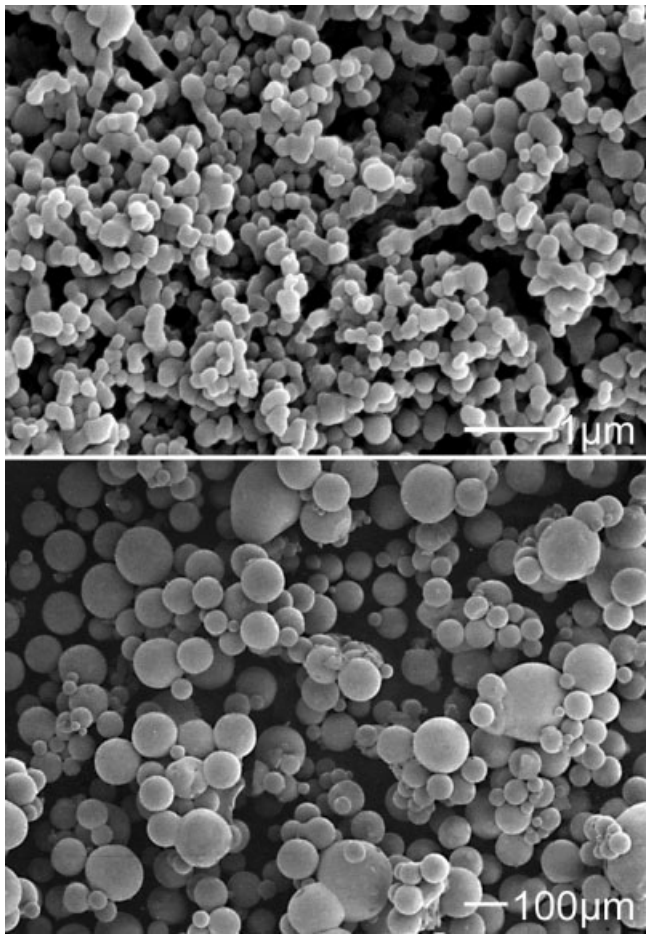

Fig. 7. SEMs of silica products synthesized from tetraethylorthosilicate (TEOS) at ~20°C, neutral pH, and atmospheric pressure. Upper: Silica nanoparticles grown in the presence of recombinantly derived silicatein β, the second-most abundant of the *Tethya aurantia* silicateins. Lower: Glassy mesoporous microspheres grown in the presence of the reduced form of the biomimetic L-Cys_{30}-L-Lys_{200} block copolypeptide (Cha et al., 2000).

of the papain superfamily of cysteine proteases. Computer-assisted alignment reveals that silicatein α, the most abundant of the axial filament protein constituents, contains nearly 50% amino acid identity to a well-known human digestive enzyme, cathepsin L (Fig. 8). Not only are all of the cysteine residues involved in the formation of intramolecular disulfide crosslinks conserved between these two molecules but are also the locations of the active site residues (cysteine, histidine, and asparagine in cathepsin L and serine, histidine, and asparagine in silicatein α), suggesting that the 3D conformations of these two proteins are highly similar (Cha et al., 1999; Shimizu et al., 1998).

Amino acid sequence analysis of the n-terminal flanking regions of the protein involved in directing cellular trafficking during protein maturation reveals an additional degree of relatedness between the biosilica associated silicatein α and the cathepsin L protease. Both hydrolases are successively modified by sequence-specific proteases involved in peptide cleavage during incorporation into membrane-bound vesi-

Fig. 8. Comparison of the amino acid sequence of *Tethya aurantia* silicatein α and human cathepsin L. The vertical bars (|) indicate identical residues shared between the two proteins and the colons (:) indicate those residues with biochemical or codon similarity. The locations of the conserved active site residues are indicated by black boxes; the cysteine residues (C), responsible for the formation of intramolecular disulfide crosslinks, are dotted [Adapted from Shimizu et al. (1998)].

cles and subsequent 3D conformational folding. These findings, the hydrolytic functionalities and their vesicular packaging, all strongly suggest that the demosponge silicateins and the papain family of lysosomal proteases have evolved from a common molecular ancestor (Cha et al., 1999; Shimizu et al., 1998; Krasko et al., 2000).

Based on these observations, we have suggested that the silicateins' putative active-site serine-histidine sidechain couple functions as a general acid-base catalyst of the rate-limiting hydrolysis of the silicon alkoxides, in a manner parallel to the catalysis of peptide bond hydrolysis by the homologous cysteine-histidine pair in the proteolytic enzymes (Fig. 9). In the proposed silicatein α-mediated reaction, hydrogen bonding between the imidizole nitrogen of the conserved histidine 165 (at position 165 in the protein chain) and the hydroxyl of the active site serine (at position 26) is postulated to increase the nucleophilicity of the serine oxygen, facilitating its attack on the silicon atom. Nucleophilic attack on the silicon atom would then displace ethanol, with the formation of a transitory covalent protein-O-Si intermediate that is structurally analogous to the enzyme-substrate intermediate formed by the protease and other hydrolases. This intermediate might be further stabilized as a pentavalent silicon species via a donor bond from the imidizole nitrogen. Addition of water would then complete the hydrolysis of the first alkoxide bond (the rate-limiting step at neutral pH), with condensation then initiated by nucleophilic attack of the released Si-OH on a second molecule of substrate (Cha et al., 1999; Shimizu et al., 1998).

