Acquisition of Structure-guiding and Structure-forming Properties during Maturation from the Pro-silicatein to the Silicatein Form*

Heinz C. Schröder‡, Xiaohong Wang‡‡, Alberto Manfrin‡, Shu-Hong Yu, Vlad A. Grebenjuk‡, Michael Korzhev‡, Matthias Wiens§, Ute Schlossmacher‡, and Werner E. G. Müller‡¶

From the ¤ ERC Advanced Grant Research Group at the Institute for Physiological Chemistry, University Medical Center of the Johannes Gutenberg University Mainz, Duesbergweg 6, D-55128 Mainz, Germany, the § National Research Center for Geoanalysis, Chinese Academy of Geological Sciences, Beijing 100037, China, and the ¶ The Cheung Kong Chair Professor, Division of Nanomaterials & Chemistry, University of Science and Technology of China, Hefei 230026, China

Background: Silicateins are proteins that form biosilica enzymatically in siliceous sponges.

Results: Silicatein from Suberites domuncula obtains the enzymatic function and the property to self-assemble after removal of the propeptide.

Conclusion: Silicatein acquires structure-guiding and structure-forming activity during its maturation.

Significance: Silicatein is a unique enzyme mediating polymerization of orthosilicate units and functions as structure-giving protein during biosilica deposition.

Silicateins are the key enzymes involved in the enzymatic polycondensation of the inorganic scaffold of the skeletal elements of the siliceous sponges, the spicules. The gene encoding pro-silicatein is inserted into the pCold TF vector, comprising the gene for the bacterial trigger factor. This hybrid gene is expressed in Escherichia coli and the synthesized fusion protein is purified. The fusion protein is split into the single proteins with thrombin by cleavage of the linker sequence present between the two proteins. At 23 °C, the 87 kDa trigger factor-pro-silicatein fusion protein is cleaved to the 51 kDa trigger factor and the 35 kDa pro-silicatein. The cleavage process proceeds and results in the release of the 23 kDa mature silicatein, a process which very likely proceeds by autocatalysis. Almost in parallel with its formation, the mature enzyme precipitates as pure 23 kDa protein. When the precipitate is dissolved in an urea buffer, the solubilized protein displays its full enzymatic activity which is enhanced multi-fold in the presence of the silicatein interactor silintaphin-1 or of poly(ethylene glycol) (PEG). The biosilica product formed increases its compactness if silicatein is supplemented with silintaphin-1 or PEG. The elastic modulus of the silicatein-mediated biosilica product increases in parallel with the addition of silintaphin-1 and/or PEG from 17 MPa (silicatein) via 61 MPa (silicatein:silintaphin-1) to 101 MPa (silicatein:silintaphin-1 and PEG). These data show that the maturation process from the pro-silicatein state to the mature form is the crucial step during which silicatein acquires its structure-guiding and structure-forming properties.

Among the metazoans the skeletal elements of the sponges (phylum Porifera), the spicules, are the architecturally most impressive and the structurally most complicated designed inorganic building components (1). Their morphologies, sizes, and shapes are characteristic for each sponge species (2), and in turn their synthesis is primarily genetically controlled (3). The spicules in sponges are formed either of silica, like in the classes Hexactinellida and Demospongia, or of calcite, like in the class of Calcarea (1). A breakthrough in the understanding of the formation of siliceous spicules was accomplished with the discovery of the major protein, the silicatein, that initiates the synthesis of the spicules (4–6). Consequently the silica component was termed biosilica (7). The proposed enzymatic feature of silicatein (5) was studied in detail (8, 9) and based on these data, a mechanism for the enzymatic reaction has been outlined (10). The silicateins, found in over 20 sponge species, are closely related to the cathepsins and grouped to the cysteine protein family (5). Two subgroups of silicatein have been described as existing in spicules from marine sponges, termed silicatein-α and silicatein-β (4). The primary protein translation product of the silicatein-α gene from the demosponge Suberites domuncula with a calculated size of 36,306 [proenzyme] is composed of a signal sequence (spanning aa1 to aa13), a propeptide (aa16 to aa112) and the mature, enzymatically active protein (aa113 to aa139). The calculated size of the propeptide together with the mature protein is 34,806 [proenzyme] and of the processed mature enzyme is 23,125 [enzyme] (6). The enzyme is localized within the axial canal of the spicule, where it forms an axial filament, and additionally also in the biosilica mantel around the axial canal (11, 12). Using S. domuncula as a model, it could be shown that the formation of the spicules starts intra-
cellularly, driven by an elongation of the axial filament, and is completed extracellularly by two concerted morphogenetic processes, firstly by an evagination of cell protrusions that direct spicule growth axially (13) and secondly by an organic mantel, formed of silicatein and collagen, that allows a radial thickening of the spicules (12).

