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Formation of silicones mediated by the sponge enzyme silicate in- α

Stephan E. Wolf,^{*a*} Ute Schlossmacher,^{*b*} Anna Pietuch,^{*a*} Bernd Mathiasch,^{*a*} Heinz-C. Schröder,^{*b*} Werner E. G. Müller^{**b*} and Wolfgang Tremel^{**a*}

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The sponge-restricted enzyme silicatein- α catalyzes *in vivo* silica formation from monomeric silicon compounds from sea water (*i.e.* silicic acid) and plays the pivotal role during synthesis of the siliceous sponge spicules. Recombinant silicatein- α , which was cloned from the demosponge *Suberites domuncula* (phylum *Porifera*), is shown to catalyze *in vitro* condensation of alkoxy silanes during a phase transfer reaction at neutral pH and ambient temperature to yield silicones like the straight-chained polydimethylsiloxane (PDMS). The reported condensation reaction is considered to be the first description of an *enzymatically enhanced organometallic condensation reaction*.

Introduction

The use of biocatalysts, employed either as isolated enzymes or whole microbial cells, offers a remarkable arsenal of highly selective transformations for state-of-the-art synthetic organic chemistry. Over the last two decades, this methodology has become an indispensable tool for asymmetric synthesis, not only at the academic level, but also on an industrial scale. Biotransformations have been applied for the synthesis and hydrolysis of esters, amides and nitriles, various reduction and oxidation reactions or carbon– carbon bond-forming systems. In particular, the combination of selectivity and mild reaction conditions offer promising perspectives for industrial and pharmaceutical applications with a key for environmentally friendly chemistry.¹⁻⁵

Only a few living taxa, *e.g.* sponges, radiolarians, some algae like diatoms and silicoflagellates, construct their skeleton from silica.⁶⁻¹⁰ Sponges (phylum *Porifera*) show the singular ability to synthesize their siliceous skeleton enzymatically,¹¹⁻¹³ in contrast to other organisms which deposit silica in a template-controlled manner.¹⁴ Some of the responsible enzymes, silicatein- α , silicase or silintaphin, were isolated and cloned from different siliceous sponges (class *Demospongiae*), *e.g. Tethya aurantium* or *Suberites domuncula*.^{11,12,15}

Silicateins were shown to undergo post-translational modifications, primarily phosphorylation¹⁶ and to share high sequencesimilarity to the endopeptidase cathepsin L.^{11,12} Cathepsins occur in lysosomes and cleave amide bonds near hydrophobic amino acid residues in P2 and P3 positions.¹⁷ They belong to the group of papain-like peptidases which are characterized by an active centre consisting of the catalytic triade histidine (His), asparagine (Asn) and cystein (Cys).^{13,18} The catalytic triade in silicateins differs by only one amino acid from the one of cathepsin L; cystein is replaced by serine (Ser).¹³ By site-directed mutagenesis, the Ser-His-Asp triad was shown to be crucial for the hydrolytic activity of the silicateins. It was further proposed that the silicon substrate undergoes a transitory pentavalent silicon species involving a donating bond of the imidazole nitrogen of histidine.^{7,19} The enzymatic parameters for silicatein were recently quantified and are close to those which had have been determined previously for the related hydrolytic reaction of cathepsin L.^{20,21}

The enzymes involved in silica metabolism, in particular silicatein and silicase,^{22,23} have attracted increasing attention because of their potential applications in the field of nano-biotechnology and biomedicine. Silica-based materials are used in many hightech products including microelectronics, optoelectronics, and catalysis. Biocatalysis of silica formation from water-soluble precursors, in particular silicatein-mediated biosilica production, occurs under mild physiological conditions and is advantageous compared to technical (chemical) production methods which require high temperatures, pressures or extremes of pH. In addition, biological organisms such as sponges and diatoms are able to fabricate their skeletons with high fidelity and in large copy number. These properties are of extreme importance for potential applications in nano-biotechnology. Silicatein remains functionally active even after immobilization of the protein onto metal or metal oxide surfaces.24

Silicatein is known to catalytically enhance the deposition of silica and related metal oxides from a variety of precursors at physiological temperature, pressure and neutral pH.²⁵⁻²⁸ Likewise, recombinant silaffin, a silica-precipitating protein from the diatom *Cylindrotheca fusiformis*, was repeatedly and successfully employed to synthesize aggregates or layers of titania nanoparticles under ambient conditions from silicium(IV) resp. titanium(IV) alkoxides.^{29,30} Actually, the R5 peptide, which is derived from repeating subunit of the silaffin gen, shows as well the capability of titania and silica synthesis at mild and ambient conditions,³¹⁻³³ and involves in fact a self-assembling process.³⁴

In this contribution we have utilized the catalytic activity of recombinant silicate α for the *in vitro* formation of silicones like the non-branched polydimethyl-siloxane (PDMS) under ambient conditions.