Site-directed mutagenesis experiments performed with recombinant silicatein DNA expressed in bacteria confirmed the suggested roles of the putative active site residues in silicatein α (Zhou et al., 1999). Replacement of the active site serine (with a hydroxyl sidechain) and/or histidine (with its H-bonding imidizole sidechain) with amino acids of significantly different structural motifs (e.g., alanine with a methyl sidechain) dramatically lowered the catalysis of silica formation from the alkoxide substrate. The requirement for histidine was further substantiated by the partial rescue of activity of the histidine-replaced mutant protein by supplementation with exogenous imidizole (Merget et al., in prep.). Similar results were also obtained by site-directed mutagenesis studies of the β subunit (Cao et al., in prep.).

Kinetic analyses confirm that silicatein α reacts with the silicon alkoxides in a typical enzyme-like manner, displaying substrate saturation with true Michaelis-Menten kinetics, although the rate of reaction is quite slow compared to that of the related proteases (Zhou et al., in prep.). In site-directed mutagenesis studies with proteolytic enzymes, replacement of either of the two residues required for activity typically results in a reduction in catalytic activity by several orders of magnitude (Carter and Wells, 1988), while in mutagenized silicatein α, the loss of activity is only on the order of ~90%. This might suggest that by sacrificing speed in the hydrolysis of the silica precursor, the protein may be able to exert a greater degree of structural control over the polymerization process. Relatively low rates of silica formation may also facilitate the precise fabrication of the superficial elaborations often observed on sponge skeletal elements (Hartman, 1981). This activity might be further regulated, either at the level of the protein or the delivery of substrate to produce pauses and reinitiation of silica deposition, resulting in the

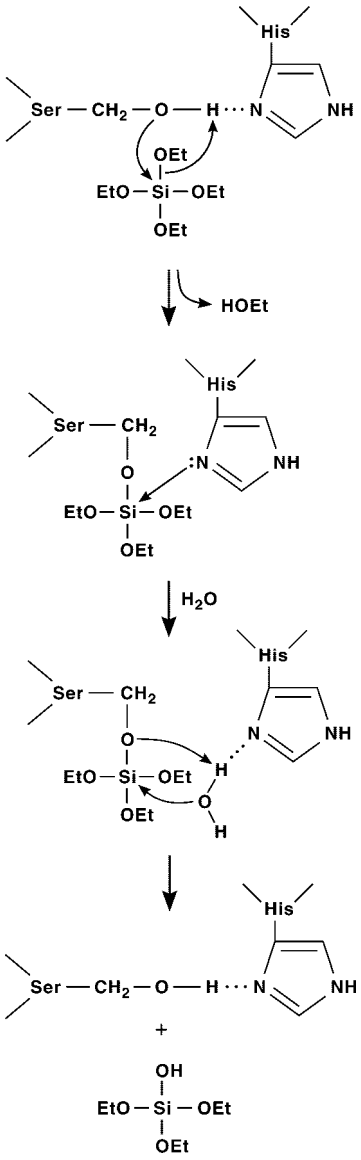

Fig. 9. Proposed mechanism of silicatein-mediated silicon alkoxide hydrolysis modeled after the known catalysis of the related proteases. This reaction illustrates the generation of the reactive silanol, the rate-limiting step in the polymerization and condensation of silica at neutral pH [Adapted from Cha et al. (1999)].

annular deposits of differentially condensed silica described by Schwab and Shore (1971) (cf. Fig. 6). This annular construction may also result in the enhanced mechanical properties of demosponge spicules, as reported for spicules from the hexactinellids, *Monorhaphis sp*. and *Rosella racovitzae*, compared to synthetic silica fibers of similar dimensional scales (Levi et al., 1989; Sarikaya et al., 2001).

Based on the identification of the catalytic residues in the silicatein molecule confirmed by the mutagenesis studies described above, we synthesized di-block copolypeptides that mimic both the catalytic and structure-directing activities of the protein (Cha et al., 2000). The block copolypeptides were synthesized using lysine and cysteine amino acid N-carboxyanhydride monomers (Deming, 1997) with a final stoichiometry of L-cysteine$_{30}$-L-lysine$_{200}$. In these synthetic peptides, the cysteine residues provide the nucleophilic sidechains and the lysine residues provide the required hydrogen-bonding amine functionalities. When in their reduced form, these polymers self-assemble into 600 nm-diameter aggregates and when combined with TEOS in a biphasic emulsion, catalyze the formation of mesoporous silica microspheres measuring ~100 μm in diameter (Fig. 7). Interestingly, when the sulfhydral sidechains of the cysteines are partially oxidized, causing the peptides to form multichain concatenates, reaction with the silicon alkoxide yields ordered bundles of fibrillar silica. These experiments suggest that the 3D architecture of these self-assembled block-copolypeptides is an important determinant of the structure of the final product. Based on these results and further mutagenesis studies, we are examining additional domains of the silicatein molecules that may prove important in controlling the order and orientation of the polymerized siloxane product (Cha et al., 2000). This information should prove useful in the development of engineered proteins, block copolypeptides, and small molecules with desired catalytic functionalities for the structurally controlled in vitro synthesis of silicas and organically substituted polysiloxanes with custom-tailored optical or electronic properties (Morse, 1999, 2001; Shimizu and Morse, 2000).