Silicatein is not only a structure-guiding protein but also a structure-forming protein. Studies with silicatein from *Tethya aurantium* revealed that monomeric silicatein self-assembles to fibrous structures via oligomer intermediates mediated by diffusion limited, fractally patterned aggregate formation (14). These authors proposed that the driving force for the self-assembly process is mediated by the interaction of hydrophobic patches, located on the surface of the silicatein molecules. In a subsequent study, and using glycerol, a viscogenic agent to destabilize protein-protein interactions (15), for extraction of native silicateins from spicules of *S. domuncula*, it was found that silicatein-α monomers form dimers and tetramers that are subsequently joined together via silicatein-β molecules to fractal-like structures, and finally to filaments (16). The hydrophobic patches within the mature silicatein molecule had been localized between aa135 to aa150, close to the N terminus of the molecule (6). Interestingly enough, this hydrophobic segment is, according to model predictions, covered by the propeptide (17). Superimposition studies of models for silicatein and cathepsin disclosed five unique hydrophobic patches that are exposed to the solvent-accessible surface of the mature silicatein (14). From studies with the recombinant, mature silicatein, it is known that this protein, after expression in *E. coli*, is highly insoluble and needs to be processed through a drastic unfolding and refolding procedure to obtain an enzyme preparation that is applicable for enzymatic studies (9). Based on these predicted calculations and experimental findings, it can be postulated that silicatein changes decisively its solubility properties during the transition from the proenzyme form to the enzyme form. This view is compatible with the previous finding that the extracellularly existing silicatein has a size of 34.7 kDa, fitting with the *M* of the proenzyme (11). In this study, it had been hypothesized that silicatein undergoes autoprocessing mediated by the intrinsic proteolytic activity of this protein (18).

To prove experimentally that during processing of silicatein to the mature enzyme the protein changes its solubility properties and precipitates, the enzyme was expressed as fusion protein in *E. coli* together with the trigger factor (19), a ribosome-associated chaperone protein. While the mature enzyme precipitates during the cleavage of the fusion protein with thrombin at 23 °C, it remains, at least partially, in solution if the reaction was performed at 4 °C. It is shown that the cleaved 23 kDa mature enzyme is catalytically active and also acts as a template for an organized biosilica deposition. Finally the elastic modulus of the biosilica formed by the recombinant silicatein, in the absence or the presence of a natural (silintaphin-1) (20) or a synthetic organic polymer, poly(ethylene glycol), (21) had been determined. Silintaphin-1 has been proposed to interact with pentamers formed of four silicatein-α and one silicatein-β molecules and, by that, stabilizes the fractal structures, initially formed (17). Both additives have previously been shown to enhance biosilica formation in vitro (20, 22). Comparing the in vitro activity of the recombinant silicatein with the extent of the in vivo biosilica formation, determined during spicule formation, it becomes evident that silicatein is the major, very likely the only, molecule that accounts for the synthesis of the inorganic polymer (9). It had been calculated that one silicatein molecule converts, under in vivo conditions, 8 × 10³ substrate molecules per 1 h. In comparison, one molecule of recombinant silicatein converts 5 × 10³ substrate molecules per 1 h and in the presence of silintaphin-1 even 28.8 × 10³ substrate molecules per 1 h. The data presented in this report qualify silicatein as a structure-guiding and structure-forming protein that acquires those properties during the processing from the proenzyme to the mature enzyme form.

**MATeRIALS AND METHODS**

**Construction of the Fusion Gene**—The segment of the *S. domuncula* silicatein-α cDNA (accession number AJ272013), which encodes the precursor of the protein, the propeptide and the mature enzyme, was obtained by means of polymerase chain reaction (PCR) using the following primers; forward primer (Fwd): 5’-gtttcatatgGCAGCCCAGCCCAAGTTTG-3’ (corresponding to nt77 to nt1024 of the cDNA, shown in capitals; in small letters the tail is shown that includes the NdeI restriction site, which is underlined) and the reverse primer (Rev): 5’-ctcacttc- cagTTATAGGGTGGGATAAGATGCATC-3’ ([stop codon in bold] nt1024 to nt1023; XhoI site in the tail). The nucleotide sequence, corresponding to nt77 to nt1029a, was amplified by PCR. The product was purified, digested with the mentioned restriction enzymes and ligated into the digested pCold TF (trigger factor) plasmid vector (Takara Bio Europe, Göttingen; Germany) as described (23). This construct has a 2361 bp long open reading frame, encoding for a fusion protein of 786 aa with a calculated size of 87.2 kDa; the N terminus has the 6×His tag followed by the trigger factor chaperone, the spacer with proteolytic cleavage sites, and the *S. domuncula* pro-silicatein-α sequence (Fig. 1). The correctness of the DNA fragments obtained by PCR was confirmed by DNA sequence analysis. The fusion gene was used for transformation of *E. coli* (One Shot BL21 pLYS competent Cells; Novagen/Merck, Darmstadt; Germany) and subsequent for protein expression.

**Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis**—Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (9, 11). The protein samples were subjected to 12% PAGE with 0.1% SDS. After size separation, the proteins were stained with Coomassie Brilliant Blue.