Experimental section

Recombinant silicatein- α was cloned in *Escherichia coli* as described previously.¹² The enzyme was stored at a concentration

^aInstitute for Inorganic Chemistry, Johannes Gutenberg-University, Duesbergweg 10–14, 55099, Mainz, Germany. E-mail: tremel@mail.unimainz.de; Fax: +49 6131 39-25605; Tel: +49 6131 39-25135

^bInstitute for Physiological Chemistry (Applied Molecular Biology), Johannes Gutenberg-University, Duesbergweg 6, 55099, Mainz, Germany. E-mail: wmueller@uni-mainz.de; Fax: +49 6131 39-25243; Tel: +49 6131 392 5910

of 100 μ g mL⁻¹ in 20 mmol L⁻¹ MOPS [3-(*N*-morpholino) propanesulfonic acid] buffer (pH 7.5, 50 mmol L⁻¹ sodium acetate, 1 mmol L⁻¹ EDTA). For control assays, heat-denatured silicatein was used (95 °C for 15 min).

Enzymatic preparation of silicones and their characterization

Silicatein- α (40 µg) dissolved in 2.5 mL MOPS buffer was covered with 2.5 ml of dimethoxy dimethylsilane (DMS, ABCR) dissolved in diethyl ether (p.a., Sigma-Aldrich, 10:1 v/v). Sampling was done after incubation periods of 1 h, 3 h, and 5 h at 20 °C under intense shaking (200 rpm, Promax 1020, Heidolph). In control assays, heat-denaturated silicatein or bovine serum albumine (BSA, Sigma-Aldrich) or no protein was added. Further experiments were performed with educt mixtures of DMS with trimethoxymethylsilane (TMMS, supplied by ABCR) or trimethoxyphenylsilane (PTMS, supplied by ABCR) at molar different ratios (1:0, 0:1, 1:4, 4:1, 1:9). The aqueous layer which contained water-soluble decomposition products, silicatein and buffer, was removed. Prior to investigation, the organic phase containing the substrate (e.g. DMS) and silicone condensates (e.g. PDMS), was dried with sodium sulfate (anhydrous, p.a., Sigma-Aldrich) to prevent further decomposition. The condensation products were characterized by EI mass spectrometry (Finnigan MAT mass spectrometer 8230, Midland, Canada) and ²⁹Si DEPT NMR (DRX400, Bruker Biospin, Rheinstetten).

²⁹Si DEPT NMR

²⁹Si DEPT NMR measurements were performed in nondeuterated ether locked on the proton signal of an external D₂Ofilled capillary and calibrated to tetramethylsilane. The data was recorded with a digital NMR spectrometer, (Advance DRX400, Bruker Biospin) operating at 9.4 T and 79.5 MHz in case of ²⁹Si. A typical run consisted of *ca*. 300 DEPT scans with J = 6.6 Hz and 2 s of relaxation time, which is efficient to characterize –SiCH_x, –SiOCH_x and even –²⁹Si–SiCH_x. The assignment of the ²⁹Si NMR signals of dimethyl silandiole and tetramethyldisilan-3-ole was validated by comparison with standards synthesized according to standard protocols.³⁵ The corresponding mixed hydrolysis products dimethyl methoxysilanole and 1-methoxy tetramethyldisilan-3-ole were prepared by quenching an analogous reaction with one equivalent of methanol.

NMR signals of the organic phase originating from two-phase reactions showed a slight shift to lower chemical displacements, which is attributed to traces of water dissolved in the organic phase because ²⁹Si signals of silanols are known to shift with increasing donor ability of the respective solvent.³⁶ NMR spectra of synthesized silicon standards recorded in ether saturated with water approved that the observed shift to lower chemical displacement is due to a trace of dissolved water. The employed water-saturated ether (approx. 1.8% v/v) was prepared by vigorously shaking diethyl ether (*p.a.*, Sigma-Aldrich) against ultrapure water (Millipore Synergy 185 with UV photo oxidation, 18.2 MΩ/cm) for several days.