The silica-precipitating proteins isolated from diatom frustules appear to be unrelated to the silicateins, and likely to have evolved independently. The silaffins (named for their affinity for silica) are found occluded within the silicified cell walls of *Cylindrotheca fusiformis* and other diatom species (Kröger et al., 1999; 2000; Kröger and Sumper, 2000). These proteins are extensively posttranslationally modified with unique polyamine and phosphate functionalities added to specific amino acid sidechains (Kröger et al., 1999, 2002; Kröger and Sumper, 2000). When added to metastable silicic acid, these peptides induce the rapid polycondensation and synthesis of silica nanospheres with fairly uniform morphologies. Similar silica polymerization activities previously had been observed for pollyallylamine and other synthetic polyamines (Mizutani et al., 1998). Recently, it has been demonstrated that in addition to the species-specific silaffins, diatom frustules also contain high levels of free (nonpeptide-linked) longchain polyamines (Kröger et al., 2000; Kröger and Sumper, 2000). Not only are these polyamines active in promoting the precipitation of silica, but the resulting condensed silica spheres exhibit a dramatic polyamine-specific size distribution. Significantly, the sizes of the silica nanoparticles produced by the silaffins and polyamines in vitro are similar to those recently revealed by AFM investigations of intact frustules (Noll et al., 2001). In addition to the dramatic structural difference between the silicateins and the silaffins, their mechanisms of action are also distinct; the demosponge silicateins are catalytic (enzymatic) while the diatom polyamines act stoichiometrically.

CONCLUSIONS AND FUTURE PROSPECTS

While silicatein α and β are by far the most abundant proteins found within axial filaments of *Tethya aurantia* strongyloxea, they are by no means the only ones. High-resolution SDS-PAGE analyses indicate traces of at least six additional minor constituents ranging in molecular weight from less than 10 kDa to greater that 80 kDa. While the sequences and functions of these proteins have yet to be determined, it is possible that they may play critical roles in the formation and stabilization of the axial filaments or the templated biosilica.

One remaining paradox in understanding demosponge spicule biosynthesis is the mechanism by which silica deposition continues once the proteinaceous axial filament has become completely covered during the initial stages of spicule formation. The silica polymerization activity reported previously for the silicateins revealed that its mechanism of action is catalytic and not stoichiometric. Based on our observations, and previous reports that this catalytic activity is retained by the individual subunits liberated after axial filament solubilization (Cha et al., 1999), it is possible that the controlled punctuated secretion of monomeric silicateins, pulses in the transitory flux of silica-precursor molecules, oscillations in the pH, ionic or other conditions of the condensation environment, or a combination of these factors may be responsible for the annular patterning and continued silica deposition during spicule biosynthesis in vivo once the axial filament has become completely covered during the early stages of silica deposition around this proteinaceous core.

As mentioned above, in addition to the demosponges, hexactinellids also synthesize siliceous spicules and many are well known for their production of truly remarkable skeletal elements. One of the best examples of this is found in specimens of the hexactinellid *Monorhaphis sp*. collected from the Indian Ocean. This deep sea sponge synthesizes the largest known silicified skeletal element of any metazoan studied to date. Skeletal support as well as benthic anchoring is provided by a single siliceous spicule, measuring up to 3 m in length and almost 1 cm in diameter (Chun, 1900). Although monolithic spicules such as those found in *Monorhaphis sp.* are rare among the Hexactinellida, there are other remarkable skeletal modifications such as those exhibited by *Euplectella aspergillum* that are frequently encountered in this sponge class. In this species, a sediment-dwelling hexactinellid from the Western Pacific, the supporting skeletal framework consists of hypersilicified hexactine megascleres that are consolidated into a 3D silicified cylindrical lattice (Fig. 10). While many lithistid demosponges also exhibit articulated siliceous networks (Hartman, 1981; Simpson, 1984), the consolidation of the spicules is one more of interdigitation* (Fig. 11) and does not typically occur to the same extent as in many of the hexactinellids.