**Expression of the Recombinant Fusion Protein**—The transformed bacteria were grown at 37 °C to a density of *A*₅₀₀ = 0.5 in Terrific Broth Medium (Roth, Karlsruhe; Germany), supplemented with 50 μg/ml carbenicillin (AppliChem, Darmstadt; Germany). Then the culture was cooled down to 15 °C, induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (Sigma, Taufkirchen; Germany), and was grown for additional 40 h at 15 °C under shaking. The cells (500 mg wet weight; collected from 100 ml culture) were harvested by centrifugation (5000 × g, 30 min, 4 °C) and after one cycle of freezing/thawing they were disrupted by ultrasound sonication (51 kHz; Branson 5200...
Maturation of Silicatein

Sonifier) in 20 ml of lysis buffer (20 mM Tris/HCl, pH 8.0; 150 mM NaCl, 0.1% (v/v) Tween 20). The lysate was cleared by centrifugation and filtered through 0.45 μm disposable filters (Roth, Karlruhe; Germany). The fusion protein was purified by immobilized metal ion affinity chromatography on a 5 ml Ni-NTA column using the Profinia Protein Purification System (Bio-Rad). The column was equilibrated with 50 mM KH₂PO₄ buffer (pH 8.0; 300 mM KCl), washed after loading with the recombinant protein with this KH₂PO₄ buffer, supplemented with 10 mM imidazole. The bound protein was eluted with the KH₂PO₄ buffer, including 250 mM imidazole. The samples eluted were analyzed by SDS-PAGE. Finally, the purified/enriched protein samples were dialyzed against 250 volumes of a 20 mM Tris/HCl buffer (pH 8.8; 150 mM NaCl) for 72 h at 4 °C. The samples were adjusted to 1 mg/ml of protein and stored at 4 °C.

Thrombin Cleavage of the Fusion Protein—The recombinant fusion protein was digested with 4 μg/ml of thrombin at 23 °C or at 4 °C for various incubation periods, as mentioned with the experiments. The samples were centrifuged (12,000 × g, 15 min, 23 °C or 4 °C), and both the sediment and the supernatant were collected. The sediment was dissolved in a 50 mM Tris/HCl buffer (pH 7.4; 1 μL urea, 150 mM NaCl) and a protein concentration of 100 μg/ml was adjusted prior to SDS-PAGE analysis. As outlined under “Results,” the mature 23 kDa silicatein precipitates if the reaction is performed at 23 °C. This enzyme sample, which was (almost) pure, if analyzed by SDS-PAGE analysis, was dissolved in the Tris/HCl/urea/NaCl buffer and used for enzyme activity testing.

Recombinant Silintaphin-1—The preparation and purification of recombinant silintaphin-1 had been described before (9, 20). The mass of silintaphin-1 is 43 kDa (20).

Enzymatic Activity Determination of Silicatein—The recombinant, purified silicatein was assayed for activity, as previously described (6, 9). Samples of 1 ml in 50 mM Tris/HCl buffer (pH 7.4; 150 mM NaCl) were supplemented with 6 μg of recombinant silicatein. Where indicated, silicatein was preincubated (10 min; 22 °C) with recombinant silintaphin-1 at a stoichiometric ratio of 1:1 or 4:1, prior addition to the enzyme assay. As substrate for the enzyme, 200 μM prehydrolyzed TEOS (tetraethyl-orthosilicate,2 Sigma-Aldrich) was added. The procedure for preparation of prehydrolyzed TEOS was given before (24). Where indicated poly(ethylene glycol) (PEG) (cat. 202398; Sigma), with an average molar mass (Mₘ) of 400 g/mol, was added at a defined molar ratio to prehydrolyzed TEOS as the last component to the assays. Following earlier studies (22) we selected a molar ratio between prehydrolyzed TEOS and PEG of 1:0.1. The enzymatic reactions were performed at 22 °C for 1 h while agitation. The biosilica formed was centrifuged (10,000 × g, 30 min, 4 °C) and, after washing with ethanol, the sedimented silica particles were treated with 2 M NaOH (30 min, 30 °C) to hydrolyze biosilica and the soluble silicic acid was determined applying the molybdenum blue colorimetric method (6) and the Silicon Test colorimetric assay kit (Merck 1.14794, Darmstadt; Germany). The absorbance values were determined at 795 nm and the absolute amounts of silicic acid were calculated after establishing a calibration curve using the silicon standard (Merck 1.09947).

For the studies to determine the mechanical properties of biosilica, formed by silicatein, the enzymatic reactions were performed in microscope culture slides with one cavity each (Sargent-Welch, Buffalo, NY). The depth of each cavity was ~1.8 mm. A 1 ml assay in 50 mM Tris/HCl buffer (pH 7.4; 150 mM NaCl) was supplemented with 6 μg of silicatein, silintaphin-1 (molar ratio to silicatein 1:4) and 400 μM prehydrolyzed TEOS. The reaction was performed for 24 h. Where indicated the samples were supplemented with PEG (molar ratio prehydrolyzed TEOS:PEG of 1:0.1). After the reaction, the samples were inspected by SEM or laser microscopy and then used for EDX or nanoindentation studies.