Dimethylsilandiole (dry ether): -7.73 (s, 1, Si) ppm. (ether, saturated with water): -6.62 (s, 1, Si) ppm. Tetramethyldisilan-3-ole (dry ether): -14.92 (s, 2, Si) ppm. (ether, saturated with water): -14.62 (s, 1, Si) ppm. Dimethylmethoxysilanole (dry ether): -5.21

(s, 1, Si) ppm. (ether, saturated with water): -4.42 (s, 1, Si) ppm. *Methoxytetramethyldisilan-3-ole* (*dry ether*): -11.79 (s, 1, Si–OH); -11.97 (d, 1, Si–OMe) ppm. (ether, saturated with water): -11.08 (s, 1, Si–OH); -11.63 (s, 1, Si–OMe) ppm.

In situ UV/VIS characterization

By substituting the *p*-aminophenoxy chromophoric group for the methoxy group of the DMS substrate, it was possible to monitor the cleavage and hydrolysis of the monomeric educt in situ. A solution containing 40 µg of silicatein-α in 2.75 mL MOPS buffer was covered with 0.75 mL of 2 mg mL⁻¹ bis(*p*-aminophenoxy)dimethylsilane (APS, ABCR) in ether. The two-phase system was stirred in Suprasil mixing cuvettes (Hellma QS-110) at 20 °C, and the absorption of the aqueous phase was monitored between 220 and 800 nm (Varian Cary 5G UV/VIS spectrophotometer, Mulgrave, Australia). Kinetic measurements were started 30 s after addition of the components. Several controls were performed: (a)in the absence of silicatein, (b) by replacing heat denaturated silicatein for the active silicatein, or (c) by employing bovine serum albumin (BSA, 50 µg in 2.75 mL MOPS buffer) instead of silicatein. In addition, the reaction was performed by adding water-soluble sodium hexafluorosilicate to the buffer solution to compete with the APS substrate.

Results

A comparison of the MALDI-MS spectra of PDMS obtained in presence and in absence of active silicatein shows a distinct increase of the product chain length. In the absence of silicatein, oligomers with up to seven repeat units were detected, whereas in the presence of active silicatein the highest mass signal could be assigned to a oligomer consisting of twelve monomer units (Fig. 1).

²⁹Si NMR analysis showed silicate in- α to enhance the monomer cleavage and the subsequent condensation to silicones considerably. In the absence of silicatein, the DMS monomer was hydrolyzed only to a negligible extent. Signals of two hydrolysis intermediates, dimethyl methoxysilanole and 1-methoxy tetramethyldisilan-3-ole, could be identified. A low constant signal ratio of 0.05 of the decomposition products to the monomer during 5 h of incubation (Fig. 2a) indicated the stability of the starting compound. In contrast, in the presence of silicate in- α the NMR spectrum changed significantly with time (Fig. 2b), and new signals of silandiols (dimethyl silandiole and tetramethyldisilan-3-ole) appeared after 1 h of incubation. The signal ratio of the hydrolysis products to the monomeric starting compound increased drastically to 0.22 after 1 h, 0.89 after 3 h, and 2.31 after 5 h of incubation with active silicatein. In particular, the NMR spectra of the sample incubated for 5 h showed obvious changes (Fig. 3) having tetramethyldisilan-3-ole as the predominant component. In control assays with denaturated silicatein and without silicatein, only traces of the hydrolysis intermediate methoxytetramethyldisilan-3-ole were found. These results demonstrate the catalytic activity of silicate in- α in the cleavage of the alkoxy bonds in alkoxy silanes.

Higher oligomers (n > 2) could not be detected by ²⁹Si NMR. Typically, species can only be detected by NMR techniques if their concentration exceeds approx. 1 mol%. But the presence of higher oligomers was demonstrated by mass spectrometry



Fig. 1 EI mass spectrum of siloxane polymers. The mass distributions of PDMS obtained in the presence of silicatein (b) differ significantly from the distribution obtained in a control reaction within silicatein (a). A distinct increase in the chain length of the product was observed in contrast to PDMS reaction that proceeded in the absence of silicatein. The mass difference of ~75 Da between the individual peaks corresponds to a single $[Si(Me)_2O]$ unit.

(*vide supra*). The amount of higher oligomers decreased significantly with chain length, and therefore reached the detection limit of the NMR technique (Fig. 1).

In additional presence of varying amounts of either TMMS or PTMS, the silicatein-enhanced polymerization of DMS is competitively inhibited. The phenylated substrate PTMS blocked the polymerization reaction completely as traceable in the detection limit of the ²⁹Si NMR technique. This can be rationalized by both (*a*) steric hindering due to the bulky phenoxy group and (*b*) by a decreased reactivity of the substrate to hydrolyze. The phenoxy residue increases electron density at the silicon centre of the substrate PTMS and thus decreases the affinity for a nucleophilic attack of silicatein at the central silicon atom of the substrate.