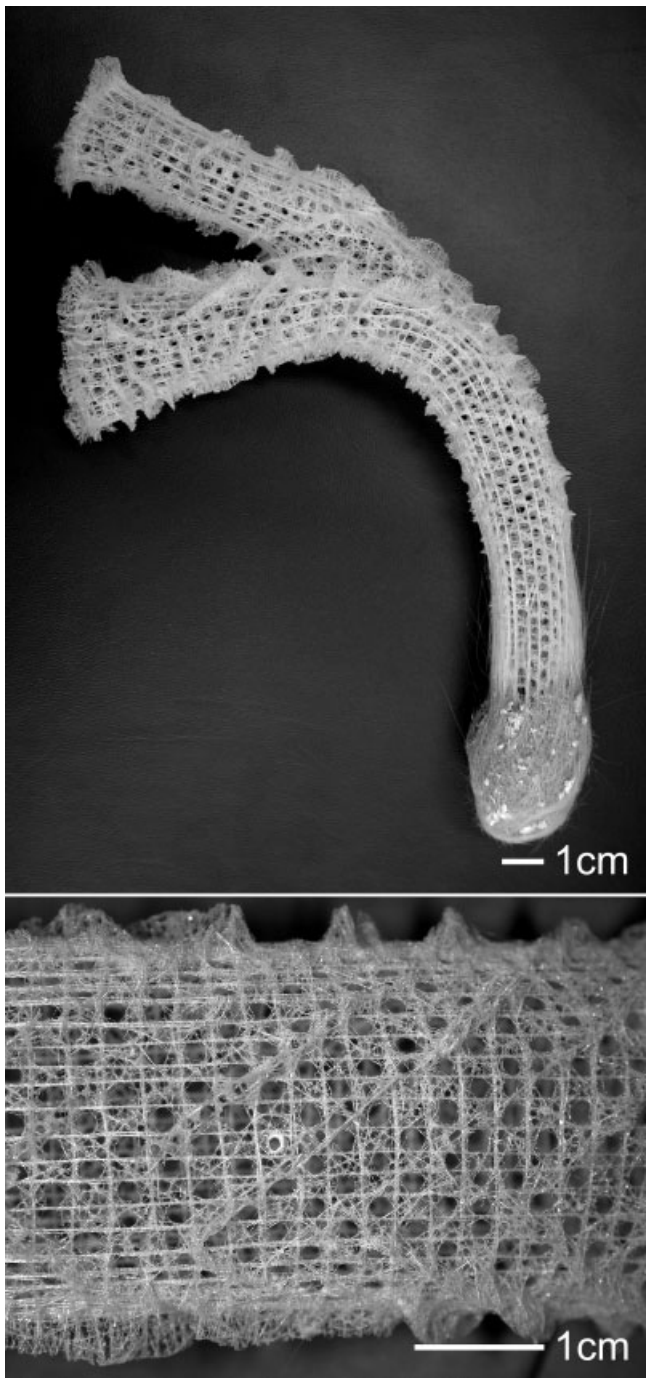

Fig. 10. Photograph of an atypical bifurcated specimen of the sediment-dwelling hexactinellid, *Euplectella aspergillum*. (Upper): A portion of the mineralized skeleton from a similar specimen illustrating the intricate consolidated spicular lattice composed almost entirely of hypersilicified hexactine megascleres (Lower).

Although the detailed interrelationships of the poriferan classes still remain unresolved (Adams et al., 1999; Cavalier-Smith et al., 1996; Kruse et al., 1998),

*The terminal branches of the desmas (antler-like spicules) and their remarkable interlocking connections (Fig. 11) are reported to extend beyond the limits of the occluded axial filaments (Simpson, 1984) and thus raise perplexing questions about the possible mechanisms controlling their mutually conforming higher-order structural organization.

recent molecular evidence from 18s rDNA sequences suggests an early divergence of the Hexactinellida from the ancestral poriferan stock that gave rise to the demosponge and the Calcarea/eumetazoan lineages (Borchiellini et al., 2001). This proposed early hexactinellid divergence is supported by the observation that the cellular anatomy of this group is so markedly different from the Calcarea and Demospongiae that some taxonomists believe this ancient lineage should be elevated to the rank of phylum (Bergquist, 1985; Borojevic, 1970). Hexactinellids exist predominately as multinucleate syncytia and although single cells, functionally comparable to amoebocytes and archeocytes of demosponges are present, they are closely associated with the syncytia and exhibit limited mobility. An additional feature of hexactinellids is that the choanocytes, which appear to be discontinuous from the syncytial networks, lack nuclei (Boury-Esnault and De Vos, 1988; Ijima, 1904; Mackie and Singla, 1983; Reiswig and Mackie, 1983; Schulze, 1880, 1899). Although it is possible that these unusual characteristics of hexactinellids are secondarily derived, their relatedness to the Demospongiae remains questionable. It has also been proposed that silica biomineralization in sponges evolved first, followed later by the evolution of calcareous biomineralization, suggesting that evolutionarily, the Calcarea are most recently diverged (Adams et al., 1999; Brasier et al., 1997).

While this may be the case, we have recently discovered a marked difference in spicule axial filament protein composition of hexactinellids compared to those obtained from demosponges. Preliminary SDS-PAGE analysis of axial filament proteins from the hexactinellid *Euplectella aspergillum* reveals that they are dominated by proteins of higher molecular weights than those isolated from demosponges. Although the amino acid composition of these protein filaments is similar to those isolated from *Tethya aurantia*, suggesting the possibility that demosponge silicateins might be truncated versions of their hexactinellid counterparts, it is at this point equally likely that further sequence analysis and structural characterization of these proteins may reveal that the formation of silicified skeletal elements has evolved independently within these two groups and that the presence of axial filaments within these spicules may be convergently derived.

Although there exists a remarkable diversity in demosponge megasclere morphology, the most intricate of the siliceous sponge skeletal elements are indeed the microscleres (Hartman, 1981) (Fig. 12). In contrast to the possible extracellular locus of megasclere formation recently observed in *Crambe crambe* (Uriz et al., 2000), microsclere biosynthesis appears to be an intracellular process and seems at least in some species to occur in dense aggregations of microsclerocytes, frequently restricted to specific regions of the sponge. Currently, many of the reported observations of microsclere biosynthesis in demosponges seem to indicate that this is a dynamic, highly variable, and species- or spicule-specific process (Garrone et al., 1981; Simpson, 1984). Because of these discrepancies, additional research is required in this area before any generalities regarding microsclere biosynthetic mechanisms can be made with confidence.