Microscopic Analyses—Scanning electron microscopy (SEM) analyses were performed with a Zeiss DSM 962 digital scanning microscope (Zeiss, Aalen, Germany) as described (11), while transmission electron microscopy (TEM) analysis of the samples was done with a Tecnai 12 microscope (FEI Electron Optics, Eindhoven, Netherlands), as outlined before (9). For instant visualization of biosilica, the samples were inspected with a color three-dimensional laser microscope (VK-8710) from Keyence (Osaka, Japan).

Energy-dispersive X-ray Spectroscopy—Energy-dispersive x-ray spectroscopy (EDX) was performed as described before (25). The determinations by SEM were performed with a HITACHI SU 8000 (Hitachi High-Technologies Europe GmbH, Krefeld, Germany) that was employed at low voltage (<1 kV; analysis of near-surface organic surfaces).

Nanoindentation Analysis—Biosilica was allowed to form on notched object slides (Sargent-Welch). The enzymatic biosilica synthesis was performed on those glass slides in 50 mM Tris/HCl buffer (pH 7.4; 150 mM NaCl) using 6 μg/ml of silicatein and 400 μM prehydrolyzed TEOS. Additionally, PEG was added at a molar ratio to prehydrolyzed TEOS of 0.1:1. Where indicated also silintaphin-1 (ratio to silicatein 1:4) was included in the enzymatic reaction. After 24 h, the biosilica product was analyzed.

For the nanoindentation experiments the TriboScope nanoindenter (Hysitron, Minneapolis, MN) mounted on an atomic force microscope (AFM) (Veeco, Santa Barbara, CA) was used following the procedure described by Li et al. (26) and Akhtar et al. (27). A spherical diamond tip of 1 μm radius was used. All experiments had been performed with biosilica samples immediately after synthesis. Indent locations were selected manually with each indent situated at least 3 μm apart. The characteristics for the properties of the glass substrate (Young’s modulus, E = 70 GPa and Poisson’s ratio, ν = 0.23) were adopted from Akhtar et al. (27). Prior to each nanoindentation test, the thermal drift was recorded and implemented in the assessment of the hardness and elastic modulus analyses.

Further Methods—Protein concentrations were routinely determined by application of the Bradford method (28).
RESULTS

Silicatein-trigger Factor Fusion Protein—Silicatein was expressed in E. coli as a proenzyme fused to the trigger factor (Fig. 1) in the pCold TF DNA vector. To allow a slow expression rate of the protein, only 0.5 mM isopropyl β-D-1-thiogalactopyranoside had been used for induction and the incubation temperature for the bacteria was lowered to 15 °C. The expressed 87.17 kDa fusion protein (786 aa) is composed of a translation enhancing element (aa1 to aa5), a hexahistidine-tag (aa6 to aa11), the 48.2 kDa E. coli trigger factor (aa12 to aa443), a spacer (aa444 to aa471) comprising the cleavage sites for thrombin, and the 34.82 kDa propeptide of silicatein-α (aa472 to aa786). From the pro-silicatein the 15 aa long signal peptide had been removed. The fusion protein by SDS-PAGE revealed an 87.2 kDa major protein (Fig. 1, lane a) that agrees with the calculated sizes of theHis-tagged pro-silicatein (34.8 kDa).

Thrombin Cleavage of the Fusion Protein: 23 °C—The recombinant silicatein-trigger factor fusion protein was digested with thrombin at 23 °C for 0 h (Fig. 2, lane a), 1 h (lane b), and 24 h (lane c). Size separation by SDS-PAGE revealed that the 87 kDa fusion protein is increasingly split into the 51 kDa His-tagged trigger factor and the 36 kDa pro-silicatein. The mature 23 kDa silicatein band already appears after an incubation period of 1 h (lane b) and increases in intensity after 24 h (lane c). This series of experiments shows that in the presence of thrombin the silicatein-trigger factor fusion protein is separated into the trigger factor and the silicatein proteins, as expected (23); in addition, and unexpected, the processing of pro-silicatein to the mature silicatein is seen as well. The latter reaction has not been described for the processing of pro-silicatein or of pro-cathepsin, again suggesting that the maturation to silicatein is an autocatalytic reaction. Furthermore, it is striking that the 51 kDa trigger factor partially degrades into a 49 kDa smaller protein. This cleavage is attributed to the enzymatic activity of silicatein, since this reaction can be inhibited by 200 μM phenylmethylsulfonyl fluoride (PMSF), a potent broad spectrum Ser- and Cys-protease inhibitor (data not shown) (32).