Replacing the methoxy group by the *p*-aminophenoxy chromophor allowed an *in situ* monitoring of the reaction by UV/VIS absorption spectroscopy. Fig. 4a shows the evolution of the absorption spectra with time by the increasing concentration of the decomposition product *p*-aminophenol. The plot of absorption at 290 nm *versus* time showed sigmoidal characteristics of the decomposition of the APS substrate (Fig. 4b), which allowed recently the extraction of the Michaelis constant $K_m = 22.7 \,\mu M.^{21}$ In the presence of silicatein- α , the increase in the absorption was accelerated considerably compared to control assays with BSA or without protein. Saturation, which we defined as 98% of the upper asymptote of a Boltzmann fit, was reached after 16 min of incubation with silicatein- α , whereas in control assays saturation was reached only after 26 min. The reaction was strongly competitively suppressed by adding the water-soluble



Fig. 2 ²⁹Si NMR spectrum of PDMS formed from the monomer DMS after 1, 3 and 5 h. The reaction was performed (**a**) in the absence of silicatein or (**b**) in the presence of the enzyme. All spectra are normalized with respect to their most intense signals.



Fig. 3 ²⁹Si NMR spectrum of PDMS formed in the presence of silicatein after 5 h of incubation with signal assignments.

substrate ammonium hexafluorosilicate to the aqueous layer. For heat-denatured silicatein a decrease of the conversion rate (Fig. 4b) was observed, even in comparison to control assays in the absence of protein or in the presence of BSA, respectively. Saturation was reached not until 50 min. From this we conclude that denaturated silicatein exhibits a surface activity which can block the phase-transfer of APS from the ether to the aqueous phase; this leads to the observed decrease in the conversion rate. One may speculate from this that heat-denatured silicatein- α exposes hydrophic regions and thus have an increased tensioactivity.



Fig. 4 a) Absorption spectrum of the aqueous layer during phase-transfer reaction in absence of silicatein. The absorption increases due to the increasing concentration of the decomposition product *p*-aminophenol. b) Time dependence of the absorption at 290 nm. The reactions were performed in the presence of silicatein- α (\bullet), heat-denaturated silicatein (\blacktriangle), BSA (\bigcirc) and in the absence of protein (\blacksquare). The increase in absorption shows a sigmoidal behaviour. In the conducted phase-transfer reaction, the amount of available substrate should remain at a high level and thus the reaction may cease by reason of an inactivation of the enzyme. The smaller absorption for inactive silicatein can be related to a blocking of the substrate phase-transfer due to tensioactivity of heat-denatured silicatein. The reaction kinetics determined in assays with BSA (\bigcirc) are comparable to those recorded in the control assays without silicatein.

Conclusion

Aqueous organisms live in an environment which is undersaturated with respect to silica. The average concentrations of dissolved silicon in seawater, which is mostly present as undissociated orthosilicic acid, Si(OH)₄, is about 70 mM.³⁷ In surface waters this concentration is even lower (< 3 mM) due to the biological consumption of Si(OH)₄.^{37,38} On the other hand, the intracellular concentrations of silicon, *e.g.* in diatoms, can reach > 100 mM,³⁹ thus exceeding the extracellular silicon concentration by > 1000-fold. This can be seen as an indication that these organisms have developed efficient mechanisms for the active uptake of silicic acid from the surrounding water to form their silica skeleton. The uptake of silicon into sponge cells and diatoms is linked to the transport of sodium.⁴⁰⁻⁴² Silicateins, which are exclusively found in sponges, catalyse *in vivo* silica formation from monomeric silicic acid esters. The enzymatic activity of recombinant silicatein was studied *in vitro* for transformation of alkoxy silanes to silicones (siloxanes) in a phase-transfer reaction at neutral pH and under physiological conditions. An extensive increase in the formation rate of oligomers and in the chain length of the silicones could be demonstrated by mass spectrometry, UV/Vis and NMR techniques. To the best of our knowledge, the formation of silicones in the presence of silicatein represents the first enzymatically driven organometallic condensation reaction. UV/Vis-measurements and ²⁹Si DEPT NMR were used as a analytical probe to determine the enzymatic activity of silicatein- α and revealed the hydrolytic activity of silicatein- α .

The extraordinary feature of silicatein to produce silica biocatalytically under physiological conditions (neutral pH, ambient temperature and pressure) can be utilized for the synthesis of silicones. Therefore this biocatalytical reaction may have an impact for the sustainable synthesis of silicones.⁴³

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