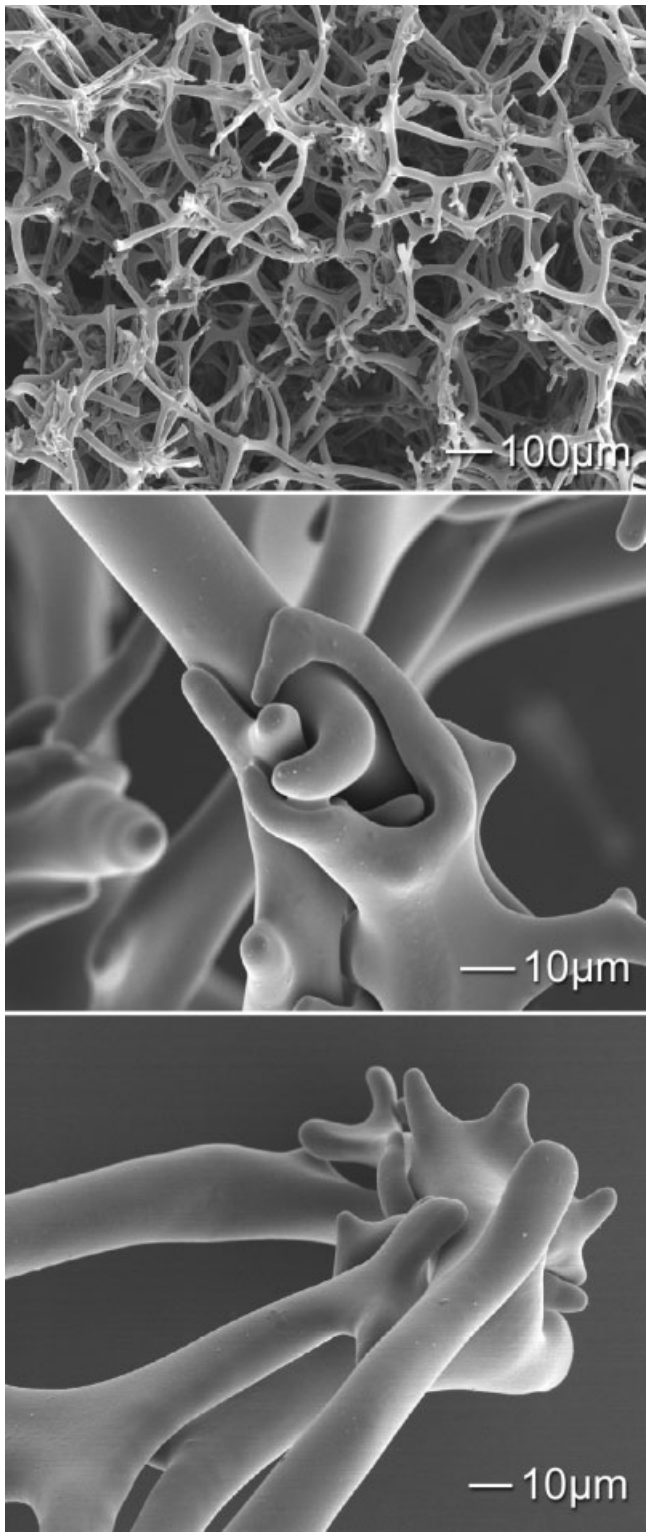

Fig. 11. Articulated 3D network of interconnected desmas (antler-like spicules) from the Caribbean lithistid demosponge *Discodermia dissoluta*. The lower two images illustrate sites of spicule interdigitation characteristic of this complex skeletal system.

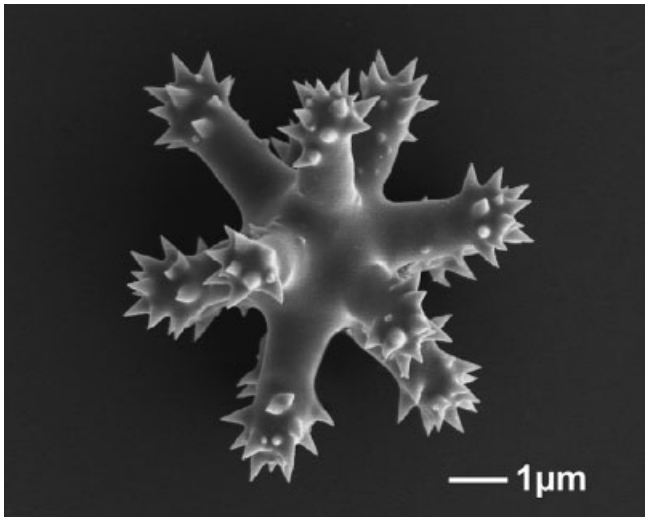

Fig. 12. SEM of an acanthaster isolated from the cortex of *Tethya aurantia*. These are the smallest and most numerous microsclere present in this species.

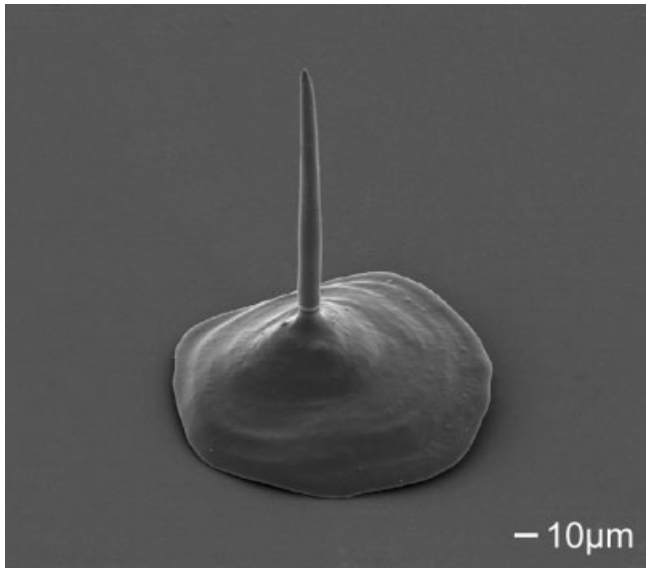

Fig. 13. Scanning electron micrograph of a discotriaene from the Caribbean lithistid demosponge *Discodermia dissoluta*.

Due to the structural complexity of many microscleres, they seem to be ideal candidates for investigating the mechanisms governing nanoscale silica deposition in 3D space. While microsclere abundance in many species can be quite high, their contribution to the total skeletal mass is often negligible. In *T. aurantia* for example, the microscleres typically comprise less than 1/1000th of 1% of the total spicule mass in this species. Although there has been some work in recent years investigating the role that environmental and micronutrient variability can exert on the synthesis of microscleres (Maldonado et al., 1999), for the most part, because of the complication associated with their manipulation, they have primarily remained items of curiosity, principally of taxonomic significance. The presence of discotriaenes (tack-like spicules) (Fig. 13), for example, proves useful for the unequivocal identification of sponges such as *Discodermia spp*.