Thrombin Cleavage of the Fusion Protein at 4 °C—In a second series of experiments, the cleavage reaction of the silicatein-trigger factor fusion protein with thrombin was performed at 4 °C. Under these conditions, the reaction of digestion of the trigger factor from pro-silicatein was slower, if compared with the kinetics seen at 23 °C. As documented in Fig. 3, the digestion of the silicatein-trigger factor fusion protein was not com-
Maturation of Silicatein

Completed at 4 °C, even after an incubation period of 144 h (lane e), while a separation into two proteins was seen at 23 °C already after 2 h (Fig. 2, lane b). Interestingly, the mature silicatein, formed after the appearance of pro-silicatein, remained in a soluble form even after an incubation period of 144 h (Fig. 3, lane e). A centrifugation of such a sample and a dissolution of the sediment with a 50 mM Tris/HCl buffer (pH 7.4; 150 mM NaCl), either in the absence of 1 M urea or the presence of this organic compound (lane f), revealed no protein band at 23 kDa; only a faint band is seen that co-migrated with the trigger factor.

Enzymatic Activity of Silicatein—The mature silicatein sample, precipitating during the thrombin cleavage reaction, was dissolved in Tris/HCl/urea/NaCl buffer and assayed for enzyme activity. If added to the assay containing prehydrolyzed TEOS as the substrate, a biosilica-forming activity of 62 ± 11 μg/ml was measured (Fig. 4). In contrast, if only silintaphin-1 or PEG was added, the ability to form biosilica was much lower with 27 ± 5 μg/ml and 11 ± 5 μg/ml, respectively. The biosilica-forming activity of silicatein was significantly increased if silintaphin-1 was added to the enzyme, as described before (9). Using in the present study a silicatein/silintaphin-1 stoichiometric molar ratio of 1:1 (4:1), the production significantly increased to 174 ± 21 (372 ± 66) μg/ml. Addition of PEG at a stoichiometric ratio between prehydrolyzed TEOS and PEG of 1:0.1 a further increase of activity to 254 ± 31 (471 ± 55) μg/ml was measured.

Self-assembly of Recombinant Silicatein and Biosilica Product Formation: TEM and SEM Analysis—Silicatein has the property to self-assemble (9). If the samples were allowed to stand in the 50 mM Tris/HCl buffer (pH 7.4; 150 mM NaCl), the protein associates within 5 min to ~20 nm sized nanoparticles, irrespectively of the presence of silintaphin-1 (Fig. 5A). If silintaphin-1 is added to silicatein at a ratio of 1:4, the same sized particles are seen (Fig. 5B). A longer incubation (30 min) of silicatein in the presence or absence of silintaphin-1 resulted in the formation of protein aggregates showing fractal-like patterns; TEM images of a silicatein sample incubated for 30 min in the absence (Fig. 5C) or presence of silintaphin-1 (Fig. 5D) are given. Addition of 50 μM prehydrolyzed TEOS during the reaction to the samples increased the contrast of the contours of the fractal figures if compared with the aggregates seen in the absence of TEOS (not shown).
If biosilica formation was performed for 24 h at 6 μg/ml of silicatein together with silintaphin-1 (ratio to silicatein 1:4), 400 μM prehydrolyzed TEOS and allowed to proceed in cavity microscope slides, small boulders of biosilica could be visualized after SEM analysis (Fig. 5E). The biosilica cubes became more compact if PEG was added together with prehydrolyzed TEOS (molar ratio of 0.1:1) to the assays. A representative image is given in Fig. 5F.

Energy-dispersive X-ray Spectroscopy—A sample of biosilica, formed in the standard reaction assay, not containing PEG, but with silicatein and silintaphin-1 (ratio of 4:1) and using 400 μM prehydrolyzed TEOS as substrate during the 24-h incubation period, was analyzed (Fig. 6). An area of 5 × 5 μm on the largely plane surface (Fig. 6A) was selected for the subsequent EDX analysis. The spectrum obtained shows dominant signals for Si and O, in addition to Na and Cl (Fig. 6B). Interesting to mention is the C peak that originates from silicatein/silintaphin-1 components in the reaction assay.

Nanoindentation Analysis—Biosilica layers were enzymatically prepared onto glass slides, as described under “Material and Methods.” The assays contained in the 50 mM Tris/HCl buffer, 400 μM prehydrolyzed TEOS together with PEG at a molar ratio of 1:0.1. If indicated, silicatein and silintaphin-1 were added as well to the assays. Laser-optical inspection of the samples revealed only small < 0.1 μm sized silica cubes/spheres in those assays which only contained prehydrolyzed TEOS and PEG (Fig. 7A). However, if silicatein together with silintaphin-1 (molar ratio 4:1) was added, larger biosilica layers are formed, even in the absence of PEG (Fig. 7B). The layers are interspersed with air bubbles. Addition of PEG to silicatein and silintaphin-1 (1:1) caused a reduction of the air bubbles both in size and in number (Fig. 7C). If the molar ratio between silicatein and silintaphin-1 is adjusted to 4:1, the number of air bubbles is even smaller (Fig. 7D).