Even more obscure than the mechanisms governing the biosynthesis of the microscleres is their functional significance. It is possible that these silicified structures might provide either small scale or transient skeletal support, their small size perhaps making them ideal for either or both of these roles (Bergquist, 1978; Hartman, 1981; Simpson, 1984). In *T. aurantia* (as is common for many sponge species), microsclere synthesis, while occurring sparsely in the choanosome, is predominantly restricted to the sponge cortex. Because of their small size, these skeletal elements may be more easily mobilized than the megascleres during the reorganization of this region of the sponge during normal sponge growth. During cellular reorganization, they may also provide small-scale support, affording localized microscale structuring before the consolidation of ordered cellular aggregates takes place. The high concentrations of microscleres in the cortex in many sponge species also might reflect the inability of the microsclerocytes to successfully compete with megasclerocytes for available dissolved silica precursors. This idea may be supported by Maldonaldo's studies with the Mediterranean demosponge *Crambe crambe*, demonstrating that small sponge cellular aggregates grown under elevated concentrations of dissolved silicate exhibited a dramatic increase in isochele biosynthesis (Maldonado et al., 1999). These results are further supported by our recent work examining spicule biosynthesis in cortical explants of *T. aurantia*. Although both rate and quantity of spheraster microscleres (Fig. 14) synthesized in intact animals are quite low, cortical explants, when grown under conditions of comparable ectosomal and choanosomal regenerative rates and at ambient concentrations of dissolved silicate, exhibit increased rates of microsclere synthesis when spatially isolated from the megasclerocytes of the sponge interior (Weaver et al., in prep.). While these findings are consistent with competitive domination by the megasclerocytes under conditions of limiting silica precursor, other possibilities have not been excluded. Müller and colleagues (Krasko et al., 2000) recently demonstrated that expression of silicatein genes is regulated by the concentration of dissolved silicate. It is possible that the threshold for synthesis activation of these and other spicule components might be differentially regulated in mega- and microsclerocytes. Additional research is needed, however, if we are to truly understand the biosynthetic regulatory mechanisms and adaptive significance of microsclere structural diversity exhibited in the Demospongiae and the Hexactinellida.

Although much has been learned in recent years regarding the mechanisms of spicule formation in sponges, there are still many unknowns concerning the molecular precursors of this synthesis, and the factors responsible for control of structure at the macro-, micro-, and nanoscale. New advances in high-resolution electron and atomic force microscopy, nuclear magnetic resonance spectroscopy, and the latest biotechnological

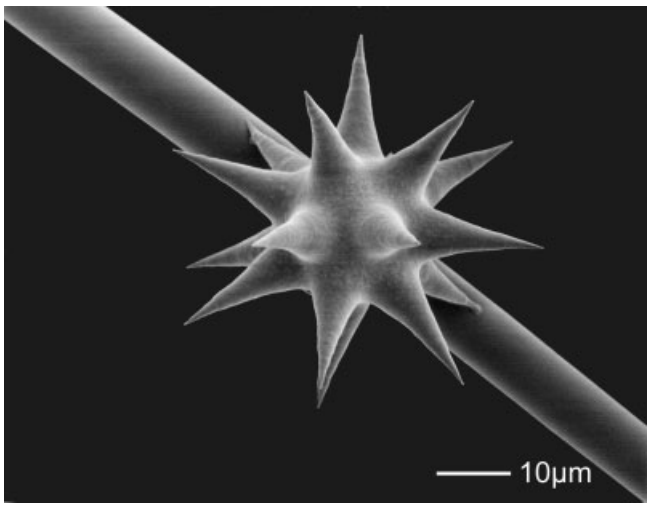

Fig. 14. SEM of a typical spheraster from *Tethya aurantia* (foreground) and an unusually small strongyloxea (background).

innovations in protein structure determination, site-directed and combinatorial mutagenesis, and immunohistochemical localization should help in future investigations of the fabrication of some of nature's truly unique engineering marvels, the mineralized skeletal elements of the Porifera.

ACKNOWLEDGMENTS

This article draws on the research of many colleagues from a number of departments. In particular, we thank Jennifer N. Cha, Katsuhiko Shimizu, Yan Zhou, Jixiang Cao, Camille Lawrence, Lía I. Pietrasanta, Jan L. Sumerel, Pablo F. Cabrera, Mark A. Brzezinski, Galen D. Stucky, Paul K. Hansma, and Brad F. Chmelka for allowing us to report our collaborative research and Larry J. Friesen for the light micrographs in Figures 3 and 4 (top image).

REFERENCES

Adams CL, McInerney JO, Kelly M. 1999. Indications of relationships between poriferan classes using full-length 18s rRNA gene sequences. Mem Queensl Mus 44:33–43.

Bhattacharyya P, Volcani BE. 1983. Isolation of silicate ionophore(s) from the apochlorotic diatom *Nitzschia alba*. Biochem Biophys Res Commun 114:365–372.

Bergquist PR. 1978. Sponges. Berkeley: University of California Press.

Bergquist PR. 1985. Poriferan relationships. In: Morris SC, George JD, Gibson R, Pratt HM, editors. The origins and relationships of lower invertebrates. Oxford: Clarendon Press. p 14–27.

Borchiellini C, Manuel M, Alivon E, Boury-Esnault N, Vacelet J, Le Parco Y. 2001. Sponge paraphyly and the origin of Metazoa. J Evol Biol 14:171–179.

Borojevic R. 1970. Différentiationcellulaire dans l'embryogénèse et la morphogénèse chez les Spongiaires. In: Fry WG, editor. The biology of the porifera. London: Academic Press. p 467–490.