A characteristic load-displacement curve is shown in Fig. 7E. The diagram represents data obtained for a biosilica sample, formed during a 24 h incubation of silicatein and silintaphin-1 (4:1) from prehydrolyzed TEOS in the presence of PEG, at a 105 μN maximum indentation force. The absence of any steps and likewise also discontinuities on the curve are taken as an indi-
Maturation of Silicatein

FIGURE 8. Elastic modulus, determined by nanoindentation, for biosilica formed in the standard assay supplemented with silicatein alone (Silic), with silicatein:silintaphin-1 (Silic:Siphn) at a molar ratio of 1:1 or 4:1, and with silicatein:silintaphin-1 at a molar ratio of 1:1 or 4:1, together with PEG. The means (n = 10) ± S.E.; *, p < 0.01.

DISCUSSION

The diverse and intricately structured spicules of the siliceous sponges, the demosponges and the hexactinellids, are the results of an enzymatic polycondensation reaction driven by silicateins. These enzymes constitute a distinguished family of proteins with regard to their properties (i) to form an inorganic polymer (biosilica) from inorganic monomeric precursors (orthosilicate) (7), (ii) to remain entrapped, after product formation, inside of the formed biosilica (34), (iii) to act as structure-guiding scaffold protein (14, 16), and (iv) also to function as structure-forming protein (35). While the enzymic parameters underlying the kinetic processes are known (8, 9), and the localization of silicatein within the spicules (34, 36) had been well documented, the mechanisms of structure-guiding and structure-forming by silicateins remained unsolved. It had been elucidated that silicatein is the main structural protein within the axial canal that harbors the axial filament and through its elongation drives ahead the spicules in the longitudinal axis during their growth (13). In a parallel process a radial growth takes place that is mediated by extracellularly localized silicatein and results in an appositional layering of silica lamellae (12). The basic property of silicatein to act as a structure-guiding and structure-forming protein can be ascribed to a tuned protein-protein interaction resulting in building of fractally patterned aggregates that ultimately proceeds to filament formation (14, 16). So the question remains of what is the controlling step during which silicatein acquires the property to recognize and to interact with a second silicatein molecule. The impressive aspect with regard to silicatein is the fact that this interaction is not restricted to homotypic interactions only (e.g. recognition of silicatein-α) but involves also heterotypic interactions between silicatein-α and silicatein-β molecules in an ordered manner (16). Usually homocomplexes are more permanent and superiorly optimized compared with heterocomplexes that are prone to nonobligatory disturbances acting from the environment (37). Experimental studies showed that silicateins [silicatein-α] form dimers and tetramers (14, 16) that are linked together by one silicatein-β molecule (16) allowing a bidirectional elongation of the filaments. Since several of those filaments formed in a 4:1 stoichiometric ratio (silicatein-α:silicatein-β) associate together and form a superstructure (38), it is crucial to identify the initial step during which the “soluble” silicatein is transferred to the “insoluble” state that allows protein:protein interactions to proceed. The first hint that silicatein acquires this property came from immunohistochemical and immunobiochemical studies which revealed that in the extracellular and there in the extraspicular space, silicatein exists as a 34.7 kDa immature enzyme that undergoes a processing to the 23 kDa mature form only very shortly before its catalytic reaction (11). Murr and Morse (14) already proposed that the surface of the silicatein molecule comprises hydrophobic stretches that might allow an interaction via protein:protein interfaces.

To elucidate experimentally that silicatein acquires its property during maturation of the proenzyme to the active enzyme, a hybrid gene between the trigger factor protein and the pro-silicatein was constructed and the protein was expressed. The pCold TF DNA vector system is suitable to obtain soluble hybrid proteins, comprising a soluble ~48 kDa tag (19). Our studies fulfilled this expectation. An ~87 kDa hybrid protein, formed by the 51 kDa trigger factor protein and the 35 kDa pro-silicatein, was recombinantly produced and purified. The insertion of the cleavage site for thrombin within the spacer region separating the two fused proteins allowed the split of these proteins, as expected (23). Unexpectedly, almost simultaneously this proteolytic cleavage reaction was followed by a processing of the 35 kDa pro-silicatein into the mature 23 kDa silicatein. The border between the propeptide and the mature peptide does not exhibit a known thrombin cleavage site. Hence it was consequent to ask the question if this processing is the result of autolysis or the result of cleavage caused by thrombin. Therefore, we used both E-64 and phenylmethylsulfonyl fluoride (PMSF) as inhibitors in the reaction assay. E-64 inhibited directly the processing of the cathepsin-related silicatein, as expected (data not shown) (31), while the maturation of prosilicatein to silicatein proceeded in the presence of PMSF. PMSF is known to be an effective inhibitor of thrombin protease (39), however, only for a very limited period of time (about...
the effect of silintaphin-1 on fractal formation during the initial of 1:0.1 (22), accelerated biosilica formation at a factor of two. Added in a stoichiometric ratio of prehydrolyzed TEOS to PEG during silica formation (21). In turn it was found that PEG, if organic polymer had been shown to promote sol-gel processes a more structured, solidified environment, we also selected into the product formed by them.