Boury-Esnault, De Vos L. 1988. *Caulophacus cyanae n. sp.*, une éponge hexactinellide des sources hydrothermales. Biogéographie du genre *Caulophacus* Schulze, 1887. Oceanol Acta 8:51–60.

Brasier M, Green O, Shields G. 1997. Ediacarian sponge spicule clusters from southwestern Mongolia and the origin of the Cambrian fauna. Geology 25:303–306.

Bütschli O. 1901. Einige Beobachtungen über Kiesel- und Kalknadeln von Spongien. Z Wiss Zool 69:235–286.

Carter P, Wells JA. 1988. Dissecting the catalytic triad of a serine protease. Nature 332:564–568.

Cavalier-Smith T, Allsopp MTEP, Chao EE, Boury-Esnault N, Vacelet J. 1996. Sponge phylogeny, animal monophyly, and the origin of the nervous system: 18s rRNA evidence. Can J Zool 74:2031–2045.

Cha JN. 2001. Lessons from nature: novel routes to biomimetic synthesis of silica based materials. Ph.D. Thesis, University of California, Santa Barbara.

Cha JN, Shimizu K, Zhou Y, Christiansen S, Chmelka BF, Stucky GD, Morse DE. 1999. Silicatein filaments and subunits from a marine sponge direct the polymerization of silica and silicones in vitro. Proc Natl Acad Sci USA 96:361–365.

Cha JN, Stucky GD, Morse DE, Demming TJ. 2000. Biomimetic synthesis of ordered silica structures mediated by block polypeptides. Nature 403:289–292.

Chisholm SW, Azam F, Eppley RW. 1978. Silicic acid incorporation in marine diatoms on light:dark cycles: use as an assay for phased cell division. Limnol Oceanogr 23:518–529.

Chun C. 1900. Aus den Tiefen des Weltmeers.

Deming TJ. 1997. Facile synthesis of block copolypeptides of defined architecture. Nature 390:386–389.

Evans DF, Par J, Coker EN. 1990. Nuclear-magnetic-resonance studies of silicon (IV) complexes in aqueous solution. I. Tris-catecholato complexes. Polyhedron 9:813–823.

Gaino E, Magnino G. 1999. Dissociated cells of the calcareous sponge *Clathrina*: a model for investigating cell adhesion and cell motility in vitro. Microsc Res Tech 44:279–292.

Garrone R. 1969. Collagen, spongin, and mineral skeleton in sponge *Haliclona rosea* (OS) (Demospongiae, Haplosclerina). J Microsc (Paris) 8:581–598.

Garrone R, Simpson TL, Pottu-Boumendil J. 1981. Ultrastructure and deposition of silica in sponges. In: Simpson TL, Volcani BE, editors. Silicon and siliceous structures in biological systems. New York: Springer. p 495–525.

Harrison CC. 1996. Evidence for intramineral macromolecules containing protein from plant silicas. Phytochemistry 41:37–42.

Harrison CC, Loton N. 1995. Novel routes to designer silicas—studies of the deposition of $(M(+))(2)[Si(C_6H_4O_2)_3] \cdot XH_2O$—Importance of M(+) identity of the kinetics of oligomerization and the structural characterization of the silicas produced. J Chem Soc Faraday Trans 91:4287–4297.

Hartman WD. 1981. Form and distribution of silica in sponges. In: Simpson TL, Volcani BE, editors. Silicon and siliceous structures in biological systems. New York: Springer. p 453–493.

Ijima I. 1904. Studies on the Hexactinellida, contribution IV (Rossellidae). J Coll Sci 18:1–303.

Iler RK. 1979. The chemistry of silica; solubility, polymerization, colloidal and surface properties, and biochemistry. New York: John Wiley & Sons.

Kinrade SD, Del Nin JW, Schach AS, Sloan TA, Wilson KL, Knight CTG. 1999a. Stable five- and six-coordinated silicate anions in aqueous solution. Science 385:1542–1545.

Kinrade SD, Maa KJ, Schach AS, Sloan TA, Knight CTG. 1999b. Silicon-29 NMR evidence of alkoxy substituted aqueous silicate anions. J Chem Soc Dalton Trans 3149–3150.

Kinrade SD, Gillson A-ME, Knight CTG. 2002. Silicon-29 NMR evidence of a transient hexavalent silicon complex in the diatom *Navicula pelliculosa*. J Chem Soc Dalton 3:307–309.

Krasko A, Lorenz B, Batel R, Schröder HC, Müller IM, Müller WEG. 2000. Expression of silicatein and collagen genes in the marine sponge *Suberites domuncula* is controlled by silicate and myotrophin. Eur J Biochem 267:1–11.

Krasko A, Schroder HC, Batel R, Grebenjuk VA, Steffen R, Müller IM, Müller WEG. 2002. Iron induces proliferation and morphogenesis in primmorphs from the marine sponge *Suberites domuncula*. DNA Cell Biol 21:67–80.

Kröger N, Sumper M. 2000. The biochemistry of silica formation in diatoms. In: Baeuerlein E, editor. Biomineralization: from biology to biotechnology and medical application. Weinheim: Wiley-VCH. 151–170.

Kröger N, Deuzmann R, Sumper M. 1999. Polycationic peptides from diatom biosilica that direct silica nanosphere formation. Science 286:1129–1132.

Kröger N, Deutzmann R, Bergsdorf C, Sumper M. 2000. Species-specific polyamines from diatoms control silica morphology. Proc Natl Acad Sci USA 97:14133–14138.

Kröger N, Lorenz S, Brunner E, Sumper M. 2002. Self-assembly of highly phosphorylated silaffins and their function in biosilica morphogenesis. Science 298:584–586.