which is indicative for a (partial) encapsulation of the proteins ing of orthosilicate and silicatein/silintaphin-1 also contained formation (9). The biosilica deposits formed in assays consist-
catalyzed biosilica formation (9). This effect had been attrib-
interactor silintaphin-1 (20), especially if given at a 4:1 stoichi-
schematic representation of the autocatalytic cleavage of pro-
sample solubilized in this buffer was found to be highly active and forms biosilica to an extent described previously for the recombinantly prepared and column-purified enzyme (9). A schematic representation of the autocatalytic cleavage of pro-silicatein to mature, assembly-competent and enzymatically active silicatein is shown in Fig. 9.

Important for the question asked here, the formation of self-assemblies of silicateins due to protein:protein interactions, is the finding that the mature 23 kDa silicatein precipitates, or a better description flocculates, out of the solution during cleavage at 23 °C, while it remains in solution at 4 °C. A temperature-dependent and reduced flocculation of proteins at lower temperature, e.g. for casein, is well known (41). The ambient temperature where the animals live in is around 14–20 °C (42), and hence under conditions favoring precipitation. The precipita-
tion is selective for the mature silicatein, while pro-silicatein remains soluble under those conditions.

In the consecutive series of experiments, the question of whether the mature silicatein, precipitating at 23 °C, is active or not, was solved. Therefore, the precipitated 23 kDa silicatein was dissolved in the chaotropic agent, urea (1 M), through direct interfering with intramolecular interactions (43). The silicatein sample solubilized in this buffer was found to be highly active and forms biosilica to an extent described previously for the recombinantly prepared and column-purified enzyme (9). A schematic representation of the autocatalytic cleavage of pro-silicatein to mature, assembly-competent and enzymatically active silicatein is shown in Fig. 9.

The activity of the silicatein sample prepared and analyzed here, was found to be strongly up-regulated by the silicatein interactor silintaphin-1 (20), especially if given at a 4:1 stoichiometric ratio of silicatein:silintaphin-1 (9). At this molar ratio silintaphin-1 was found to stimulate maximally the silicatein-catalyzed biosilica formation (9). This effect had been attributed to the property of silintaphin-1 to form compact structures that could act as matrices for a facilitated poly-biosilica formation (9). The biosilica deposits formed in assays consisting of orthosilicate and silicatein/silintaphin-1 also contained carbon (C) in those cubes, besides of the elements Si and O, which is indicative for a (partial) encapsulation of the proteins into the product formed by them.

In line with this thought, an increased biosilica formation in a more structured, solidified environment, we also selected PEG as an additive for the silicatein assays. Recently, this organic polymer had been shown to promote sol-gel processes during silica formation (21). In turn it was found that PEG, if added in a stoichiometric ratio of prehydrolyzed TEOS to PEG of 1:0.1 (22), accelerated biosilica formation at a factor of two.

Electron microscopic studies were performed to elucidate the effect of silintaphin-1 on fractal formation during the initial

self-assembly process of silicatein molecules. During the first 30 min of co-incubation only a slightly stabilizing effect of silintaphin-1 was seen in assays composed of silicatein. However, addition of PEG to silicatein/silintaphin-1 assays strongly increased the compactness of the biosilica cubes formed. The biosilica deposits, synthesized by silicatein in the presence of silintaphin-1 and also of PEG, had been more compact and their surfaces more plane, compared with the assays, lacking PEG. Light optical studies confirmed that biosilica in the absence of silintaphin-1 and PEG is less solid and more interspersed with air bubbles. This increase in compactness of the silicatein-synthesized biosilica allowed an assessment of the elastic modulus of biosilica.

The nanoindentation analyses were performed with a nanoindenter coupled with an AFM. Load-displacement studies were conducted to demonstrate that the biosilica samples prepared did not contain measurable fractures and cracks. The subsequent determinations of the elastic moduli of the different biosilica products revealed increased values for the elastic modulus if silintaphin-1 and PEG were added to the silicatein-driven polycondensation. The effect was quite pronounced. The values for the synthesized biosilica, formed in assays with 400 μM prehydrolyzed TEOS as substrate and allowing silicatein to be enzymatically active for 24 h at 23 °C, are around 20 MPa, and hence in the range of solidified agarose (44). This value substantially increases to about 60 MPa especially if silintaphin-1 is added at a ratio of 4:1 (silicatein:silintaphin-1), and even more significant to around 100 MPa if PEG is added as a further component. These values measured for the silicatein-
mediated biosilica deposits in vitro are by a factor of 1000 lower than the ones measured for spicules formed in vivo. Analyzing the Antarctic hexactinellid Rosella racovitzae as a model, an elastic modulus of 38 GPa was found (45); this value reflects a higher flexibility than those measured for optical fibers (63 GPa) or fused silica (70 GPa) (46).

CONCLUSION

The data presented here demonstrate that silicatein during its maturation to the active enzyme undergoes a conformational change during which the molecule becomes less soluble and precipitates. This effect which is seen at higher temperature (around 20 °C) around those in which the specimens live in their environment, results in the formation of self-assembled structures of silicatein, which pass a fractal stage. The precipitated silicatein molecules can be re-dissolved in the presence of urea, without losing the biological activity. Those self-assembly formations are stabilized by addition of silintaphin-1, a natural interactor of silicatein, and of PEG, a synthetic polymer. Under natural conditions aliphatic carbohydrate molecules are proposed to interact with biosilica (47). Adding the latter two components to the silicatein-mediated enzymic reaction results in the formation of biosilica cubes; those deposits become flat/planar allowing already elastic modulus determination. The values determined are about 1000-fold lower than those measured for spicules formed in vivo. The decreased flexibility of natural spicules can be attributed to a hardening process (48), for which a process of syneresis had been attributed to. A detailed description of the process of syneresis had been given recently (48). It had been outlined that the reaction water, which accumulates during the polycondensation of ortho-silicate to polysilicate in the extracellular space, is removed by an aquaporin-driven cellular process (48); Fig. 9.

REFERENCES

1. Uritz, M. J. (2006) Mineral spiculogenesis in sponges. Can. J. Zool. 84, 322–356.
2. Boury-Ésnault, N., and Rützler, K. (1997) Thesaurus of sponge morphol-
ogy. Smithsonian Contrib. Zool. 596, 1–55.
3. Wiens, M., Wrede, P., Grebenjuk, V. A., Kaluzhnaya, O. V., Belikov, S. I., Schrör, H. C., and Müller, W. E. G. (2009) in Biosilica in Evolution, Morphogenesis, and Nanobiotechnology. Prog. Mol. Subcell Biol. [Marine Molecular Biotechnology] (Müller, W. E. G., and Grachev, M. A., eds), Vol. 47, pp. 111–144, Springer-Verlag, Berlin Heidelberg
4. Shimizu, K., Cha, J., Stucky, G. D., and Morse, D. E. (1998) Silicatein α: cathepsin L-like protein in sponge biosilica. Proc. Natl. Acad. Sci. U.S.A. 95, 6234–6238.
5. Cha, J. N., Shimizu, K., Zhou, Y., Christiansen, S. C., Chmelka, B. F., Stucky, G. D., and Morse, D. E. (1999) Silicatein filaments and subunits from a marine sponge direct the polymerization of silica and silicones in vitro. Proc. Natl. Acad. Sci. U.S.A. 96, 361–365.
6. Krasko, A., Lorenz, B., Batel, R., Schröder, H. C., Müller, I. M., and Müller, W. E. G. (2000) Expression of silicatein and collagen genes in the marine sponge Suberites domuncula: relevance to bio-silica formation and maturation during spicule formation in sponges. PLoS ONE 6, e20523.
7. Murr, M. M., and Morse, D. E. G. (2005) Fractal intermediates in the self-assembly of silicatein filaments. Proc. Natl. Acad. Sci. U.S.A. 102, 11657–11662.
8. Meng, F., Park, Y., and Zhou, H. (2001) Role of proline, glycerol, and heparin as protein folding aids during refolding of rabbit muscle creatine kinase. Int. J. Biochem. Cell Biol. 33, 701–709.
9. Müller, W. E. G., Rothenberger, M., Boreiko, A., Tremel, W., Reiber, A., and Schröder, H. C. (2005) Formation of siliceous spicules in the marine demosponge Suberites domuncula. Cell Tissue Res. 321, 285–297.
10. Schröder, H. C., Wiens, M., Schlossmacher, U., Brandt, D., and Müller, W. E. G. (2012) Silicatein-mediated polycondensation of orthosilicic acid: Modeling of a catalytic mechanism involving ring formation. Silicon 4, 33–38.
11. Müller, W. E. G., Rothenberger, M., Boreiko, A., Tremel, W., Reiber, A., and Schröder, H. C. (2005) Formation of siliceous spicules in the marine demosponge Suberites domuncula. Cell Tissue Res. 321, 285–297.
12. Schröder, H. C., Wiens, M., Schlossmacher, U., Psigiano, D., Jochum, K. P., and Müller, W. E. G. (2011) Evagination of cells controls silica-silica formation and maturation during spicule formation in sponges. PLoS ONE 6, e20523.
13. Wang, X., Wiens, M., Schröder, H. C., Schlossmacher, U., Psigiano, D., Jochum, K. P., and Müller, W. E. G. (2011) Evagination of cells controls silica-silica formation and maturation during spicule formation in sponges. PLoS ONE 6, e20523.
Maturation of Silicatein

JUNE 22, 2012 • VOLUME 287 • NUMBER 26
JOURNAL OF BIOLOGICAL CHEMISTRY

22205