Kruse M, Leys SP, Müller IM, Müller WEG. 1998. Phylogenetic position of the hexactinellida within the phylum porifera based on the amino acid sequence of the protein kinase C from *Rhabdocalyptus dawsoni*. J Mol Evol 46:721–728.

Levi C, Barton JL, Guillemet C, Lebras E, Lehuede P. 1989. A remarkably strong natural glassy rod—the anchoring spicule of the *Monorhaphis* sponge. J Mat Sci Lett 8:337–339.

Li HY, Matsunaga S, Fusetani N. 1998. Antifungal metabolites from marine sponges. Curr Org Chem 2:649–682.

Mackie GO, Singla CL. 1983. Studies on hexactinellid sponges. I. Histology of *Rhabdocalyptus dawsoni* (Lambe, 1873). Philos Trans R Soc Lond 301:365–400.

Maldonado M, Carmona MC, Uriz MJ, Cruzado A. 1999. Decline in Mesozoic reef-building sponges explained by silicon limitation. Nature 401:785–788.

Mizutani T, Nagase H, Fujiwara N, Ogoshi H. 1998. Silicic acid polymerization catalyzed by amines and polyamines. Bull Chem Soc Jpn 71:2017–2022.

Morse DE. 1999. Silicon biotechnology: harnessing biological silica production to construct new materials. Trends Biotechnol 17:230–232.

Morse DE. 2001. Biotechnology reveals new routes to synthesis and structural control of silica and polysilsesquioxanes. In: The chemistry of organic silicon compounds. New York: John Wiley & Sons.

Müller WEG, Wiens M, Batel R, Steffen R, Schröder HC, Borojevic R, Custodio MR. 1999. Establishment of a primary cell culture from a sponge: primmorphs from *Suberites domuncula*. Mar Ecol-Prog Ser 178:205–219.

Noll F, Sumper M, Hampp N. 2002. Nanostructure of diatom silica surfaces and of biomimetic analogues. Nano Lett 2:91–95.

Perry CC, Yun LJ. 1992. Preparation of silicas from silicon complexes–role of cellulose in polymerization and aggregation control. J Chem Soc Faraday Trans 88:2915–2921.

Reiswig HM. 1971. Axial symmetry of sponge spicules and its phylogenetic significance. Cah Biol Mar 12:505–514.

Reiswig HM, Mackie GO. 1983. Studies on Hexactinellid sponges. III. The taxonomic status of Hexactinellida within the Porifera. Philos Trans R Soc Lond 301:419–428.

Rützler K, Smith KP. 1993. The genus *Terpios* (Suberitidae) and new species in the "Lobiceps" complex. In: Uriz MJ, Rützler K, editors. Recent advances in ecology and systematics of sponges. Barcelona: Scientia Marina. p 381–393.

Sarikaya M, Fong H, Sunderland N, Flinn BD, Mayer G, Mescher A, Gaino E. 2001. Biomimetic model of a sponge-spicular optical fiber—mechanical properties and structure. J Mater Res 16:1420–1428.

Schulze FE. 1880. On the structure and arrangement of the soft parts in *Euplectella aspergillum*. Trans R Soc Edinb 29:661–673.

Schulze FE. 1899. Zur Histologie der Hexactinelliden. Sitz Ber K Pr Akad Wiss Berlin 14:198–209.

Schwab DW, Shore RE. 1971. Mechanism of internal stratification of siliceous sponge spicules. Nature 232:501–502.

Shimizu K, Morse DE. 2000. The Biological and biomimetic synthesis of silica and other polysiloxanes. In: Baeuerlein E, editor. Biomineralization: from biology to biotechnology and medical application. Weinheim: Wiley-VCH. 207–219.

Shimizu K, Cha JN, Stucky GD, Morse DE. 1998. Silicatein α: cathepsin L-like protein in sponge biosilica. Proc Natl Acad Sci USA 95:6234–6238.

Shore RE. 1972. Axial filament of siliceous sponge spicules, its organic components and synthesis. Biol Bull 143:125–136.

Simpson TL. 1984. The cell biology of sponges. New York: Springer.

Simpson TL, Vaccaro CA. 1974. An ultrastructural study of silica deposition in the freshwater sponge *Spongilla lacustris*. J Ultrastruct Res 47:296–309.

Simpson TL, Langenbruch P-F, Scalera-Liaci L. 1985. Silica spicules and axial filaments of the marine sponge *Stelletta grubii* (Porifera, Demospongiae). Zoomorphology 105:375–382.

Sullivan CW. 1986. Silicification in diatoms. In: Silicon biochemistry. Chichester, UK: John Wiley & Sons.

Urban S, Hickford SJH, Blunt JW, Munro MHG. 2000. Bioactive marine alkaloids. Curr Org Chem 4:765–807.

Uriz MJ, Turon X, Becerro MA. 2000. Silica deposition in demosponges: spiculogenesis in *Crambe crambe*. Cell Tiss Res 301: 299–309.

Weissenfels N, Landschoff HW. 1977. Bau und Funktion des Suβwasserschwamms *Ephydatia fluvatilis* L. (Porifera). IV. Die Entwicklung der Monaxialen SiO_2 Nadeln in Sandwich-Kulturen. Zool Jb Anat Bd 98:355–371.

Wilkinson CR, Garrone R. 1980. Ultrastructure of siliceous spicules and microsclerocytes in the marine sponge *Neofibularia irata n. sp.* J Morphol 166:51–64.

Zhou Y, Shimizu K, Cha JN, Stucky GD, Morse DE. 1999. Efficient catalysis of polysiloxane synthesis by silicatein a requires specific hydroxyl and imidizole functionalities. Angew Chem Int Ed Eng 38:780–782.